ORIGINAL ARTICLE

Significance of *SYT8* for the Detection, Prediction, and Treatment of Peritoneal Metastasis from Gastric Cancer

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Short running head: *SYT8* and Peritoneal Metastasis of GC

ABSTRACT

Objective: To develop novel diagnostic and therapeutic targets specific for peritoneal metastasis of gastric cancer (GC).

Summary Background Data: Advanced GC frequently recurs because of undetected micrometastases even after curative resection. Peritoneal metastasis has been the most frequent recurrent pattern after gastrectomy and is incurable.

Methods: We conducted a recurrence pattern-specific transcriptome analysis in an independent cohort of 16 patients with stage III GC who underwent curative gastrectomy and adjuvant S-1 for screening candidate molecules specific for peritoneal metastasis of GC. Next, another 340 patients were allocated to discovery and validation sets (1:2) to evaluate the diagnostic and predictive value of the candidate molecule. The results of quantitative reverse-transcription PCR and immunohistochemical analysis were correlated with clinical characteristics and survival. The effects of siRNA-mediated knockdown on phenotype and fluorouracil sensitivity of GC cells were evaluated in vitro, and the therapeutic effects of siRNAs were evaluated using a mouse xenograft model.

Results: Synaptotagmin VIII (*SYT8*) was identified as a candidate biomarker specific to peritoneal metastasis. In the discovery set, the optimal cutoff of *SYT8* expression was established as 0.005. Expression levels of *SYT8* mRNA in GC tissues were elevated in the validation set comprising patients with peritoneal recurrence or metastasis. *SYT8* levels above the cutoff value were significantly and specifically associated with peritoneal metastasis, and served as an independent

prognostic marker for peritoneal recurrence-free survival of patients with stage II/III GC. The survival difference between patients with *SYT8* levels above and below the cutoff was associated with patients who received adjuvant chemotherapy. Inhibition of *SYT8* expression by GC cells correlated with decreased invasion, migration, and fluorouracil resistance. Intraperitoneal administration of *SYT8*-siRNA inhibited the growth of peritoneal nodules and prolonged survival of mice engrafted with GC cells.

Conclusions: *SYT8* represents a promising target for the detection, prediction and treatment of peritoneal metastasis of GC.

Keywords: biomarker, gastric cancer, peritoneal metastasis, synaptotagmin VIII

MINI-ABSTRACT

Gastric cancer with peritoneal metastasis is invariably fatal, thus requiring biomarkers and therapeutic targets. Comparative transcriptome analysis detected gastric cancer-specific expression of synaptotagmin VIII (*SYT8*). We identified *SYT8* expression as a significant biomarker for predicting and detecting peritoneal metastasis and as a potential therapeutic target.

Gastric cancer (GC) is the fourth most common cancer worldwide.[1] Excellent prognosis is expected when endoscopic or surgical resection is performed in patients with early stage GC.[2, 3] However, the disease diagnosed at more advanced stage frequently recurs after surgical resection with curative intent, presumably through growth of the occult micrometastases.[4, 5] Whereas D2 dissection followed by adjuvant chemotherapy (S-1 monotherapy for 12 months or a combination of capecitabine and oxaliplatin for six months) serves as standard treatment in East Asia, gastrectomy with perioperative chemotherapy or postoperative chemoradiotherapy is the standard in Western countries, and the overall outcome remains unsatisfactory.[6-10] Furthermore, prognosis of the patients already with distant metastasis at the time of diagnosis is dismal with the median survival time of 6 to 13 months.[3, 11]

The peritoneum is a common site of distant metastasis and also of the disease recurrence, and peritoneal metastasis is the leading cause of death among patients with advanced GC.[3, 12, 13] Although essentially an incurable disease, significant reduction in the incidence of peritoneal disease among the postoperative adjuvant chemotherapy arm compared with the surgery alone arm in a phase III adjuvant trial implicates that patients with peritoneal micrometastasis could benefit from chemotherapy. In addition, detection of small peritoneal deposits which would alter the whole treatment strategy is often difficult with imaging studies and requires staging laparoscopy and biopsy for confirmation.[14, 15]Thus, identification of biomarkers that accurately predict the risk of peritoneal metastasis is warranted to facilitate appropriate clinical decisions in addition to providing candidates for targeted therapies.

The heterogeneity of GC presents a formidable obstacle to defining molecular pathogenesis as well as developing sensitive biomarkers and specific molecular targeting agents.[16, 17] GC cells metastasize through peritoneal, lymphatic, and hematogenous pathways. Each stage of metastasis requires a complex set of cellular functions mediated by stage-specific characteristics of the target organ.[18, 19] Therefore, identifying the molecular mechanisms specific to each pathway is required for discovering specific biomarkers that will facilitate management of patients with metastasis through that pathway.

Here, we conducted a recurrence pattern-specific transcriptome analysis for peritoneal metastasis and identified synaptotagmin VIII (*SYT8*) as a candidate molecule associated with the peritoneal disease from GC. The aim of this study was to evaluate the diagnostic and therapeutic potential of *SYT8* expression for patients with GC peritoneal metastasis.

PATIENTS AND METHODS

Transcriptome Analysis

To identify molecules that are specifically overexpressed in patients with peritoneal metastasis, global expression profiling in which the expression levels of 57,761 genes including splicing variants were evaluated was conducted using the HiSeq System (Illumina, San Diego, CA) to analyze primary GC tissues from a cohort of 16 patients with stage III GC who underwent curative gastrectomy and adjuvant S-1 (an oral fluoropyrimidine derivative) monotherapy. This patient cohort consisted of the following four groups (4 patients per a group): 1) no recurrences for longer

than 5 years, 2) peritoneal recurrences within 2 years after surgery, 3) liver-confined recurrences within 2 years after surgery, and 4) distant nodal recurrences within 2 years after surgery. Through comprehensive expression analysis of the samples from the no recurrence group and those from the three recurrent groups, 14 molecules with high expression exclusively in the peritoneal recurrence group were identified, including *SYT8*.

Analysis of Discovery and Validation Sets

Next, mRNA levels of the *SYT8* was evaluated in the following patient cohort. Between November 2001 and April 2015, 987 patients underwent surgery for GC at the Department of Gastroenterological Surgery, Nagoya University. Of these, 340 patients that fulfill the following criteria were selected for inclusion in the analysis: no preoperative treatment, availability of paired (cancerous and adjacent non-cancerous) gastric tissues obtained from surgical specimens, and availability of all relevant patient information in the departmental database. A written informed consent form for the use of clinical samples and data was obtained from each patient as required by the Institutional Review Board of Nagoya University.

Since 2006, adjuvant chemotherapy using S-1 has been used to treat all patients with UICC stage II–III GC unless contraindicated by the patient's condition.[20] We designed a two-step evaluation protocol of the diagnostic and predictive value of *SYT8* expression. Using a table of random numbers, 340 patients were allocated in a 1:2 ratio to the discovery and validation sets. An optimal cutoff of the *SYT8* expression level for peritoneal metastasis was determined using the

discovery set, and the diagnostic and predictive value of *SYT8* expression was subsequently evaluated in the validation set.

Expression Analysis of Primary GC Tissues

SYT8 mRNA levels of triplicate technical replicates were determined using a quantitative real-time reverse-transcription PCR (qRT-PCR) assay performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). The *SYT8* primers were as follows: sense 5′-GCTTCTCTCTCCGGTACGTG-3′ and antisense 5′-AGGAAGGTGAAGGCCTCATT-3′. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA served as an endogenous control. The expression level of *SYT8* mRNA in each sample is shown as the value of *SYT8* divided by that of *GAPDH*.

Immunohistochemical analysis of the in situ localization and expression patterns of SYT8 was performed using a rabbit polyclonal antibody raised against SYT8 (LS-C161657, LifeSpan Biosciences, Seattle, WA) diluted 1:150 in antibody diluent. We analyzed 54 representative formalin-fixed, paraffin-embedded sections of well-preserved tissues as described previously.[21] SYT8 protein expression was graded depending on the percentage of stained cells at the cancerous components as follows: no staining, minimal (<25 %), focal (25–50 %), and abundant (>50 %). No staining and minimal were categorized as negative SYT8, and focal and abundant were categorized as positive SYT8.[15, 22] To avoid subjectivity, the specimens were randomized and coded before analysis by two independent observers uninformed about the nature of the samples. Each observer

evaluated all specimens (positive or negative SYT8 at the GC component) at least twice within a given time interval to minimize intraobserver variation.

Effects of Inhibiting *SYT8* Expression on the Phenotype and the 5-Fluorouracil (5-FU) Sensitivity of GC Cells

Ten GC cell lines were used in this study. MKN1, MKN45, MKN74, NUGC2, NUGC3, NUGC4 and SC-6-JCK were obtained from the Japanese Collection of Research Bio Resources Cell Bank (JCRB, Osaka, Japan), and AGS, KATOIII and N87 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A control, non-tumorigenic epithelial cell line (FHs74) was purchased from ATCC. After qRT-PCR assay for SYT8 mRNA expression in all cell lines, MKN45, which has been broadly used in investigations on peritoneal metastasis of GC, and MKN1 were selected for functional analyses. Cells were cultured in a 24-well plate (5×10^4 cells/mL) and transiently transfected with either of 30 nM of two siRNAs specific for *SYT8* (A-019166-13 and A-019166-15, Accell siRNA, GE Healthcare Dharmacon Inc., Lafayette, CO) or a control siRNA (siControl) (5′-GUACCUUGACAGUACCGAUdTdT-3′) and then incubated for 72 h. We evaluated the effects of inhibiting *SYT8* expression on cell proliferation, invasion, and migration as described previously.[23, 24] In brief, cell proliferation was evaluated using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cells (5×10^3 cells per well) were incubated and the optical density of the solution in each well was measured on days 1, 3 and 5 following the addition of 10 mL of Cell Counting Kit-8 solution. The ability of GC cells to

invade Matrigel was determined using BioCoat Matrigel invasion chambers (BD Biosciences, Bedford, MA) according to the manufacturer's protocol. Invading cells in eight randomly selected fields were counted using a microscope (200× magnification).[25] The migration ability of cells was evaluated using wound-healing assays. The width of the wound was measured at 100-mm intervals (20 measurements per well, 40× magnification).[25] To detect the influence of *SYT8* knockdown on the resistance of GC cells to 5-FU, MKN45 cells (5×10^3 per well, five wells for each condition) were treated for 72 h with 5-FU at final concentrations of 0 (control), 0.5, 4, 32, and 256 mg/L. Cell viability was measured using the Cell Counting Kit-8 (Dojindo Laboratories), and the cell viability ratio was defined as the absorbance at 450 nm of the sample divided by the absorbance of control.

Intraperitoneal Intervention using a Mouse Xenograft Model

MKN45-Luc cells (1×10^6) that stably expressed luciferase were transfected with si*SYT8*, siControl, or treated with glucose (vehicle) and implanted into the abdominal cavity of 12-week-old male BALBc$^{nu/nu}$ mice to analyze the peritoneal dissemination of the xenografts. Mice (n = 8 each) were intraperitoneally injected with 500 μL of vehicle, siControl (50 μg), or si*SYT8* (50 μg) twice weekly for 6 weeks after implantation. An in vivo Imaging System (IVIS) Lumina (Xenogen, Alameda, CA, USA) was employed to non-invasively measure the volumes of peritoneal metastasis 2, 4, and 6 weeks after cell implantation, and Living Image Ver.2.6 (Xenogen) software was used to acquire and analyze the data.[26, 27] All animal experiments described here were approved by the Animal Research Committee of Nagoya University.

Statistical Analysis

Goodness-of-fit was assessed by calculating the area under the curve (AUC) of the receiver operating characteristic (ROC) curve, and the optimal cutoff value was determined using the Youden index. The qualitative χ^2 and quantitative Mann–Whitney tests were used to compare the two groups. Survival rates were calculated using the Kaplan–Meier method, and the difference between curves was analyzed using the log-rank test. The univariate Cox proportional hazards model was used to evaluate the hazard ratio of peritoneal recurrence-free survival associated with each variable. Variables with $P<0.05$ were included in the multivariate analysis to identify independent factors. Statistical analysis was performed using JMP 10 software (SAS Institute Inc., NC). $P < 0.05$ indicates a statistically significant difference.

RESULTS

Evaluation of *SYT8* as a Specific Biomarker for Peritoneal Recurrence

Transcriptome analysis identified 14 genes that were expressed at significantly higher levels exclusively in the peritoneal recurrence group compared with the non-recurrence group (Table 1). We focused on *SYT8* here for the following reasons: 1) ranked among the highest five $\log_2$ ratios, 2) high specificity for peritoneal recurrence indicated by lack of increased expression in the hepatic and nodal recurrence groups, 3) no published data related to *SYT8* expression in GC, and 4) recognized as a transmembrane protein associated with the transport of growth factors and

anticancer drugs.

Optimal Cutoff Value

Patients were randomly allocated to discovery (n = 113) and validation (n = 227) sets. Except for age, there were no significant differences in demographics and clinical characteristics between the two sets (Supplemental Table 1). First, we used the discovery set to determine the optimal cutoff value of *SYT8* mRNA levels in primary GC tissues to detect and predict peritoneal metastasis using qRT-PCR assay. ROC curve analysis revealed that the AUC value of *SYT8* levels was 0.771 for detection of peritoneal metastasis or peritoneal recurrence within 2 years after surgery, and the optimal cutoff value was 0.0050 (sensitivity 67%, specificity 83%) (Fig. 1A). When we divided patients into the high (above the cutoff value) or low (below the cutoff value) *SYT8* groups, overall survival of the *SYT8*-high group was significantly lower in the *SYT8*-low group (5-year survival rates, 45% and 77%, respectively) (Fig. 1B)

Validation of the Cutoff Value and the Clinical Significance of *SYT8* Levels

The diagnostic and predictive values of *SYT8* mRNA levels were evaluated using the validation set. Similar levels of *SYT8* mRNA were detected in normal gastric tissues adjacent to stage I GC tissues (n = 71). The levels of *SYT8* expression were significantly higher in patients with stage II/III GC who experienced peritoneal recurrence following curative gastrectomy (n = 10) compared with those who did not (n = 91) (mean mRNA expression levels 0.0097 and 0.0049, respectively). In

patients harboring distant metastasis at the time of surgery (stage IV), patients with peritoneal metastasis (n = 41) had high *SYT8* levels in primary GC tissues compared with those without (n = 11) (mean mRNA expression levels 0.0114 and 0.0053, respectively; Fig. 2A). These results indicated that the elevated *SYT8* expression was characteristic of the primary GC tissues with the potential to metastasize to the peritoneal surface.

When we used this cutoff value to classify patients into high and low *SYT8* expression groups, high *SYT8* mRNA levels in the primary GC tissues were significantly associated with Borrmann type 4/5, T4 tumor, undifferentiated tumor, pathological invasive growth, and advanced disease stage (Table 2). High *SYT8* levels were associated with peritoneal metastasis but not with hepatic and distant lymph node metastasis. The Kaplan–Meier curve analysis revealed that the *SYT8*-high group to suffer from significantly lower overall survival (Fig. 2B). The primary lesions from the patients with stage II/III GC were then categorized into the *SYT8*-positive or negative groups based on immunohistochemical staining with *SYT8* (Fig. 2C). Incidence of peritoneal recurrence after gastrectomy was significantly higher in the *SYT8*-positive group compared with that of the *SYT8*-negative group (35% and 6%, respectively, $P = 0.006$) (Fig. 2C). A positive correlation was detected between the expression level of *SYT8* mRNA and the staining intensity of SYT8 in GC tissues (Supplemental Fig. 1A). These results indicated that immunohistochemical analysis of *SYT8* expression is also useful for predicting peritoneal recurrence.

Subgroup Analyses according to Disease Stage and Adjuvant Chemotherapy

To further evaluate the performance of *SYT8* in predicting disease recurrences, we conducted a subgroup analysis of 104 patients with stage II/III GC who underwent curative gastrectomy. The overall survival of the *SYT8*-high group was significantly lower compared with that of the *SYT8*-low group (Supplemental Fig. 1B), although difference in progression-free survival was marginal (Supplemental Fig. 1C). The cumulative incidence of peritoneal recurrence was significantly higher in the *SYT8*-high group compared with the *SYT8*-low group (33% and 4%, respectively, $P < 0.003$) (Fig. 3A). Multivariable Cox proportional hazards model analysis revealed that *SYT8*-high expression in GC tissues had the largest hazard ratio and was identified as an independent prognostic factor for peritoneal recurrence-free survival (hazard ratio, 8.81; 95% confidence interval, 2.36–42.0; $P = 0.001$) (Table 3).

Influence of the SYT8 expression on survival depended on whether the patients received postoperative adjuvant chemotherapy or not. For 45 patients who underwent surgery alone, the prognosis was similar regardless of whether the cancer tissue was SYT8-high or SYT8-low, the 5-year survival rates for each group being 56% and 58%, respectively, $P = 0.514$ (Fig. 3B). In striking contrast, there was a significant difference between the *SYT8*-high and *SYT8*-low groups (56% and 80%, $P = 0.005$) among 59 patients who received adjuvant chemotherapy following curative gastrectomy (Fig. 3B). These results suggest that *SYT8* expression reflects resistance to adjuvant chemotherapy as well as a propensity to form peritoneal metastasis.

Effects of Inhibiting *SYT8* Expression on the Phenotype and 5-FU resistance of GC Cells

Expression levels of *SYT8* mRNA were heterogeneous among GC cell lines, whereas the control FHs74 cell had a low *SYT8* mRNA level (Supplemental Fig. 2A). We conducted experiments using MKN45 and MKN1, which had relatively high *SYT8* mRNA levels, to determine the molecular basis of *SYT8* expression associated with the emergence of peritoneal metastasis. For this purpose, we transfected MKN45 and MKN1 cells with *SYT8*-siRNA and found that although the proliferation rate was not altered, knockdown of *SYT8* significantly decreased cell invasion and migration compared with controls (Fig. 4AB and Supplemental Fig. 2B for MKN45,and Supplemental Fig 3 for MKN1). Cell viability was evaluated to investigate the influence of *SYT8* level on the drug sensitivity of GC cells to 5-FU. Dose-dependent decreases in cell viability were observed for si*SYT8*-transfected and control cells, and si*SYT8*-transfected cells exhibited increased sensitivity to 5-FU compared with the controls (Fig. 4C).

Therapeutic Effect of Intraperitoneal Administration of si*SYT8* on Mice Injected with MKN45 Cells

MKN45-Luc cells were transfected with vehicle, siControl or si*SYT8* and implanted to the mice. To evaluate the potential therapeutic effects of inhibiting *SYT8* expression, either vehicle, siControl or si*SYT8* was intraperitoneally injected twice weekly for 6 weeks to mice inoculated with MKN45-Luc cells. In the vehicle and siControl groups, the photon flux detected by the IVIS

gradually increased in all mice, indicating the growth of intraperitoneal tumors. In contrast, photon flux was constant during treatment of the si*SYT8* group (Fig. 5A). No significant signs of toxicity were observed that accompanied the intraperitoneal administration of siRNAs. Loss of body weight during treatment was lower in the si*SYT8* group (Fig. 5B). Mice treated with si*SYT8* survived significantly longer compared with those administered vehicle (median survival times, 61 and 108 days, respectively, $P = 0.025$) (Fig. 5C).

DISCUSSION

Peritoneal metastasis remains incurable for the following reasons: 1) difficulty in early detection using imaging studies, 2) difficulty in complete resection including the micrometastases, 3) poor radiosensitivity, and 4) limited efficacy of systemic chemotherapy because of poor drug delivery to the peritoneal surface. [14, 28, 29] Therefore, novel biomarkers specific for peritoneal metastasis of GC, particularly predictive markers, are required to implement adequate risk assessment and early delivery of effective cytotoxic therapy. Furthermore, identification of a novel molecular target will likely contribute to efforts to develop and implement effective therapeutic strategies. The transcriptome analysis of recurrence pattern-specific molecules performed here identified *SYT8* as a candidate biomarker associated with peritoneal metastasis of GC. Synaptotagmins are transmembrane proteins that mediate neurotransmission and hormone secretion, which are involved in regulated exocytosis.[30, 31] *SYT8* contributes to the trafficking and exocytosis of secretory vesicles in non-neuronal tissues, and *SYT8* expression in human pancreatic islets is associated with the

activity of the promoter of the insulin gene.[31-33] It remains to be determined if *SYT8* is oncogenic.

To assess whether the *SYT8* levels in GC tissues served as a diagnostic and predictive marker for peritoneal metastasis of GC, we determined an optimal cutoff value for this purpose by analyzing *SYT8* mRNA expression in two independent patient cohorts. First, we found a close association in the discovery set between *SYT8* levels and peritoneal metastasis/recurrence (AUC, 0.771), and the optimal cutoff value was defined. We next determined the reproducibility of results through further analyses using the validation set. *SYT8* expression levels in primary GC tissues were significantly higher in patients with peritoneal recurrence or metastasis compared with those without, indicating that evaluation of *SYT8* levels identified patients who likely suffered from synchronous and metachronous peritoneal metastasis. Elevated *SYT8* expression was significantly associated with the presence of peritoneal metastasis and risk factors such as Borrmann type 4/5, pT4, undifferentiated tumor, and pathological invasive growth. These findings support the hypothesis that *SYT8* expression reflects the potential of primary tumor cells to metastasize to the peritoneal cavity. After confirming the reproducibility of the data of the validation set, we then conducted a subgroup analysis of patients with stage II/III GC to evaluate the significance of *SYT8* expression for predicting peritoneal recurrences after curative gastrectomy. Our compelling findings provide support for the use of *SYT8* expression as a novel diagnostic and predictive biomarker for peritoneal metastasis in patients with GC and therefore may be useful for preoperative staging, disease monitoring, and selection of optimal multimodal management strategies.

Immunohistochemistry is a versatile methods to evaluate in situ protein expression, because the

procedure is simple, and stably archived formalin-fixed paraffin-embedded samples are often available.[34] Our results indicate that immunohistochemistry can serve as an alternative method for predicting peritoneal recurrences in stage II/III GC and may enable physicians to stratify patients at risk for peritoneal metastasis at the time of endoscopic biopsy or surgical resection of the primary GC.

We were motivated by our encouraging expression analyses to uncover the underlying molecular mechanisms of the contribution of *SYT8* in the peritoneal metastasis of GC. In vitro studies revealed that inhibition of *SYT8* expression inhibited the migration and invasive properties of GC cell lines, which are important for free GC cells present in the peritoneum to adhere to the distant peritoneum and form nodules by invading the sub-peritoneal space.[35-37] When we next used a mouse xenograft model, we found that intraperitoneal administration of *SYT8*-siRNA significantly reduced the loss of body weight, inhibited the growth of peritoneal tumors, and prolonged survival. These findings support the conclusion that *SYT8* serves as a biomarker as well as a therapeutic target for peritoneal metastasis of GC. However, inhibition of the *SYT8* expression did not significantly affect cell proliferation. Therefore, inhibition of *SYT8* expression alone may be insufficient to eliminate disseminated GC cells, even if attachment of free cancer cells to the peritoneal tissue could be inhibited. Intraperitoneal paclitaxel shows promise for controlling peritoneal metastasis of GC.[38, 39] Further, Ishigami et al. conducted a phase II study to evaluate the efficacy and tolerability of weekly intravenous and intraperitoneal paclitaxel combined with S-1 in patients with peritoneal metastasis of GC.[28] They found that the regimen is safe with median

survival time of 22.5 months. Together, these findings support the hypothesis that intraperitoneal administration of *SYT8* inhibitors combined with cytotoxic anticancer drugs will provide an efficient strategy for treating peritoneal metastasis.[38, 39]

In our transcriptome data, *SYT8* was specifically overexpressed in patients with peritoneal recurrences even after adjuvant treatment with S-1. Thus, we hypothesized that *SYT8* plays a role in the resistance to FU-based anticancer drugs. Survival analysis of a subset according to the administration of FU-based postoperative adjuvant therapy revealed a significant improvement in overall survival of the *SYT8*-low group. In marked contrast, adjuvant therapy had little survival benefit for the *SYT8*-high group, suggesting that *SYT8* affected the therapeutic effect of FU on micrometastasis in some way. To support this clinical observation, we evaluated the influence of *SYT8* on the resistance of GC cells to 5-FU and found that knockdown of *SYT8* expression increased sensitivity to 5-FU. Postoperative adjuvant chemotherapy using S-1 monotherapy or XELOX for patients with stage II/III leaves room for improvement to prevent tumor recurrence.[2, 3] Therefore, *SYT8* may serve as a biomarker to select patients eligible for aggressive postoperative adjuvant chemotherapy or perioperative chemotherapy.

The present study includes some limitations. Transcriptome data was available only in 16 patients. Another limitation of this study is its retrospective design. A prospective large-scale observational study will be required for translation to the clinic, although here we employed a two-step evaluation. Particularly, statistical power might be insufficient due to limited number of patients in subgroup analyses according to disease stage and adjuvant chemotherapy. The value of

SYT8 expression for the prognosis of peritoneal metastasis will likely be enhanced by the development of assays to detect *SYT8* expression in serum samples that will facilitate continuous monitoring. To develop *SYT8*-targeted therapy, the dose, frequency, route, and length of administration of si*SYT8* as well as selection of cytotoxic agents for combination therapy should be optimized.

CONCLUSION

SYT8 is a promising diagnostic and predictive biomarker for peritoneal metastasis of GC and may affect sensitivity of gastric cancer cells to 5FU.

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Figures

Figure 1. Determination of the optimal cutoff value of *SYT8* mRNA expression levels using discovery set. (A) ROC curve analysis of *SYT8* expression levels that predict peritoneal metastasis. (B) Overall survival of patients with high *SYT8* expression levels categorized according to the cutoff value was significantly shorter compared with patients with low *SYT8* expression levels.

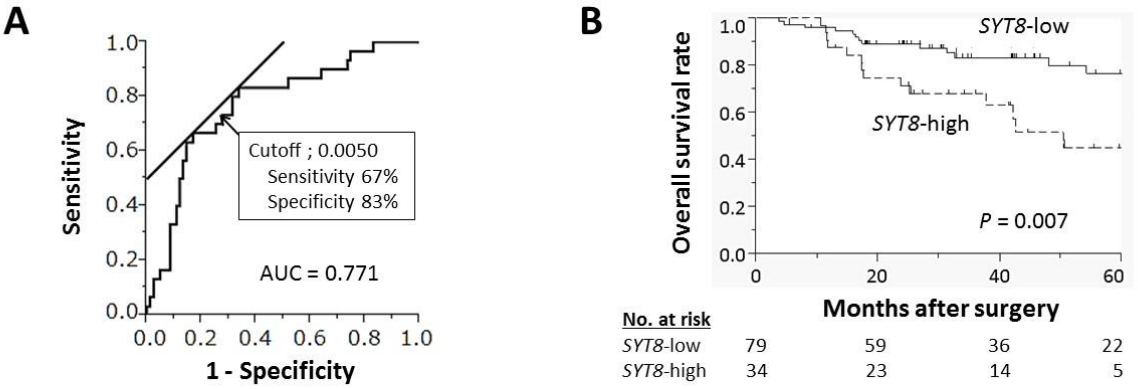


Figure 2. Evaluation of *SYT8* levels in the validation set. (A) The levels of *SYT8* mRNA in corresponding adjacent non-cancerous and GC tissues according to disease stage and the presence or absence of peritoneal recurrence/metastasis. GC, gastric cancer; P-rec, peritoneal recurrence; P/CY, peritoneal metastasis or positive cytology; AUC, area under the curve. (B) The *SYT8*-high group was more likely to have a worse prognosis compared with that of the *SYT8*-low group. (C) Representative results of immunohistochemistry analysis of *SYT8*. Upper and middle panels, *SYT8* expression in the cancerous component. Lower panel, tissue section with undetectable *SYT8* expression. The prevalence of peritoneal recurrences was significantly higher in patients with *SYT8* expression compared with those with undetectable *SYT8* expression. P-rec, peritoneal recurrence.

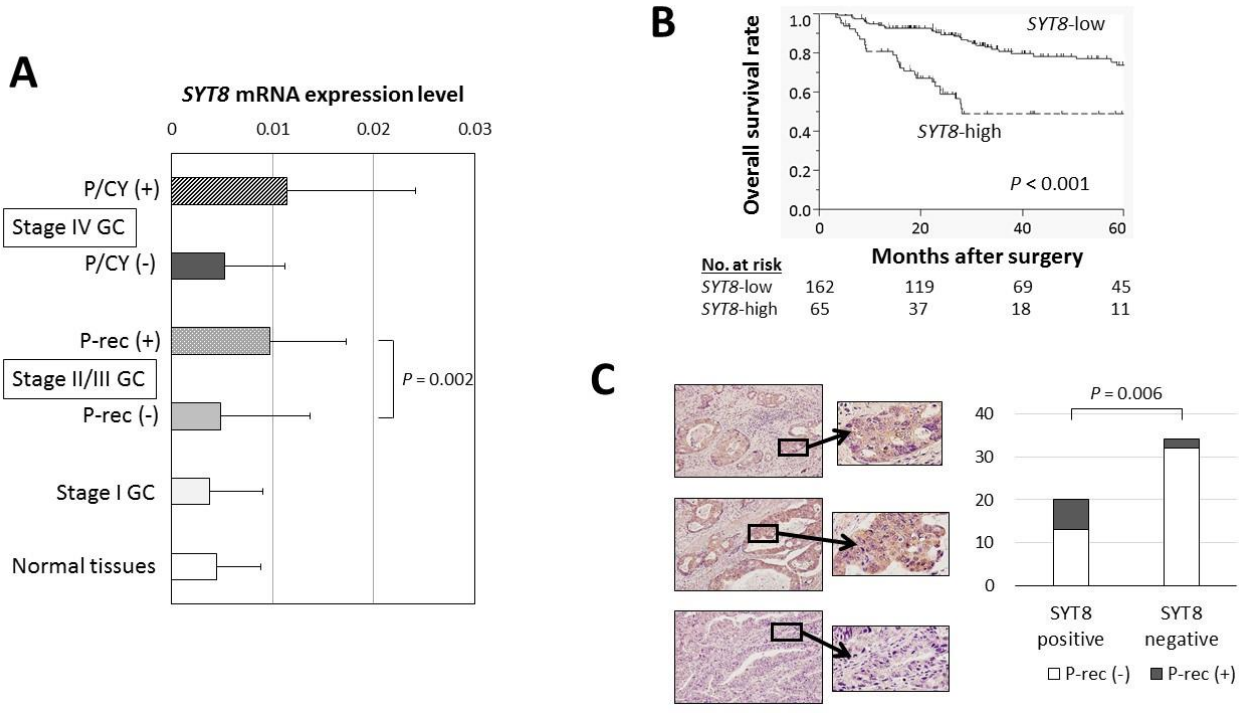


Figure 3. Predictive significance of *SYT8* expression in patients with stage II/III GC. (A) The cumulative incidence of peritoneal recurrences was significantly higher in the *SYT8*-high group. (B) Overall survival rates in subgroups according to administration of adjuvant chemotherapy.

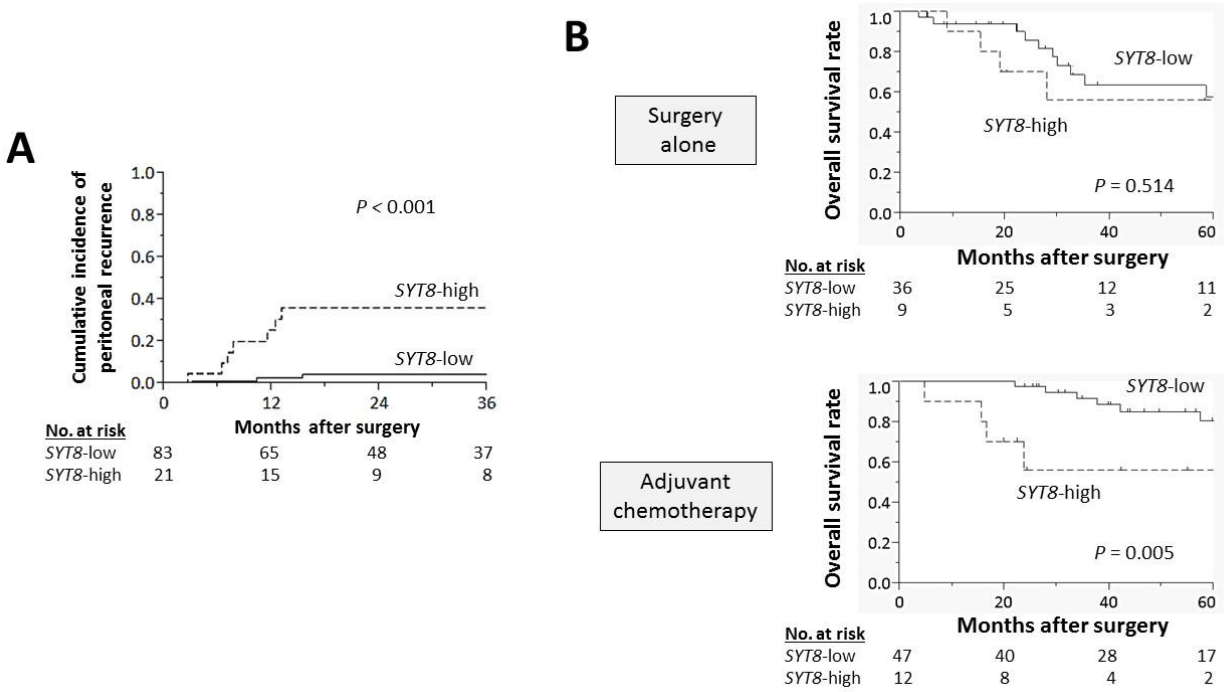


Figure 4. Effect of inhibition of *SYT8* on MKN45 cell phenotype and sensitivity to fluorouracil (5-FU). siRNA-mediated knockdown of *SYT8* significantly decreased cell migration (A) and invasion (B). (C) Effect of 5-FU concentration on cell viability. Knockdown of *SYT8* significantly decreased cell viability in the presence of 0.5 mg/L and 4 mg/L 5-FU administrations. **P* < 0.05.

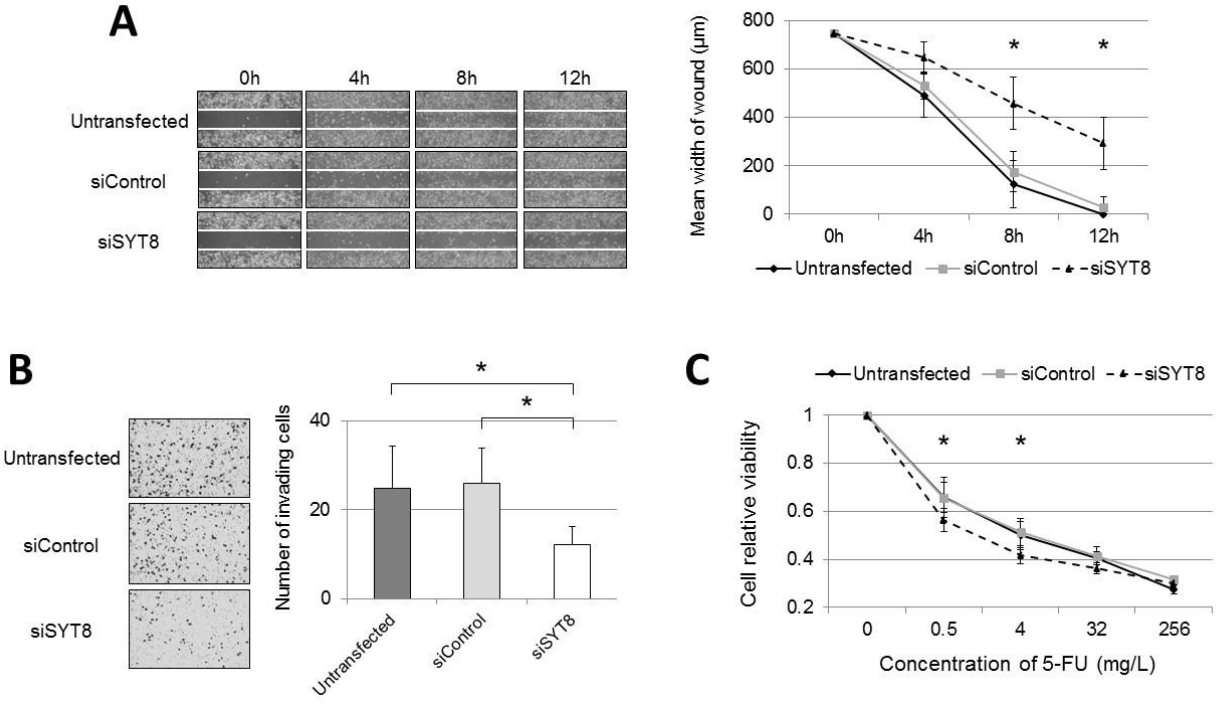
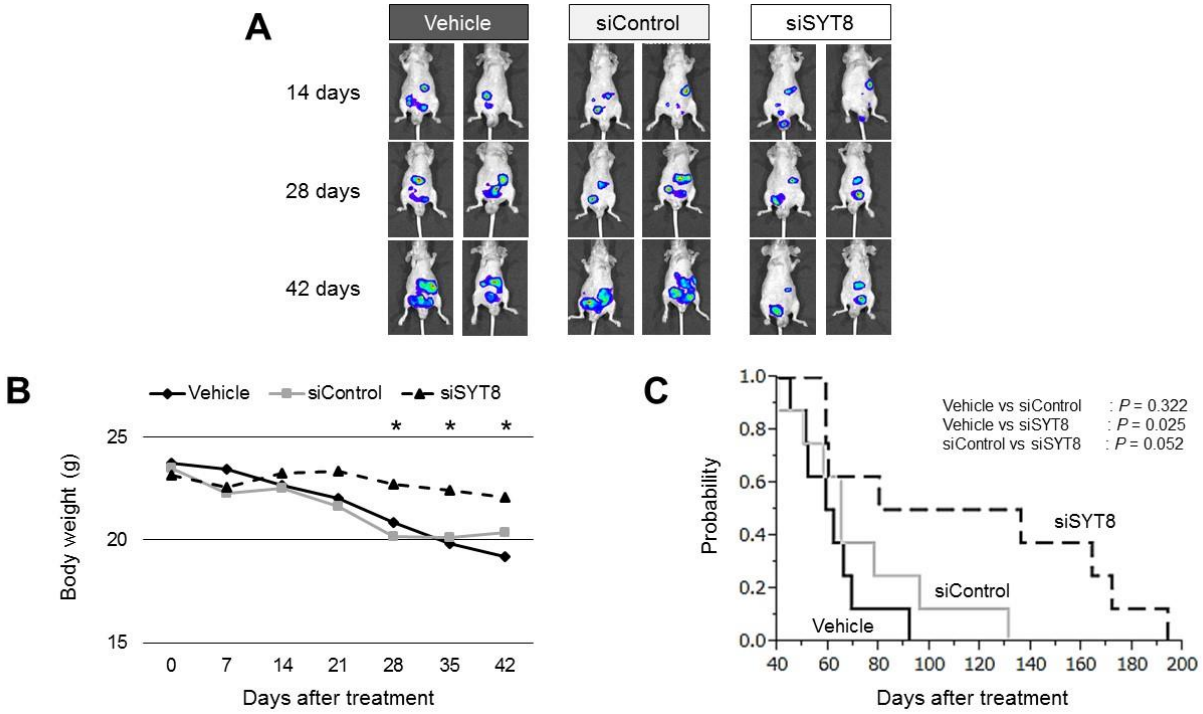
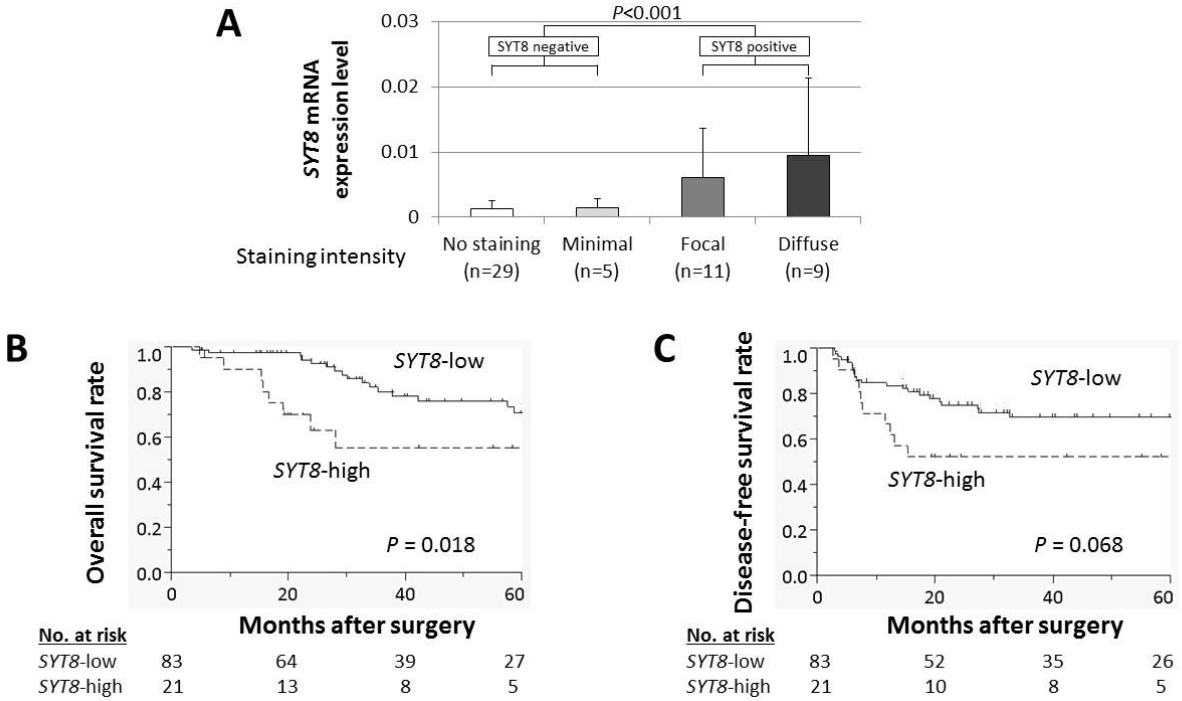


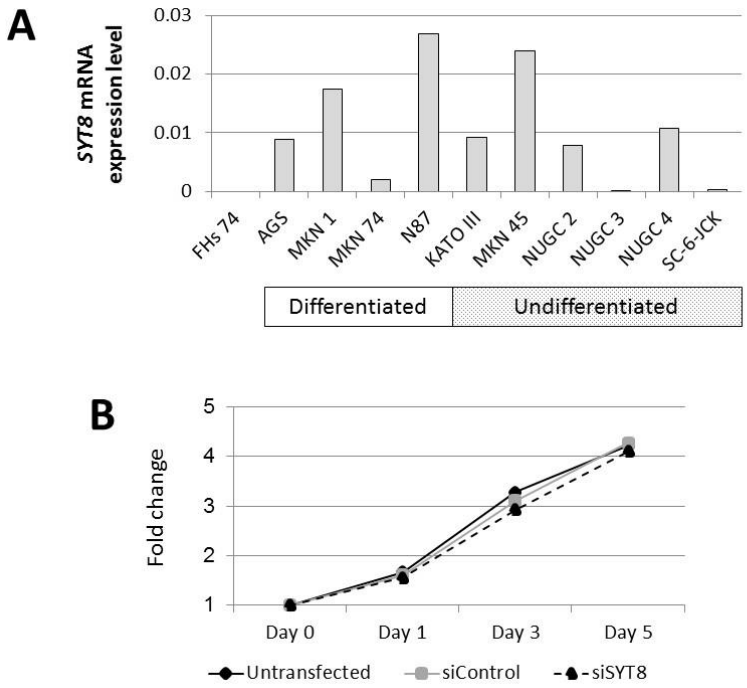
Figure 5. Therapeutic effects of intraperitoneal administration of an *SYT8*-specific siRNA. (A) IVIS analysis of representative mice engrafted with MKN45-Luc cells in each treatment group. (B) Changes in the body weight during the treatment. *$P < 0.05$, vehicle vs si*SYT8* groups. (C) Survival curves of mice administered vehicle, siControl, or si*SYT8*.



Supplemental Figure 1. (A) Expression levels of *SYT8* mRNA in GC tissues according to each staining intensity of SYT8 protein. (B) Overall survival of patients with stage II/III GC. (C) Recurrence-free survival of patients with stage II/III GC.



Supplemental Figure 2. (A) *SYT8* mRNA expression levels in GC cell lines and the control FHs74 cell analyzed using qRT-PCR. (B) Effect of inhibition of *SYT8* on proliferation ability of MKN45 cell.



Supplemental Figure 3. Effect of inhibition of *SYT8* on MKN1 cell phenotype. *$P < 0.05$.

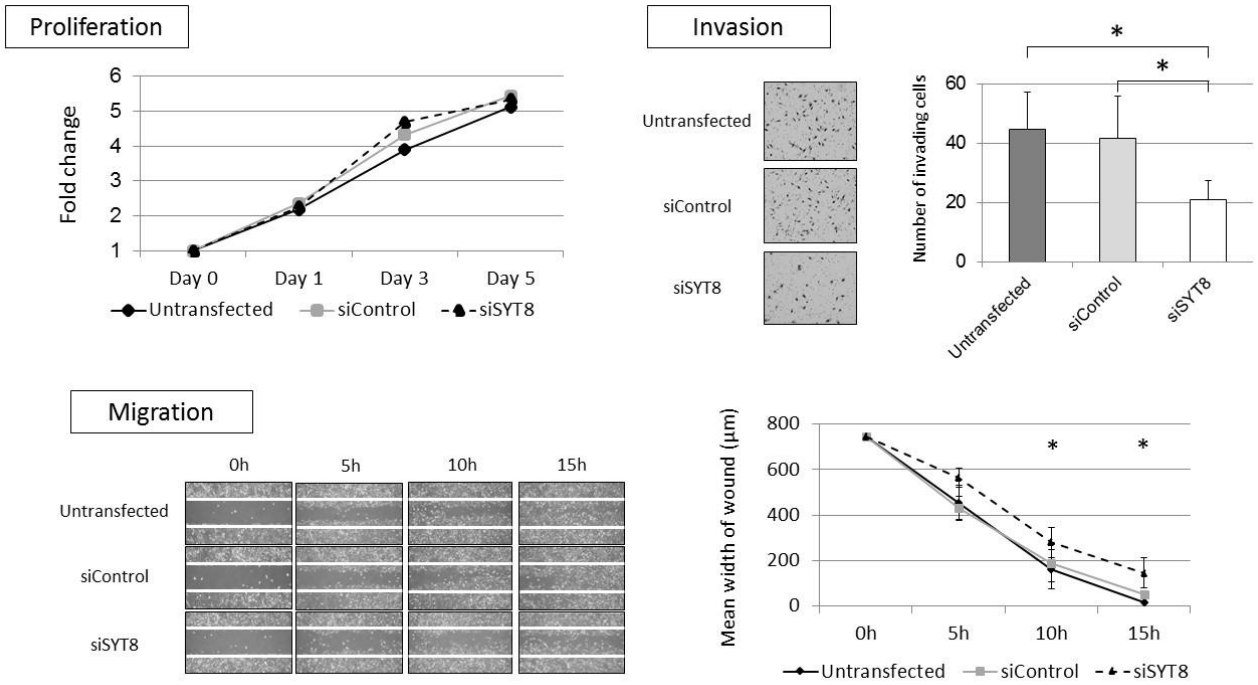


TABLE 1. List of candidate genes expressed at higher levels in gastric cancer tissues specifically in patients with peritoneal recurrences

Symbol	P-rec / Non-rec		Full name	Location	Function	H-rec / Non-rec	N-rec / Non-rec
	Log_2	*P*				Log_2	Log_2
FGFR2	5.97	<0.0001	fibroblast growth factor receptor 2	10q26	Fibroblast growth factor receptor	-0.34	0.18
TNNI2	4.88	<0.0001	troponin I type 2	11p15.5	Skeletal muscle protein	0.33	0.16
CALB2	3.74	0.0001	calbindin 2	16q22.2	Intracellular signal transducer	-0.35	-0.52
DUSP2	3.31	0.0003	dual specificity phosphatase 2	2q11	Intracellular signal transducer	-0.85	0.30
SYT8	3.25	<0.0001	synaptotagmin VIII	11p15.5	Membrane trafficking protein	-0.42	-0.46
COL28A1	2.84	<0.0001	collagen, type XXVIII, alpha 1	7p21.3	Collagen	-0.50	0.64
MSLN	2.50	<0.0001	mesothelin	16p13.3	Cell adhesion factor	1.35	1.47
SYT13	2.48	<0.0001	synaptotagmin XIII	11p15.5	Membrane trafficking protein	-0.10	1.68
DSG3	2.48	0.0003	desmoglein 3	18q12.1	Cell-cell junction protein	-1.29	1.62
IDO1	2.39	0.0003	indoleamine 2,3-dioxygenase 1	8p12-p11	Metabolic enzyme	1.93	0.74
CKS2	2.37	0.0003	CDC28 protein kinase regulatory subunit 2	9q22	Cell cycle modulator	1.01	1.50
NUPR1	2.20	0.0003	nuclear protein, transcriptional regulator, 1	16p11.2	Transcription factor	-0.07	1.82
VNN1	2.15	0.0001	vanin 1	6q23-q24	Hematopoietic cell trafficking	-1.26	-1.33
KLK10	2.12	0.0001	kallikrein-related peptidase 10	19q13	Metabolic enzyme	-0.13	1.11

P-rec, peritoneal recurrence group; Non-rec, no recurrence group; H-rec, hepatic recurrence group; N-rec, nodal recurrence group.

TABLE 2. Association between *SYT8* mRNA levels and clinicopathological characteristics of patients included in the validation set

Variables	**High *SYT8* (n=65)**	**Low *SYT8* (n=162)**	***P***
Age			
< 65 year	32	73	0.596
≥ 65 year	33	88	
Sex			
Male	42	125	0.057
Female	23	37	
CEA (ng/ml)			
≤ 5	49	137	0.112
> 5	16	25	
CA19-9 (IU/ml)			
≤ 37	45	134	0.028
> 37	20	28	
Tumor location			
Entire	9	10	
Upper third	17	35	0.028
Middle third	12	59	
Lower third	27	58	
Tumor size (mm)			
< 50	23	86	0.015
≥ 50	42	76	
Macroscopic type			
Borrmann type 4/5	18	11	<0.001
Others	47	151	
Tumor depth (UICC)			
pT1-3	25	111	<0.001
pT4	40	51	
Differentiation			
Differentiated	21	81	0.014
Undifferentiated	44	81	
Lymphatic involvement			
Absent	8	33	0.141
Present	57	129	
Vessel invasion			
Absent	22	74	0.100
Present	43	88	
Infiltrative growth type			<0.001

Invasive growth	37	38	
Expansive growth	28	124	
Regional lymph node metastasis			
Absent	18	73	0.014
Present	47	89	
Peritoneal metastasis / cytology			
Negative	34	149	<0.001
Positive	31	13	
Synchronous liver metastasis			
Absent	61	158	0.195
Present	4	4	
Distant lymph node metastasis			
Absent	64	161	0.523
Present	1	1	
UICC stage			
I	11	60	
II	6	32	<0.001
III	15	51	
IV	33	19	

*Statistically significant ($P < 0.05$). *Abbreviations*: *CEA*, carcinoembryonic antigen; *CA19-9*, carbohydrate antigen 19-9; *UICC*, Union for International Cancer Control.

TABLE 3. Univariate and multivariable analysis of peritoneal recurrence-free survival in 104 patients with stage II/III gastric cancer

Variables	Univariate			Multivariable*		
	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
Age (≥ 65)	0.47	0.10–1.69	0.254			
Gender (female)	1.20	0.26–4.31	0.797			
CEA (> 5 ng/ml)	1.05	0.20–19.4	0.965			
CA19-9 (> 37 IU/ml)	1.55	0.33–5.57	0.541			
Tumor location (lower third)	0.66	0.10–2.62	0.578			
Tumor size (≥ 50 mm)	1.02	0.29–3.98	0.977			
Multifocal lesions	1.42	0.08–7.55	0.752			
Tumor depth (pT4, UICC)	1.96	0.56–7.68	0.289			
Tumor differentiation (undifferentiated)	1.32	0.37–6.11	0.686			
Lymphatic involvement	1.69	0.09–8.99	0.643			
Vessel invasion	1.06	0.29–4.91	0.936			
Invasive growth	3.05	0.87–11.9	0.081			
Lymph node metastasis	2.69	0.51–49.6	0.284			
UICC stage III	6.08	1.14–112	0.043	4.90	0.76–96.0	0.101
Adjuvant chemotherapy	0.63	0.17–2.26	0.466			
High *SYT8*	10.0	2.78–46.6	<0.001	8.81	2.36–42.0	0.001

Abbreviations: CI, confidence interval; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; UICC, Union for International Cancer Control. *Adjusted by age and gender.

Supplementary TABLE 1. Characteristics of patients in the discovery and validation sets

		Discovery set (n=113)	Validation set (n=227)	*P*-value
Age (years), mean ± SD		65.0 ± 12.5	64.5 ± 12.6	0.012
Sex (male/female)		86 / 27	167 / 60	0.612
Tumor location	Upper third	20	52	0.187
	Middle third	27	71	
	Lower third	54	85	
	Entire	12	19	
Type of resection				0.914
Total gastrectomy		40	79	
Partial gastrectomy		73	148	
UICC pStage	I	39	71	0.674
	II	19	38	
	III	26	66	
	IV	29	52	
Adjuvant chemotherapy*		53%	57%	0.702
Follow up months, mean ± SD		43.9 ± 28.4	48.2 ± 33.6	0.770

SD, standard deviation. *Patients with stage II/III gastric cancer.