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ORIGINAL ARTICLE

# Increased levels of SLP-2 correlate with poor prognosis in gastric cancer

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**Abstract**
*Background* Stomatin-like protein 2 (SLP-2) is a member of the highly conserved stomatin protein family whose homologues span from Archaea to humans and include stomatin, SLP-1, and SLP-3. Several studies have indicated that overexpression of SLP-2 is strongly associated with adhesion and migration in several human cancers. The aim of the present study was to evaluate SLP-2 expression at the mRNA and protein level in patients with gastric cancer (GC) and to examine the relationships between SLP-2 expression, clinicopathological features, and prognosis.
*Methods* We investigated SLP-2 expression in primary GC and paired normal gastric tissue by real-time PCR (RT-PCR; $n = 16$) and Western blot analysis ($n = 32$). Additionally, we performed immunohistochemistry (IHC) on 113 paraffin-embedded GC specimens, 30 matched normal specimens, and 30 paired metastatic lymph node samples.
*Results* SLP-2 is overexpressed in GC compared with the adjacent normal gastric epithelium ($p < 0.001$), and high-level SLP-2 expression is significantly correlated with the depth of invasion, lymph node metastasis, distant metastasis, and American Joint Committee on Cancer (AJCC) stage. Furthermore, elevated SLP-2 expression is an independent prognostic factor in multivariate analysis using the Cox regression model ($p = 0.005$).
*Conclusions* Overexpression of SLP-2 may contribute to the progression and poor prognosis of GC.

**Keywords** Gastric cancer · Stomatin-like protein 2 · Prognosis

## Introduction

Gastric cancer (GC) is one of the most lethal common cancers, with a 5-year overall survival rate of less than 35 % and more than 750,000 deaths annually worldwide [1, 2]. In China, the mean annual mortality from GC is estimated to be as high as 16 per 100,000 people and accounts for a large percentage of the cancer-related deaths in China [3]. Despite significant improvement in the treatment of GC during the past few decades as the result of the introduction of new surgical techniques, superior radiotherapy techniques, and the use of chemotherapy, the prognosis of advanced GC is still poor. One major reason for the poor clinical outcome is lack of a specific early diagnosis method. Deregulation of cell adhesion molecules such as connective tissue growth factor (CTGF) [4], tumor suppressor genes such as p53 and p16 [5], or oncogenes such as c-Myc and c-ErbB2 [6] may be associated with the poor prognosis of GC patients. However, the identification of novel cancer-specific biomarkers associated with GC has the potential to provide more accurate prognostic information, increase our understanding of the molecular mechanisms underlying GC development, and lead to new therapeutic targets.

D. Liu and L. Zhang contributed equally to this work.

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Human stomatin, originally identified as a membrane protein in human red blood cells, is associated with a variety of diseases, such as kidney failure and anemia [7]. Stomatin-like protein 2 (STOML2/SLP-2) shares a similar signature sequence with stomatin but does not contain an $NH_2$-terminal hydrophobic domain, which distinguishes it from other members of the stomatin family [8]. Recently, SLP-2 has been shown to be upregulated and involved in progression and development in several types of cancer, including esophageal squamous cell carcinoma (ESCC), endometrial adenocarcinoma, laryngeal squamous cell carcinoma (LSCC), pulmonary squamous cell carcinoma (PSCC), breast cancer, and glioma [4, 8–14]. In ESCC, SLP-2 is one of the most differentially expressed cancer-related genes compared with paired adjacent normal tissues, and knockdown of SLP-2 reduces the growth rate of ESCC cells in vitro and in vivo and inhibits cell attachment [8, 9]. SLP-2 is also associated with cancer cell survival, because silencing SLP-2 enhances the sensitivity of cancer cells to chemotherapeutics [4]. In addition, SLP-2 is strongly correlated with clinical stage as well as prognostic characteristics in LSCC, suggesting that SLP-2 could be important in determining prognosis and thus in selecting treatment [11]. In PSCC, breast cancer, and glioma, SLP-2 overexpression is significantly associated with distant tumor metastasis and might represent an independent prognostic factor [12–14]. However, the molecular mechanisms of SLP-2 in GC remain largely unknown.

In this study, we investigated SLP-2 expression by real-time polymerase chain reaction (RT-PCR) and Western blot analysis in a large number of GC samples. We also performed immunohistochemical (IHC) analyses on a larger panel of GCs, matched normal gastric epithelial tissues, and paired metastatic lymph nodes. Finally, we evaluated the relationship between SLP-2 expression and the clinicopathological parameters and prognoses of gastric carcinoma patients. The results suggest that overexpression of SLP-2 may be associated with tumor progression and serve as a prognostic biomarker in patients with GC.

## Materials and methods

### Tissue specimens and clinical information

Thirty-two fresh-frozen GC and corresponding normal mucosa tissue samples (more than 10 cm away from the edge of the GC) were taken from patients with GC within 30 min after resection and immediately stored in liquid nitrogen until use. Polyformaldehyde-fixed and paraffin-embedded GC tissue blocks ($n = 113$) were obtained from the stored files of the Department of General Surgery, Nangfang Hospital, Southern Medical University between January 2002 and December 2006. Additionally, 30 control samples from matched normal gastric tissues taken from the distal resection margin and 30 paired metastatic lymph nodes were collected. The 113 patients included 51 men and 62 women aged 28–92 years (mean, 62.5 years). No patients had received chemotherapy or radiotherapy before surgery, and 29 patients had synchronous distant metastasis. The various clinicopathological parameters (age, gender, tumor size, tumor location, differentiation status, Lauren classification, depth of invasion, lymph node metastasis, and distant metastasis) were obtained from histopathology records. The stage of gastric cancer was described according to the 2010 tumor node metastasis (TNM) classification of malignant tumors by the American Joint Committee on Cancer (AJCC). The patients were followed until death or the last follow-up date (30 November 2011). Complete follow-up, ranging from 4 to 115 months, was available for all patients, and the median patient survival was 60 months.

### Ethics

The Nangfang Hospital Ethical Committee approved the usage of GC specimens, matched normal specimens, and paired metastatic lymph node samples for this study.

### RNA extraction and real-time PCR

Tissues were minced and total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies) and reverse transcribed to first-strand cDNA with the TaqMan Reverse Transcription Kit (Applied Biosystems). Then, 0.5- to 1-μl aliquots of the cDNA were used as the template to amplify the *SLP-2* fragment (forward: 5′-GTGACTCTCGACAA TGTAAC-3′; reverse: 5′-TGATCTCATAACGGAGGC AG-3′) under the following conditions: 95 °C for 5 min; 28 cycles of 95 °C for 30 s; 57 °C for 30 s; and 72 °C for 30 s; and 72 °C for 5 min.

PCR and data collection were performed on an iCycler (Bio-Rad). The expression level of *SLP-2* was normalized to *GAPDH*.

### Western blotting analysis

Western blotting was performed according to standard methods as described previously [13]. Briefly, tissues were ground and lysed with buffer (1 % sodium dodecyl sulfate; 10 mmol/l Tris-Cl, pH 7.6; 150 mmol/l NaCl; 20 g/l aprotinin; 20 g/l leupeptin; and 1 mmol/l phenylmethanesulfonyl fluoride). Protein concentrations were determined using the Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL, USA). Fifty micrograms protein was separated electrophoretically on 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to a

polyvinylidene difluoride membrane. After blocking, the membrane was incubated with a rabbit polyclonal anti-SLP-2 antibody (Proteintech Group, Chicago, IL, USA) at a 1:1,000 dilution at 4 °C overnight. After washing, the membranes were incubated with a secondary antibody at a dilution of 1:6,000 at room temperature for 70 min. Proteins were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA), and a mouse anti-β-actin antibody (1:1,000 dilution; Sigma, St. Louis, MO, USA) was used as a loading control.

### Immunohistochemical assay

The immunohistochemical (IHC) assay was performed as previously described [15]. Briefly, the slides were dewaxed with xylene and rehydrated through an ethanol gradient into water. After endogenous peroxidase activity was quenched with 3 % $H_2O_2$, sections were digested with 0.1 % trypsin. After washing with phosphate-buffered saline (PBS), nonspecific antibody binding was blocked by incubating the slides with 10 % normal goat nonimmune serum. The sections were incubated at 4 °C overnight with the rabbit polyclonal SLP-2 antibody (Proteintech Group) at a 1:400 dilution. After PBS washing, sections were incubated with biotinylated secondary antibody followed by horseradish peroxidase-labeled streptavidin and then washed in PBS again. The sections were then developed using 3,3-*V*-diaminobenzidine (Sigma), washed in running tap water, and lightly counterstained with hematoxylin before dehydration and coverslip mounting. Negative control experiments were conducted by replacing the primary antibody with phosphate-buffered saline.

Immunostaining was scored semiquantitatively by two independent observers (N.H. and L.Z.) who were blinded to the patients' outcome and other clinicopathological parameters. The SLP-2 detection system has been described previously [12]. Each sample was assigned by the extent of immunoreactivity to one of the following categories: 0, 0 %; 1, <25 %; 2, 25–50 %; 3, 51–75 %; or 4, >75 %. Staining intensity was categorized as 0, negative; 1, weak; 2, moderate; 3, strong. For each case, the immunostaining score, also known as the staining index (SI), was calculated by multiplying the percentage of positive cells with the staining intensity score, yielding a value between 0 and 12. For this study, an optimal cutoff value was identified as follows: an SI score of 8 or higher was used to define tumors with high SLP-2 protein expression, and an SI score of less than 8 was used to indicate low SLP-2 expression [11].

### Statistical analysis

Statistical analysis was performed using the SPSS statistical software package (SPSS version 13.0; SPSS, Chicago, IL, USA). Student's *t* test was used to analyze real-time (RT)-PCR data. For the SI scores obtained by immunohistochemical analysis, the Mann–Whitney *U* test was used to compare GC and corresponding normal mucosa tissue. The correlations between SLP-2 expression and clinicopathological characteristics were analyzed using the Pearson chi-square test. Survival rates were calculated according to the Kaplan–Meier method, and differences were evaluated using the log-rank test. The Cox proportional hazards regression model was used to assess the hazard ratio (HR) and identify factors that independently predict survival. Differences were considered significant if the $p$ value from a two-tailed test was $<0.05$.

## Results

### Overexpression of SLP-2 in human GC

To elucidate the role of SLP-2 in the initiation and progression of GC, we first analyzed its expression in GC and matched adjacent normal tissues at the mRNA level. RT-PCR analysis of *SLP-2* expression in matched normal and tumor tissues showed that *SLP-2* was upregulated in the majority of GC tissues compared with their normal

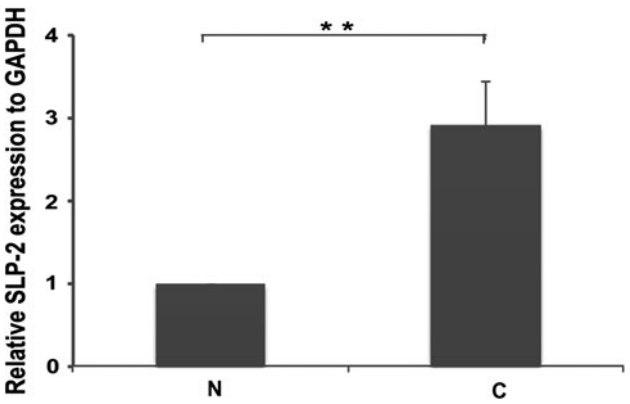


**Fig. 1** Overexpression of stomatin-like protein 2 (*SLP-2*) in gastric cancer (GC) as detected by real-time PCR. *SLP-2* expression in each sample was normalized to *GAPDH*. Relative expression of *SLP-2* in GC samples ($n = 16$) was compared with adjacent matched noncancerous gastric tissue. *N* normal tissue, *C* patient-matched tumor tissue. $^{**}p < 0.01$ (Student's *t* test)

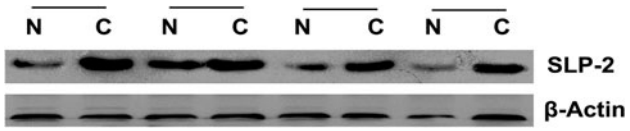


**Fig. 2** Overexpression of SLP-2 in GC as detected by Western blot analysis. Data presented here are representative of all the samples. β-Actin was used as a loading control. *N* normal tissue, *C* patient-matched tumor tissue

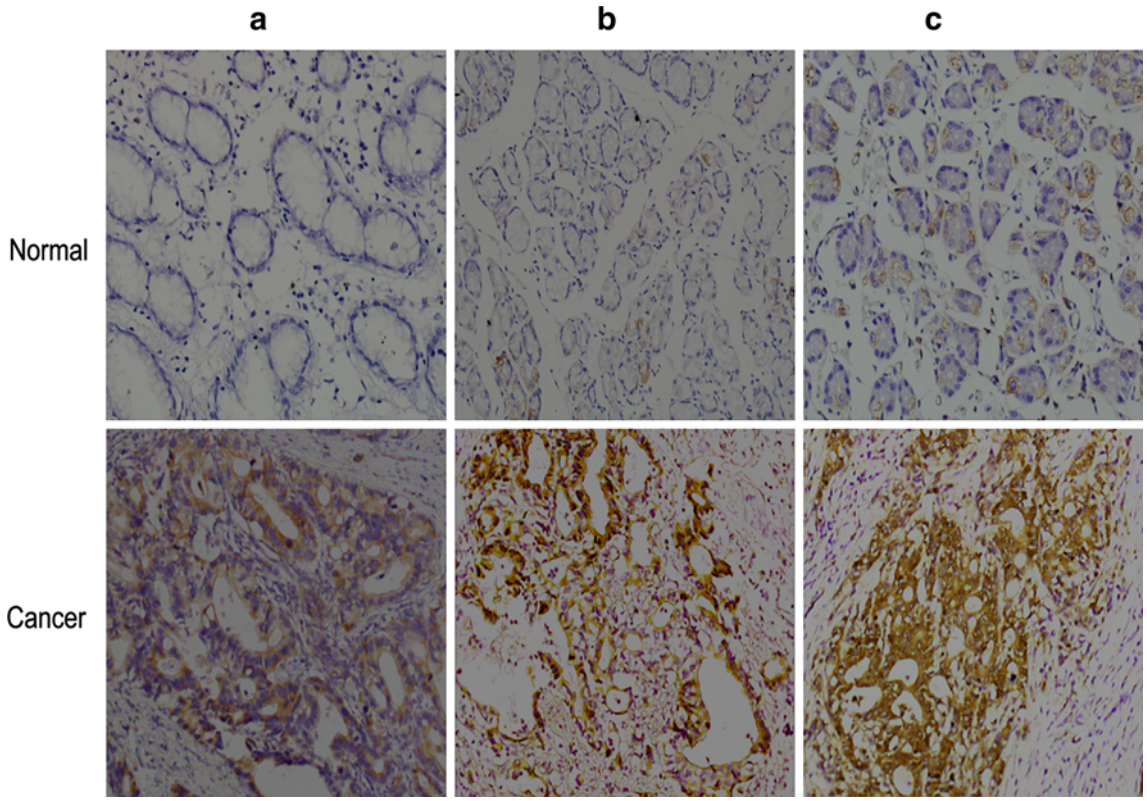


**Fig. 3** Immunochemistry (IHC) analysis of SLP-2 expression in GC. **a** Weak staining. **b** Moderate staining. **c** Strong staining. ×200

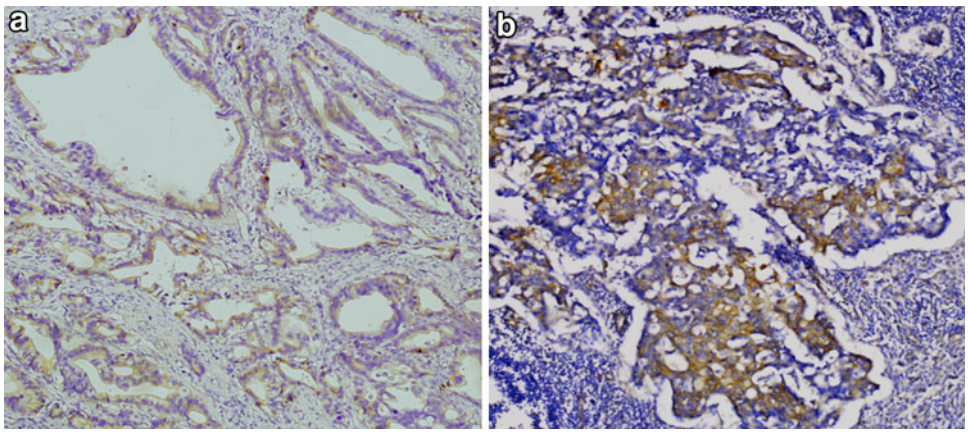


**Fig. 4** Example of GC sample with weak SLP-2 expression (**a**) that had a metastatic lymph node with strong SLP-2 expression (**b**). ×100

counterparts (12/16; 75 %) (Fig. 1). SLP-2 was similarly overexpressed at the protein level in GC, as shown by Western blot analysis and immunohistochemistry (IHC). The Western blot analysis showed that SLP-2 protein levels were elevated in 26 of 32 (81.25 %) GCs compared with normal tissues (Fig. 2). Paraffin-embedded blocks ($n = 173$) from 113 GC patients were evaluated for SLP-2 protein expression by IHC. SLP-2 protein expression was absent (7/30; 23.33 %) or low (23/30; 76.67 %) in normal gastric mucosa (Fig. 3). In contrast, cytoplasmic and occasional cell membrane SLP-2 staining was expressed in all GC tissues: 61 (53.98 %) showed strong SLP-2 expression, 35 (30.97 %) showed moderate SLP-2 expression, and 17 (15.05 %) showed weak SLP-2 expression (Fig. 3). Absent or low staining (SI $< 8$) was noted in 42 cases (37.16 %), and high staining (SI $\geq 8$) was observed in 71 cases (62.84 %). SLP-2 expression was significantly higher in GC tissues ($n = 30$; SI $= 7.92$) compared with matched normal gastric tissues ($n = 30$; SI $= 3.20$; $p < 0.001$). Additionally, all GC cells in metastatic lymph nodes ($n = 30$) showed strong expression of SLP-2 regardless of whether their primary tumors had high or low SLP-2 expression (Fig. 4).

### Association of SLP-2 expression with clinicopathological characteristics

Expression of SLP-2 in tumor tissue was not significantly associated with gender, age, tumor size, tumor location, differentiation status, or Lauren classification (Table 1). However, elevated SLP-2 expression was strongly correlated with the depth of invasion ($p = 0.016$), lymph node metastasis ($p = 0.002$), distant metastasis ($p = 0.033$), and AJCC stage ($p = 0.004$) (Table 1).

**Table 1** Correlation of SLP-2 expression with clinicopathological characteristics of patients with gastric cancer

| Variable | *N* | SLP-2 expression | | *p* value[a] |
|---|---|---|---|---|
| | | Low (%) | High (%) | |
| Gender | | | | 0.683 |
| Male | 51 | 20 (39.2) | 31 (60.8) | |
| Female | 62 | 22 (35.5) | 40 (64.5) | |
| Age (years) | | | | 0.969 |
| <55 | 46 | 17 (36.9) | 29 (63.1) | |
| ≥55 | 67 | 25 (37.3) | 42 (62.7) | |
| Tumor size (cm) | | | | 0.540 |
| <4 | 39 | 13 (33.3) | 26 (36.7) | |
| ≥4 | 74 | 29 (39.1) | 45 (60.9) | |
| Tumor location | | | | 0.980 |
| Cardia of stomach | 28 | 10 (35.7) | 18 (64.3) | |
| Body of stomach | 23 | 8 (34.7) | 15 (65.3) | |
| Antrum of stomach | 43 | 17 (39.5) | 26 (60.5) | |
| Whole | 19 | 7 (36.8) | 12 (63.2) | |
| Differentiation status | | | | 0.237 |
| Well | 37 | 17 (45.9) | 20 (54.1) | |
| Moderate | 39 | 15 (38.4) | 24 (61.6) | |
| Poor and undifferentiated | 37 | 10 (27.0) | 27 (73.0) | |
| Lauren classification | | | | 0.683 |
| Intestinal type | 70 | 25 (35.7) | 45 (64.3) | |
| Diffuse type | 43 | 17 (39.5) | 26 (60.5) | |
| Depth of invasion | | | | 0.016 |
| T1 + T2 | 43 | 22 (51.1) | 21 (48.9) | |
| T3 + T4 | 70 | 20 (28.5) | 50 (71.5) | |
| Lymph node metastasis | | | | 0.002 |
| Absent (N0) | 41 | 23 (56.1) | 18 (43.9) | |
| Present (N1–3) | 72 | 19 (26.3) | 53 (73.7) | |
| Distant metastasis | | | | 0.033 |
| Absent (M0) | 84 | 36 (42.8) | 48 (47.2) | |
| Present (M1) | 29 | 6 (20.7) | 23 (79.3) | |
| AJCC stage | | | | 0.004 |
| I | 21 | 14 (66.7) | 7 (33.3) | |
| II | 23 | 10 (43.5) | 13 (56.5) | |
| III | 40 | 13 (32.5) | 27 (67.5) | |
| IV | 29 | 5 (17.2) | 24 (82.8) | |

*SLP-2* stomatin-like protein 2, *AJCC* American Joint Committee on Cancer

[a] *p* value when expression levels were compared using Pearson chi-square tests; distant metastases included the peritoneum, liver, transverse colon, pancreas, and bone

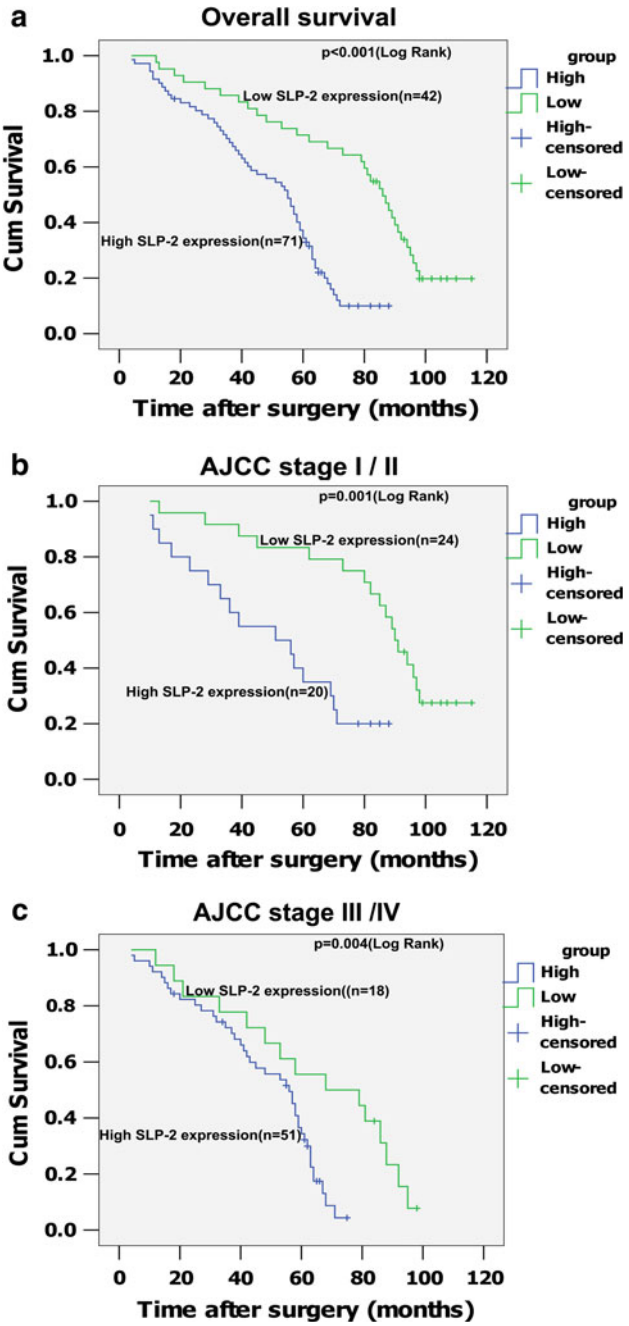


**Fig. 5** Kaplan–Meier survival analysis in patient group according to SLP-2 expression levels. **a** Patients with higher SLP-2 expression had a shorter overall survival time, whereas patients with lower SLP-2 expression had a better survival time ($p < 0.001$). The difference between survival curves of SLP-2 high-expressing and low-expressing patients was compared within subgroups of AJCC stages I + II ($p = 0.001$) (**b**) and III + IV ($p = 0.004$) (**c**)

**Table 2** Univariate and multivariate analyses of individual parameters for correlations with overall survival rate

| Variables | Univariate[a] | | | Multivariate[b] | | |
|---|---|---|---|---|---|---|
| | HR | CI (95 %) | *p* value | HR | CI (95 %) | *p* value |
| Gender | 1.095 | 0.725–1.653 | 0.667 | | | |
| Age | 0.992 | 0.975–1.010 | 0.380 | | | |
| Tumor size | 1.002 | 0.992–1.013 | 0.658 | | | |
| Tumor location | 0.891 | 0.733–1.084 | 0.248 | | | |
| Differentiation status | 1.226 | 0.957–1.571 | 0.107 | | | |
| Lauren classification | 0.973 | 0.641–1.476 | 0.897 | | | |
| Depth of invasion | 2.879 | 1.815–4.568 | <0.001 | 1.527 | 0.927–2.517 | 0.097 |
| Lymph node metastasis | 2.987 | 1.845–4.834 | <0.001 | 1.617 | 0.937–2.792 | 0.084 |
| Distant metastasis | 5.589 | 3.643–9.541 | <0.001 | 3.014 | 1.730–5.252 | 0.000 |
| AJCC stage | 2.702 | 2.065–3.536 | <0.001 | 2.023 | 1.475–2.773 | 0.000 |
| SLP-2 expression | 3.587 | 2.112–6.094 | <0.001 | 2.248 | 1.283–3.939 | 0.005 |

*HR* hazard ratio, *CI* confidence interval, *AJCC* American Joint Committee on Cancer, *SLP-2* stomatin-like protein 2

[a] Hazard ratios in univariate models

[b] Hazard ratios in multivariable models

### Survival analysis

A Kaplan–Meier analysis and log-rank test showed that patients whose tumors had high SLP-2 levels had a significantly shorter overall survival than patients whose tumors expressed low levels of SLP-2 ($n = 113$; $p < 0.001$; Fig. 5a). Patients with tumors exhibiting high SLP-2 expression also had a significantly shorter overall survival time than those with low expression of SLP-2 in either the AJCC stage I + II subgroup ($n = 44$; $p = 0.001$; Fig. 5b) or the AJCC stage III + IV subgroup ($n = 69$; $p = 0.004$; Fig. 5c).

Univariate analysis showed a significant relationship between overall survival and depth of invasion [hazard ratio (HR) = 2.879; 95 % confidence interval (95 % CI) = 1.815–4.568; $p < 0.001$], lymph node metastasis (HR = 2.987; 95 % CI = 1.845–4.834; $p < 0.001$), distant metastasis (HR = 5.589; 95 % CI = 3.643–9.541; $p < 0.001$), AJCC stage (HR = 2.702; 95 % CI = 2.065–3.536; $p < 0.001$), and high SLP-2 expression (HR = 3.587; 95 % CI = 2.112–6.094; $p < 0.001$), but not with gender, age, tumor size, tumor location, differentiation status, or Lauren classification (Table 2).

Multivariate analysis using the aforementioned five significant parameters identified distant metastasis (HR = 3.014; 95 % CI = 1.730–5.252; $p < 0.001$) as the poorest prognostic factor, followed by high SLP-2 expression (HR = 2.248; 95 % CI = 1.283–3.939; $p = 0.005$) and AJCC stage (HR = 2.023; 95 % CI = 1.475–2.773; $p < 0.001$) (Table 2).

## Discussion

The tumorigenesis and progression of GC are poorly understood, complex, multistage processes involving multiple gene and protein alterations. It is important to identify and understand the molecular mechanisms of tumor formation and progression to develop rational approaches to the early diagnosis and treatment of GC. In this study, we investigated the SLP-2 expression status in GC at both the mRNA and protein levels and found that SLP-2 was markedly overexpressed in human GC tissues compared with normal gastric epithelium. Furthermore, a high level of SLP-2 expression was significantly associated with the depth of invasion, lymph node metastasis, distant metastasis, and TNM stage in GC, similar to previous findings in LSCC and breast cancer [11–13], and all cancer cells in metastatic lymph nodes of 30 GC cases showed strong expression of SLP-2. Taken together, these results suggest that SLP-2 may be involved in the progression of human GC.

We also show for the first time that SLP-2 expression is a strong predictor of poor prognosis for GC, similar to findings in breast cancer, PSCC, and glioma [12–14]. In the present study, survival analysis showed that distant metastasis, AJCC stage, and SLP-2 expression independently predicted poor overall survival. Moreover, although distant metastasis and AJCC stage were the best predictors with hazard ratios of more than or about 2.0, SLP-2 expression had a hazard ratio of 2.2 and showed a similar risk of death from GC and thus was an equally important prognostic factor. Obvious correlations between these factors exist, which together might promote the progression of GC.

SLP-2 is a novel and unusual member of the stomatin gene superfamily [16, 17]. The human stomatin protein family consists of five members, including stomatin, SLP-1, SLP-2, SLP-3, and the kidney-specific protein podocin. SLP-2 and stomatin share a stomatin-homology region and are part of the much larger SPFH (stomatin-prohibitin-flotillin-HflK/C) superfamily. However, SLP-2 lacks the typical amino-terminal transmembrane domain present in other stomatins [18]. It has been suggested that stomatins are involved in the organization of the peripheral cytoskeleton, the formation of sphingolipid- and cholesterol-rich lipid rafts, and the assembly of ion channels and mechanosensation receptors [19, 20]. SLP-2 expression is upregulated in multiple tumor types, including ESCC, endometrial adenocarcinoma, LSCC, breast cancer, PSCC, and glioma [4, 9–14], but the mechanisms leading to SLP-2 overexpression in human tumors are not well known. SLP-2 has been shown to enhance invasiveness in glioma cells, potentially through the upregulation of MMP-9. Moreover, a luciferase reporter assay indicated that SLP-2 regulates MMP-9 expression in an NF-κB-dependent manner, suggesting that SLP-2 may be a component of the NF-κB signaling pathway [14]. Consistent with this possibility, fibronectin 1, a downstream target of NF-κB, is downregulated following SLP-2 knockdown in ESCC cells, and fibronectin 1 and SLP-2 expression levels are significantly correlated in ESCC tissues [9]. Future studies to determine the cellular mechanisms by which SLP-2 promotes GC progression are warranted.

In conclusion, we have shown that SLP-2 expression is upregulated in GC and correlates with poor clinicopathological characteristics and poor overall patient survival, suggesting that it may contribute to the malignant potential of GC. SLP-2 could therefore serve as a valuable new biomarker for predicting prognosis and directing clinical decision making for patients with GC.

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**Conflict of interest** We declare that we have no conflict of interest.

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