Expression profiling of CPS1 in Correa's cascade and its association with gastric cancer prognosis

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Abstract. Carbamoyl phosphate synthetase 1 (CPS1), which is the antigen for the hepatocyte paraffin 1 antibody, exhibits focal immunoreactivity in adenocarcinoma from the gastrointestinal tract, but its expression profiles and roles in gastric cancer (GC) remain largely unknown. The present study aimed to determine the expression pattern and prognostic value of CPS1 in Correa's cascade using tissues from 32 patients with chronic atrophic gastritis with intestinal metaplasia (IM), 62 patients with low- or high-grade intraepithelial neoplasia (IN) and 401 patients with GC. The expression of CPS1 was diffuse and strongly positive in 32 cases (100%) of IM of the glandular epithelium, and gradually downregulated in Correa's cascade, with a strongly positive ratio of 21 (70%) in low-grade IN and 4 (12.5%) in high-grade IN. The levels of CPS1 expression were significantly higher in diffuse-type GC, with 37 (26%) cases strongly positive for CPS1, compared with 14 (8%) in intestinal-type and 11 (13%) cases in mixed-type GC. In intestinal-type GC, CPS1 expression was completely lost in 107 (62%) of cases, which was associated with an advanced Tumor-Node-Metastasis stage (P=0.031) and depth of invasion (P=0.037). Kaplan-Meier analysis suggested that low CPS1 expression levels were independently associated with a short overall survival (OS) time in the three types of GC (P<0.001 in intestinal-type, P=0.003 in diffuse-type and P=0.018 in mixed-type GC). Furthermore, low levels of CPS1 mRNA and high methylation levels in the CPS1 promoter were associated with a short OS time in patients with GC. These results suggested that the expression of CPS1 was progressively downregulated in Correa's cascade, and that CPS1 may serve as a prognostic marker for patients with GC, regardless of tumor type.

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Introduction

Carbamoyl phosphate synthetase 1 (CPS1) is a rate-limiting enzyme of the urea cycle (1). In the mitochondria, CPS1 produces carbamoyl phosphate from ammonia, which initiates nitrogen disposal (2). CPS1 is the antigen of the hepatocyte paraffin 1 (Hep-Par 1) antibody, a specific marker of hepatogenic differentiation used in clinical pathology (3). CPS1 has also been identified as a diagnostic marker for intestinal metaplasia (IM) in Barrett's esophagus (BE), as well as IM associated with chronic gastritis (4). Ocak *et al* (5) have reported that the level of CPS1 gradually decreases in incomplete types of IM, suggesting that CPS1 may be used as a diagnostic aid in IM subtype classification. CPS1 is highly expressed in IM of the stomach (4), and is thus a sensitive indicator of small intestine differentiation. CPS1 in expressed in normal small intestinal, but not colonic mucosa (6). Notably, CPS1 expression is completely lost in invasive small intestinal adenocarcinoma, but gained in colorectal polyps with dysplasia (7). Nemolato *et al* (8) have demonstrated that CPS1 immunoreactivity in human colon carcinogenesis is associated with the progression from low- to high-grade dysplasia and adenocarcinoma. A study by Abu-Zeid and Farid (6) also supports the concept that CPS1 serves an active role in the initiation of dysplasia and the progression of multistep colorectal carcinogenesis. However, CPS1 may also serve a suppressive role in tumor invasion and dissemination, as its expression is inversely associated with a number of conventional prognostic parameters, including tumor type, grade and lymph node metastasis (6). Similar to its role in colorectal cancer, CPS1 is highly expressed in dysplasia and BE, with low expression levels in esophageal adenocarcinoma (9). These observations suggest that CPS1 expression may be down- or upregulated at different stages of dysplasia, independent of cancer type, although it tends to be downregulated in advanced tumors, at least in small intestinal, colorectal and liver cancer as well as

esophageal adenocarcinoma (10). This may be due to the poor differentiation of invasive cancer.

By contrast, a number of studies have reported that CPS1 is upregulated during carcinogenesis. For example, in rectal cancer, Lee *et al* (11) have demonstrated that high levels of CPS1 are associated with poor therapeutic responses and adverse outcomes in patients receiving neoadjuvant concurrent chemo-radiotherapy. In addition, two independent studies have revealed that the levels of CPS1 are upregulated in lung adenocarcinoma compared with those in adjacent non-cancerous tissues (12,13), and that CPS1 knockdown results in a reduction in cell proliferation and the levels of metabolites associated with the cross-compartmental pathway of pyrimidine metabolism (14). Li *et al* (15) have demonstrated that P53 inhibits the occurrence of uremia and the clearance of ammonia by downregulating CPS1 transcription. Thus, during carcinogenesis, the frequently lost function of p53 results in the upregulation of CPS1 and is associated with poor prognosis (15). These observations suggest a potential role for CPS1 in the occurrence of malignancy and cell transformation. In gastric cancer (GC), CPS1 has been reported to be associated with a subtype of hepatoid adenocarcinomas with diffuse positive expression (1). Although CPS1 is highly expressed in IM-associated chronic gastritis, its role in GC development and progression is largely unknown. Therefore, the present study aimed to assess the levels of CPS1 expression in Correa's cascade and to evaluate its prognostic value in patients with GC.

Materials and methods

Patient recruitment. Pre-carcinoma gastric mucosa tissues were obtained from patients who underwent routine gastroscopic biopsy examination at Ruijin Hospital (Shanghai, China) between January 2018 and January 2020. Of these patients, 10 were diagnosed with chronic non-atrophic gastritis, 32 with chronic atrophic gastritis with IM, 30 with low-grade intraepithelial neoplasia (IN) and 32 with high-grade IN. According to mucin expression patterns, the cases were classified as IM type I or II. All recruited patients were receiving gastroscopic examinations for the first time. Patients were excluded if they had received proton pump inhibitor therapy at the time of biopsy or had a history of other malignancies. The tissues were routinely fixed at room temperature overnight in 10% neutral formalin, embedded in paraffin and stored at room temperature until use. The study was approved by the Ruijin Hospital Ethics Committee, and patients with precancerous lesions provided written consent to use their medical information and biological samples.

Data from patients with GC recruited at the Nantong Tumor Hospital (Nantong, China) between December 2000 and April 2005 were also included. The majority of data from these patients have been reported in a previous study (16). The patients were diagnosed with GC for the first time and had not previously received treatment. Patients were excluded if they had a history of other malignancies or had previously received chemotherapy or surgery. All cases were confirmed by pathological examination after surgery, and baseline personal information was obtained from admission records. Clinicopathological data were extracted from medical records and further assessed by clinicians based on laboratory tests and image evaluation. A total of 401 patients met the full inclusion criteria, and the latest follow-up data were available until April 2014. All patients with GC had provided written consent to use their medical information and biological samples, and the study was approved by the Ethics Committees of Ruijin Hospital and Nantong Tumor Hospital.

Tissue microarray (TMA) construction and immunohistochemical staining of CPS1. TMAs were constructed using histologically confirmed formalin-fixed, paraffin-embedded gastric mucosa, GC and adjacent normal gastric tissue samples by the National Engineering Center for Biochip at Shanghai. All samples used for TMA construction were obtained from Nantong Tumor Hospital. Immunohistochemistry was performed using a commercially available anti-CPS1 antibody (cat. no. ab128942; 1:200; Abcam), and CPS1 expression was evaluated using the Leica Bond III full-automatic immunostainer (Leica Microsystems GmbH). The sections were incubated with a peroxidase-polymer labeled rabbit secondary antibody (cat. no. PV-6001; 1:500; OriGene Technologies, Inc.) at room temperature for 1 h. Heat-induced epitope retrieval was performed using citrate buffer solution, followed by visualization of the antigen using a DAB detection kit (cat. no. ab64238; Abcam). The slides were counterstained with hematoxylin for 20 min at room temperature. Concurrently immunostained slides containing liver tissue known to exhibit a positive reaction with CPS1 antibody were selected as positive controls.

CPS1 is a mitochondrial protein that exhibits discrete granular cytoplasmic staining (9), which was assessed based on the staining intensity and distribution in positive cells. The staining intensity was scored between 0 and 3 as follows: No staining, 1; faint staining, 1; moderate staining, 2; and dark staining, 3; and the percentage of positive cells was scored as follows: No positive cells, 0; ≤25%, 1; 25-49%, 2; 50-75%, 3; and >75%, 4. The immunoreactive score (IRS) was determined as the staining density multiplied by the percentage of positively stained cells. The samples were then divided into four categories according to these scores: i) Strong positive, IRS, 8-12; ii) focal positive, IRS, 4-6; iii) weak positive, IRS, 2-3; and iv) negative, IRS, 0-1. Samples with an IRS score of 2-12 were assigned to the CPS1-high group, whereas those with an IRS score of 0 or 1 were assigned to the CPS1-low group.

Bioinformatics analysis. The Kaplan-Meier plotter tool (https://kmplot.com/analysis/) was used to evaluate the association between CPS1 mRNA expression levels (low vs. high) at best cut-off point and the overall survival (OS) or progression-free survival (PFS) of patients with GC (17). The Kaplan-Meier plotter database of patients with gastric cancer included 1,065 histologically confirmed samples from seven independent datasets, in which the CPS1 mRNA levels were evaluated using a microarray platform (17). CPS1 mRNA levels were detected using two probes (204920_s and 217564_s_at); the OS data were available for 881 patients, and the PFS data were available for 503 patients. Kaplan-Meier survival plots were generated, and the hazard ratio with 95% confidence intervals (CI) and log-rank P-values were calculated and plotted in R using Bioconductor packages (18). The false discovery rate was determined to correct for multiple testing

using the 'brainwaver' library in R (https://CRAN.R-project.org/package=brainwaver), with the cutoff set at 5%. CPS1 status was determined using the probe set 204920_s and 217564_s_at. MethSurv is a web tool used to perform multivariable survival analysis using DNA methylation data (https://biit.cs.ut.ee/methsurv/) (19) that utilizes methylome data from 395 histologically confirmed gastric adenocarcinoma patients recruited by The Cancer Genome Atlas (TCGA) consortium, and uses the Cox proportional hazards model to develop an interactive web interface for survival analysis. In the present study, CPS1 methylation status was evaluated using the CpG site cg21967368.

Statistical analysis. Categorical data are presented as the count number and percentage. Associations between the CPS1 expression levels in GC tissues and patient clinicopathological characteristics were assessed using the χ^2 or Fisher's exact test as indicated. The OS of patients with GC was defined as the time from surgical treatment to the time of death from any cause, and the data were censored as the last follow-up point. The Kaplan-Meier plot along with the log-rank and Gehan-Breslow-Wilcoxon (weighted for early time points) test was used to compare the OS of patients with high or low CPS1 expression. Univariate and multivariate Cox proportional hazards regression models were used to assess the association between CPS1 expression and the OS of patients with GC. The distribution of the methylation level of CpG site cg21967368 at different clinical stages is presented as combined violin and box plots. All statistical analyses were performed using GraphPad prism version 8.4.0 (GraphPad Software, Inc.) and/or R software (version 4.0.2) (18). A two-sided P<0.05 was considered to indicate a statistically significant difference.

Results

CPS1 is a specific marker of IM in the gastric mucosa. In the present study, CPS1 was not expressed in the epithelial cells of patients with non-atrophic gastritis, but diffuse strong expression was observed in epithelial cells of patients with IM in 32 cases (100%) of chronic atrophic gastritis. Both IM type I and II exhibited strong positive CPS1 staining (Fig. 1A and B). CPS1 was also strongly expressed in all 102 (100%) cases of IM from IN or GC, including 30 cases of low-grade IN with IM, 28 cases of high-grade IN with IM, and 44 cases of GC with IM.

CPS1 expression is gradually downregulated in Correa's cascade. In low-grade IN, 21 (70%) and 9 (30%) cases exhibited strong and focal positive expression, respectively. The expression of CPS1 was significantly lower in high-grade IN and intestinal-type GC compared with that in low-grade IN. In high-grade IN, only 4 cases (12.5%) exhibited strong expression, 7 (21.8%) presented with focal positive staining, 7 (21.8%) had weak expression, and no expression was observed in 14 (43.8%) cases (Fig. 1C-H). In 172 cases of intestinal-type GC, only 14 (8%) retained strong-positive staining, 14 (8%) presented with focal positive staining, 37 (22%) exhibited weak expression, and 107 (62%) had no expression. These results suggested that CPS1 expression was downregulated as Correa's cascade progressed, and the differences in expression were statistically significant (P<0.05; Table I; Fig. S1).

Expression patterns of CPS1 differ according to gastric tumor type. In cases of diffuse-type GC, 37 (26%) exhibited strong positive CPS1 expression, 10 (7%) were focal positive, 22 (15%) presented with weak positive expression, and 75 (52%) had no CPS1 expression. For mixed-type GC, 11 cases (13%) were strong positive, 4 (5%) were focal positive, 19 (22%) exhibited weak positive expression, and 51 (60%) were CPS1-negative. Among the three types of tumor, a relatively high proportion of patients with diffuse-type GC exhibited strong positive CPS1 expression (26%; P<0.001; Table I; Fig. 2).

Association between CPS1 expression and the clinicopathological characteristics of patients with GC. Low CPS1 expression levels were significantly associated with an advanced TNM stage (P=0.031) and the depth of invasion (P=0.037) in intestinal-type GC, but was not associated with age, sex, lymph node metastasis or distant metastasis. Furthermore, CPS1 expression was not significantly associated with any clinical parameters in patients with diffuse- or mixed-type GC (Table II).

Low CPS1 expression levels are associated with a poor prognosis in the three GC types. The follow-up times of the 401 patients in the Nantong cohort ranged between 0.3 and 113 months, with a median time of 45.3 months; 125 of these patients succumbed to the disease, and 276 patients were alive at the last follow-up. The results of univariate analysis demonstrated that the depth of invasion, lymph node metastasis and TNM stage were associated with the OS of patients with intestinal-type GC. Lymph node metastasis and TNM stage were also associated with the OS of patients with diffuse-type GC, whereas lymph node metastasis and distant metastasis were associated with the OS of patients with mixed-type GC (Table III). Low CPS1 expression levels were associated with a short OS time in patients with intestinal-type (log-rank and Gehan-Breslow-Wilcoxon test, P<0.001), diffuse-type (log-rank and Gehan-Breslow-Wilcoxon test, P<0.001) and mixed-type GC (log-rank test, P=0.010; Gehan-Breslow-Wilcoxon test, P=0.004) (Fig. 3). Univariate Cox regression analysis revealed hazard ratio (HR) values of 2.49 (95% CI, 1.59-3.88; P<0.001) for intestinal-type, 1.89 (95% CI, 1.28-2.78; P=0.001) for diffuse-type and 2.04 (95% CI, 1.19-3.49; P=0.011) for mixed-type GC. Furthermore, the results of the multivariate Cox regression analysis demonstrated that CPS1 expression was independently associated with the OS of patients in the Nantong cohort (intestinal-type GC, HR=2.38; 95% CI, 1.53-3.69; P<0.001; diffuse-type GC, HR=1.81; 95% CI, 1.22-2.71; P=0.003; and mixed-type GC, HR=1.92; 95% CI, 1.09-3.37; P=0.018; Table IV).

Low CPS1 mRNA expression levels are associated with a short OS time in patients with GC. Survival analysis of the association between the survival outcome and CPS1 expression levels in GC was performed using the Kaplan-Meier plotter database data from seven independent patient cohorts. In this pooled patient cohort, low CPS1 mRNA expression levels were associated with a poor prognosis in patients with GC. The results demonstrated that low CPS1 mRNA

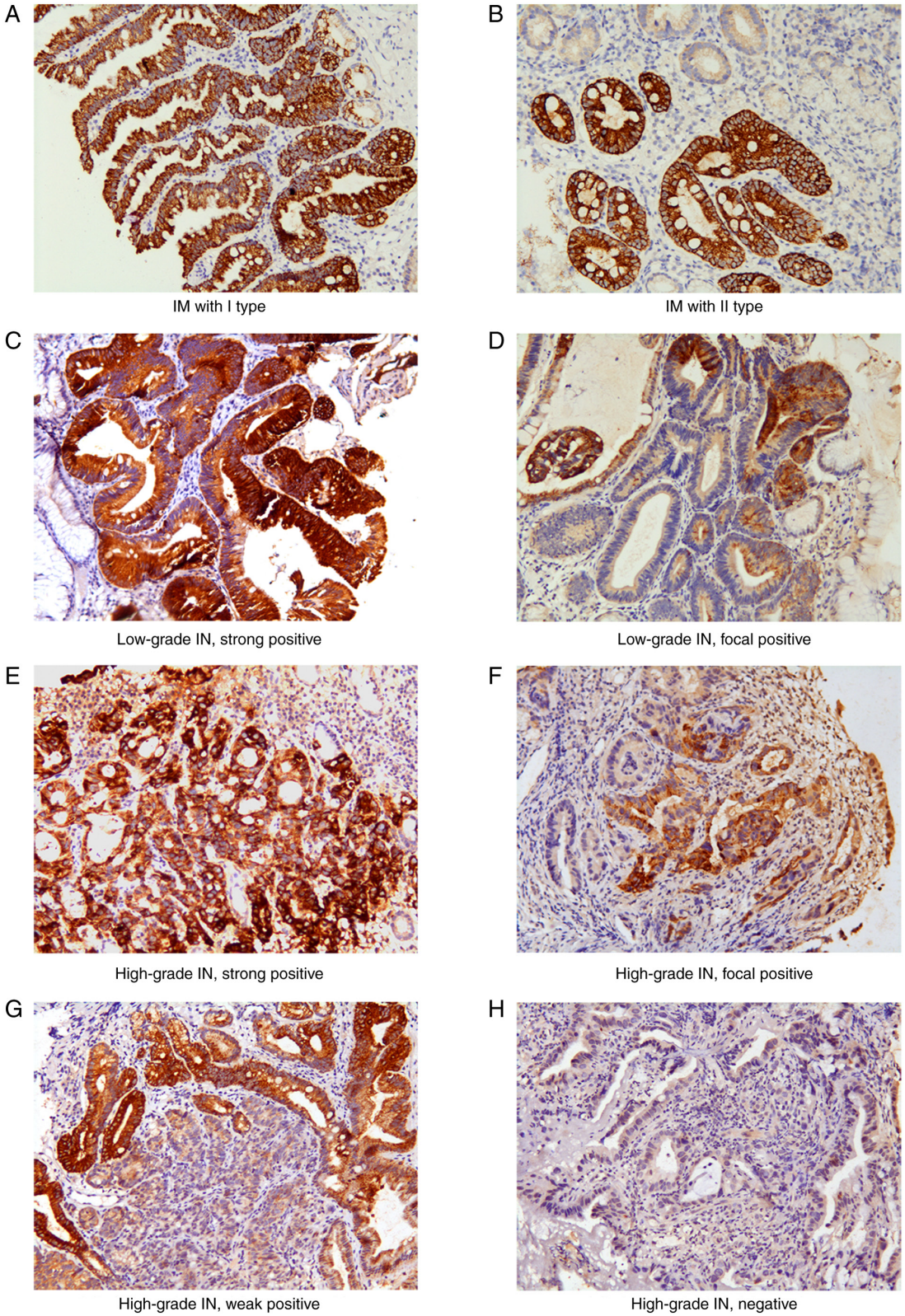

Figure 1. Representative images of CPS1 staining in different types of IM and gastric tumor tissues. (A) IM type I, strong positive; (B) IM type II, strong positive; (C) low-grade IN, strong positive; (D) low-grade IN, focal positive; (E) high-grade IN, strong positive; (F) high-grade IN, focal positive; (G) high-grade IN, weak positive; (H) high-grade IN, negative CPS1 expression. Original magnification, x200. CPS1, carbamoyl phosphate synthetase 1; IM, intestinal metaplasia; IN, intraepithelial neoplasia.

levels were associated with a short OS time, as determined using both probes (Figs. 4A-C and S2). In addition, low mRNA expression levels of CPS1 were also associated with a short PFS time in data from both gene detection probes (probe 204920_s: Total GC, HR=1.38; 95% CI, 1.13-1.69; P=0.001; intestinal-type GC, HR=1.63; 95% CI, 1.15-2.34; P=0.005; and diffuse-type GC, HR=1.78; 95% CI, 1.25-2.56; P=0.001; Fig. 4D-F). A similar trend was observed using

Table I. Expression of CPS1 in different types of intestinal metaplasia, IN and GC.

Immunoreactive score	Gastric mucosa (n=10)	Intestinal metaplasia (n=32)	Low-grade IN (n=40)	High-grade IN (n=60)	Intestinal-type GC (n=172)	Diffuse-type GC (n=144)	Mixed-type GC (n=172)
Strong (8-12)	0	32 (100)	21 (70)	4 (12.5)	14 (8)	37 (26)	11 (13)
Focal (4-6)	0	0	9 (30)	7 (21.8)	14 (8)	10 (7)	4 (5)
Weak (2-3)	0	0	0	7 (21.8)	37 (22)	22 (15)	19 (22)
Negative (0-1)	10 (100)	0	0	14 (43.8)	107 (62)	75 (52)	51 (60)
P-value		<0.001[a]	<0.001[b]	<0.001[c]	0.047[d]	0.005[e]	0.652[f]

[a]Intestinal metaplasia vs. gastric mucosa, Fisher's exact test; [b]low-grade IN vs. intestinal metaplasia, Fisher's exact test; [c]high-grade IN vs. low-grade IN, Fisher's exact test; [d]intestinal-type GC vs. high-grade IN, χ^2 test; [e]diffuse-type GC vs. intestinal-type GC, χ^2 test; [f]mixed-type GC vs. intestinal-type GC, χ^2 test. CPS1, carbamoyl phosphate synthetase 1; IN, intraepithelial neoplasia; GC, gastric cancer.

probe 217564_s_at (total GC, HR=1.41; 95% CI, 1.15-1.73; P=0.007; intestinal-type GC, HR=1.61; 95% CI, 1.14-2.28; P=0.007; and diffuse-type GC, HR=1.96; 95% CI, 1.34-2.87; P=0.004; Fig. S2).

The MethSurv database was used to assess the association between the survival outcome and CpG methylation status of the CPS1 promoter region in patients with GC. Among 395 patients from the TCGA stomach adenocarcinoma datasets released in March 2017, a high methylation level of CPS1 at cg21967368 was associated with a short OS time (HR=1.63; 95% CI, 1.13-2.36; P=0.027), and the methylation level of cg21967368 was increased in clinical stages III and IV compared with that in stages I and II (Fig. 4G-H).

Discussion

In the present study, the expression of CPS1 was not observed in the epithelial cells of chronic non-atrophic gastritis lesions; however, strongly positive expression was observed in the epithelia of the gastric mucosa of patients with IM. These results suggested that diffusely and strongly positive expression of CPS1 may be one of the primary characteristics of IM, which may be a useful diagnostic marker in the clinic. Notably, the expression levels of CPS1 were progressively downregulated during gastric carcinogenesis from IM to low-grade IN, high-grade IN and intestinal GC. These results suggested that the loss of CPS1 expression in dysplasia may be a malignant transformation marker of IM, which was consistent with the results of previous studies that have reported downregulation of CPS1 during esophageal adenocarcinoma (9) and colorectal cancer (6) progression.

Whether CPS1 is the initiating factor of IM or is simply an associated phenomenon remains to be elucidated. Molecular markers of IM can be divided into two categories according to the differentiation direction; the first category comprises gastric differentiation-related proteins, such as transcription factor SOX2 (20), mucin-5AC and mucin-6 (21), whereas the second category includes intestinal differentiation-related proteins, such as intestinal specific transcription factor homeobox protein CDX2 (22), mucin-2 and VILLIN (23). By contrast, CPS1 is typically a hepatogenic differentiation marker (3). Although CPS1 is primarily expressed in the mucosa of the small intestine, its expression and enzyme activity is 10-20-fold higher in the liver (24). The stomach, intestine, liver and pancreas all originate from endodermal progenitors, and previous studies have reported that human gastric epithelial cells or hepatocytes differentiate into multipotent endodermal progenitors using defined small molecules, such as A83-01 and hepatocyte growth factor (25,26). Thus, we hypothesize that gastric epithelial cells may also differentiate into multipotent endodermal progenitor cells under the stimulation of *Helicobacter pylori* infection or bile acid, two of the most common environmental factors for IM induction. Although only the intestinal GC phenotype can be morphologically observed, the potential to identify other phenotypes at the molecular level cannot be excluded. Fatty acid binding protein-1 (FABP-1), a type of FABP present in the liver, is highly expressed in BE (9). A previous study has revealed that FABP-1 is a more reliable diagnostic marker of BE

Table II. Association of CPS1 expression and patient clinical characteristics in various types of GC according to Lauren's classification.

	Intestinal-type GC, n=172			Diffuse-type GC, n=144			Mixed-type GC, n=85		
Variable	Low, n (%)	High, n (%)	P-value	Low, n (%)	High, n (%)	P-value	Low, n (%)	High, n (%)	P-value
All patients	107 (62.2)	65 (37.8)		75 (52.1)	69 (47.9)		51 (60.0)	34 (40.0)	
Age, years			0.9134			0.287			0.064
<65	60 (56.1)	37 (56.9)		52 (69.3)	42 (60.9)		37 (72.5)	18 (52.9)	
≥65	47 (43.9)	28 (43.1)		23 (30.7)	27 (39.1)		14 (27.5)	16 (47.1)	
Sex			0.8267			0.211			0.852
Male	79 (73.8)	47 (72.3)		14 (20.3)	14 (20.3)		34 (66.7)	22 (64.7)	
Female	28 (26.2)	18 (27.7)		55 (79.7)	55 (79.7)		17 (33.3)	12 (35.3)	
Depth of invasion			0.037[a]			0.586			0.544
T1-2	17 (15.9)	19 (29.2)		11 (14.7)	8 (11.6)		4 (7.8)	4 (11.8)	
T3-4	90 (84.1)	46 (70.8)		64 (85.3)	61 (88.4)		47 (92.2)	30 (88.2)	
Lymph node metastasis			0.091			0.656			0.708[b]
N0	31 (29.0)	27 (41.5)		11 (14.7)	12 (17.4)		9 (17.6)	10 (29.4)	
N1/N2/N3	76 (71.0)	38 (58.5)		64 (85.3)	57 (82.6)		42 (82.4)	24 (70.6)	
Distant metastasis			0.506[b]			0.104			0.271[b]
M0	102 (95.3)	60 (92.3)		63 (84.0)	64 (92.8)		48 (94.1)	34 (100)	
M1	5 (4.7)	5 (7.7)		12 (16.0)	5 (7.2)		3 (5.9)	0 (0)	
TNM stage			0.031[a]			0.965			0.182
I-II	32 (29.9)	30 (46.2)		15 (20.0)	14 (20.3)		10 (19.6)	11 (32.4)	
III-IV	75 (70.1)	35 (53.8)		60 (80.0)	55 (79.7)		41 (80.4)	23 (67.6)	

[a]P<0.05; [b]Fisher's exact test; all other data were analyzed by the χ^2 test. CPS1, carbamoyl phosphate synthetase 1; GC, gastric cancer; low, CPS1 immunoreactive score 0 or 1; high, CPS1 immunoreactive score 2-12; T, Tumor; N, Node; M, Metastasis.

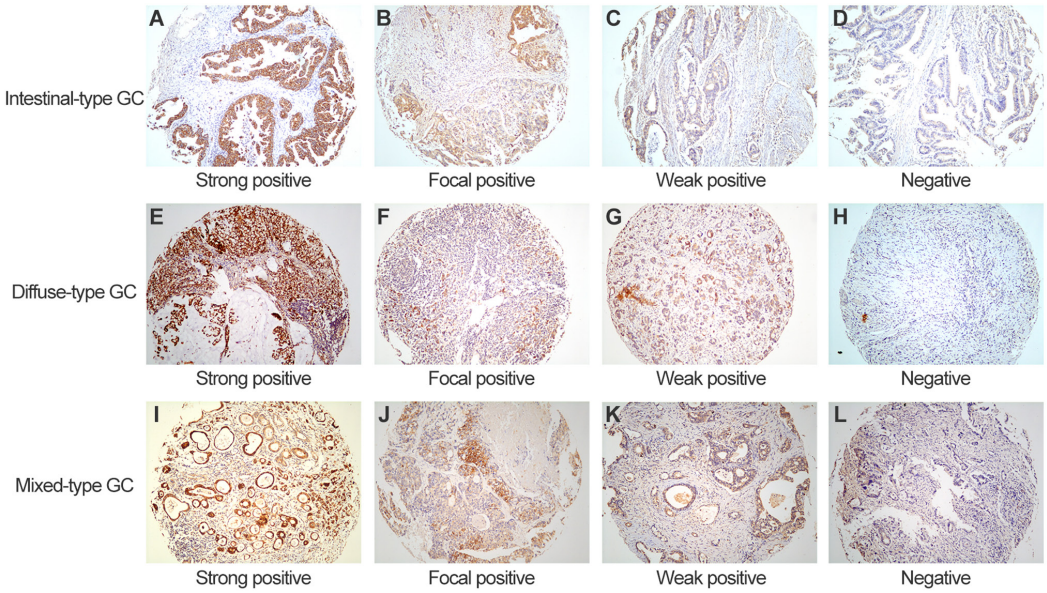

Figure 2. Representative images of CPS1 staining in intestinal-, diffuse- and mixed-type GC tissues. (A-D) Intestinal-type GC samples with (A) strong positive, (B) focal positive, (C) weak positive and (D) negative CPS1 expression. (E-H) Diffuse-type GC samples with (E) strong positive, (F) focal positive, (G) weak positive and (H) negative CPS1 expression. (I-L) Mixed-type GC samples with (I) strong positive, (J) focal positive, (K) weak positive and (L) negative CPS1 expression. Original magnification, x100. CPS1, carbamoyl phosphate synthetase 1; GC, gastric cancer; IHC, immunohistochemistry.

compared with CDX2, and that its expression levels are downregulated at the dysplasia stage of adenocarcinoma (9). Hepatocyte nuclear factor 4α (HNF4α), a liver-enriched transcription factor, is one of the major regulators of hepatocyte

Table III. Univariate analysis of the overall survival and clinical characteristics of patients with GC in the Nantong Cohort.

	Intestinal-type GC (n=172)		Diffuse-type GC (n=144)		Mixed-type GC (n=85)	
Variable	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (≥65 vs. <65 years)	1.01 (0.68-1.49)	0.968	0.87 (0.59-1.3)	0.508	1.22 (0.54-2.74)	0.633
Sex (Female vs. male)	1.05 (0.68-1.62)	0.816	0.72 (0.49-1.07)	0.102	0.81 (0.47-1.42)	0.464
Depth of invasion	1.73 (1.01-2.96)	0.045[a]	1.58 (0.86-2.88)	0.14	1.80 (0.65-4.98)	0.257
Lymph node metastasis	2.27 (1.43-3.59)	<0.001[a]	2.55 (1.36-4.78)	0.003[a]	2.13 (1.07-4.22)	0.031[a]
Distant metastasis	1.54 (0.75-3.18)	0.240	1.22 (0.69-2.13)	0.49	3.39 (1.05-10.94)	0.041[a]
TNM stage	2.18 (1.39-3.40)	<0.001[a]	1.76 (1.05-2.95)	0.033[a]	1.63 (0.88-3.02)	0.122
CPS1 (High vs. low)	2.49 (1.59-3.88)	<0.001[a]	1.89 (1.28-2.78)	0.001[a]	2.04 (1.19-3.49)	0.011[a]

[a]P<0.05. CPS1, carbamoyl phosphate synthetase 1; GC, gastric cancer; HR, hazard ratio; CI, confidence interval; TNM, Tumor-Node-Metastasis.

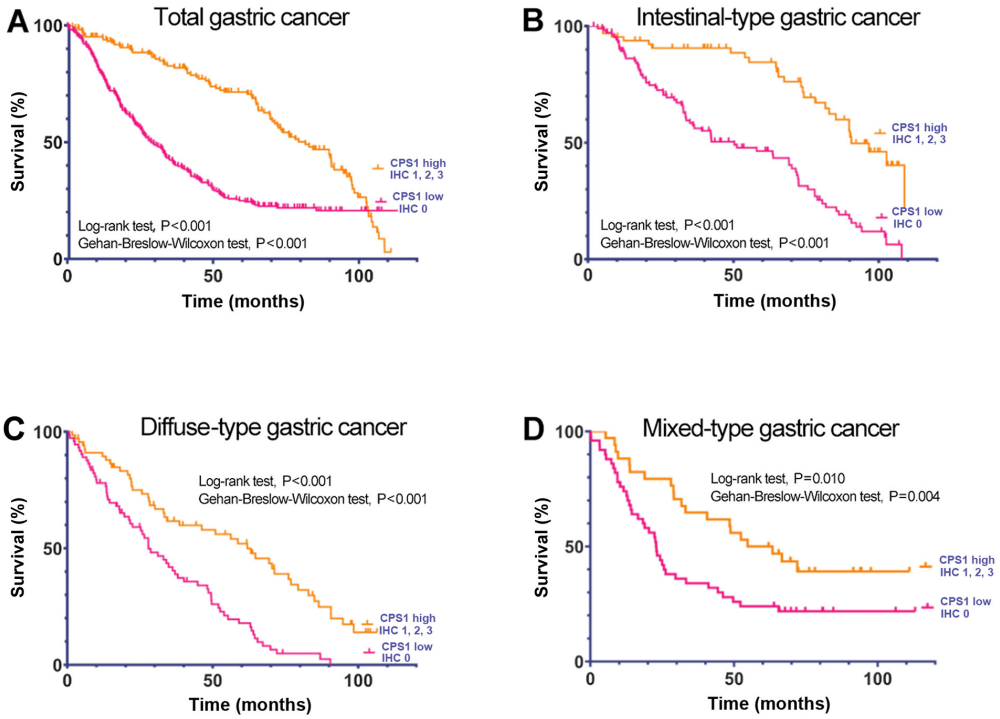

Figure 3. Overall survival of patients with GC in the Nantong cohort based on CPS1 protein expression levels. (A) Overall survival of all patients. (B) Overall survival of patients with intestinal-type GC (log-rank and Gehan-Breslow-Wilcoxon test, P<0.001). (C) Overall survival of patients with diffuse-type GC (log-rank and Gehan-Breslow-Wilcoxon test, P<0.001). (D) Overall survival of patients with mixed-type GC (log-rank test, P=0.010; and Gehan-Breslow-Wilcoxon test, P=0.004). CPS1, carbamoyl phosphate synthetase 1; GC, gastric cancer; IHC, immunohistochemistry.

differentiation and proliferation (27). At present, HNF4α is considered to be a key early component in the development of BE (28). The ectopic expression of HNF4α in the esophagus generates columnar metaplasia (28). HNF4α has recently been identified as a crucial regulator of intestinal regeneration in an organoid model of intestinal damage and repair (29). Therefore, partial hepatocyte differentiation may occur during the process of IM at the early stage of carcinogenesis, but the expression of hepatocyte differentiation genes may be lost at the stage of malignant transformation or development into invasive cancer. However, further investigation is required to confirm this hypothesis.

In the present study, CPS1 expression levels were associated with the tumor invasion depth and clinical stage in intestinal-type GC, and the loss of CPS1 expression was associated with a short OS time following surgical treatment, regardless of tumor type. These results suggested that CPS1 downregulation may be involved in the pathogenesis and progression of GC, and that CPS1 may serve as a potential prognostic biomarker or therapeutic target for patients with GC. The results of the prognostic analysis in the present study were consistent with those from a study of hepatocellular carcinoma, which suggested that low levels of CPS1 were associated with a poor OS rate (30). Similar findings in GC have been reported by Fan *et al* (31), indicating that CPS1 expression levels are negatively associated with the depth of invasion and may be a potential independent prognostic indicator. By contrast, we hypothesize that

Table IV. Multivariate analysis of the overall survival and clinical characteristics of patients with GC in the Nantong Cohort.

	Intestinal-type GC (n=172)		Diffuse-type GC (n=144)		Mixed-type GC (n=85)	
Variable	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (≥65 vs. <65 years)	0.95 (0.64-1.42)	0.812	0.91 (0.61-1.37)	0.659	1.19 (0.69-2.07)	0.516
Sex (Female vs. male)	1.23 (0.79-1.91)	0.357	0.81 (0.54-1.21)	0.301	0.86 (0.49-1.51)	0.603
TNM stage	2.06 (1.30-3.23)	0.001[a]	1.68 (0.99-2.84)	0.053	1.49 (0.78-2.84)	0.222
CPS1 (High vs. low)	2.38 (1.53-3.69)	0.000[a]	1.81 (1.22-2.71)	0.003[a]	1.92 (1.09-3.37)	0.018[a]

[a]P<0.05. CPS1, carbamoyl phosphate synthetase 1; GC, gastric cancer; HR, hazard ratio; CI, confidence interval; TNM, Tumor-Node-Metastasis.

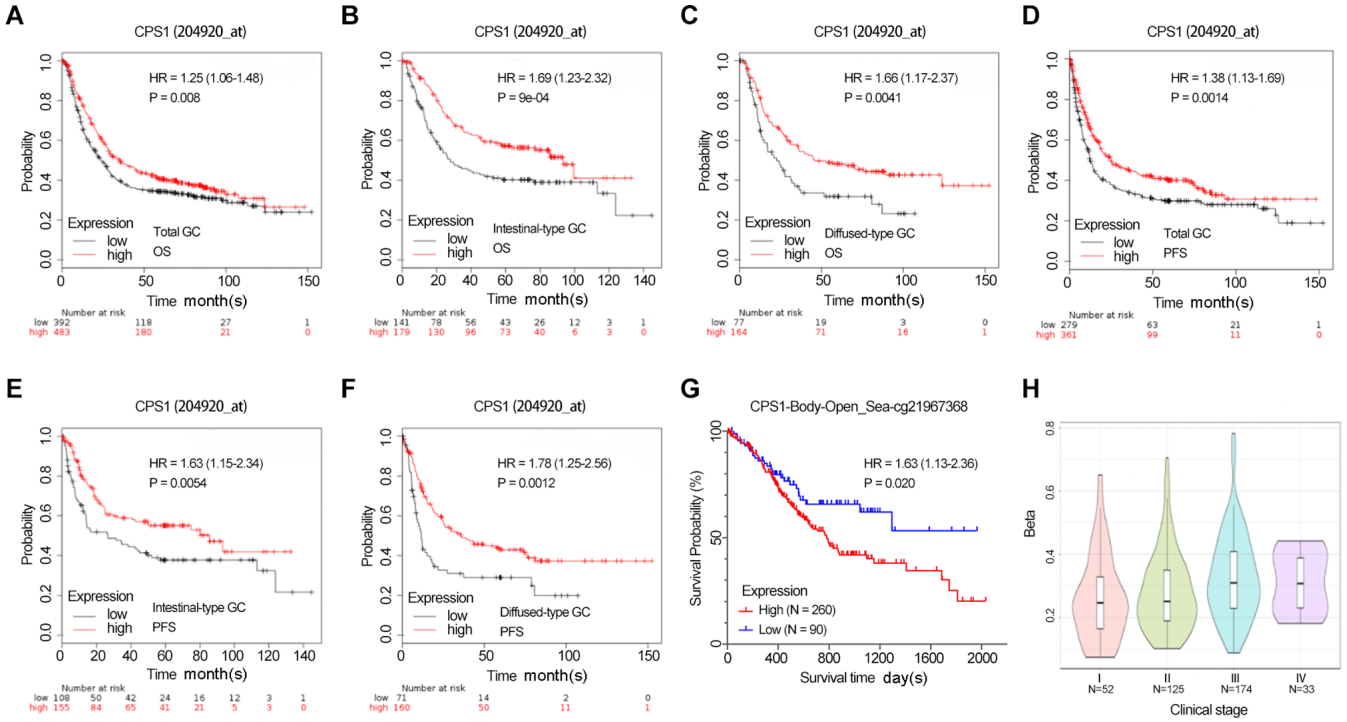

Figure 4. Overall survival of patients with GC based on CPS1 mRNA expression or DNA methylation level. (A-C) OS of patients with (A) total, (B) intestinal-type and (C) diffuse-type GC according to CPS1 mRNA expression using probe 204920_s from the KMplot database. (D-F) PFS of patients with (D) total, (E) intestinal-type and (F) diffuse-type GC according to CPS1 mRNA expression using probe 204920_s from the KMplot database. (G) Overall survival of all patients with GC grouped according to the CPS1 methylation level using probe cg21967368 from the MethSurv database. (H) The methylation profile of probe cg21967368 based on clinical stage from the MethSurv database. CPS1, carbamoyl phosphate synthetase 1; GC, gastric cancer; KMplot, the Kaplan-Meier plotter; OS, overall survival; PFS, progression-free survival; HR, hazard ratio.

CPS1 expression is likely to be lost rather than upregulated in intestinal-type GC, as this subtype originates from the IM mucosa, and diffuse strong CPS1 expression was observed at the IM stage in the present study. In addition, the results of the present study suggested that diffuse-type GC with signet ring cells presented with a higher rate of strong positive CPS1 expression compared with that in intestinal-type GC, which was in contrast to the results of a previous study by Fan *et al* (31). However, similar to the present study, Maitra *et al* (32) demonstrated that strong positive CPS1 expression was observed in 3/10 gastric tumors, all of which were poorly differentiated signet-ring or mixed intestinal-signet-ring cell carcinomas. Since intestinal-type and diffuse-type tumors have distinct origins, the role of CPS1 may differ between them.

Previous studies have reported that CPS1 is regulated by liver-enriched transcription factors. For example, Chen *et al* (33) have demonstrated that HNF3β serves an essential role in CPS1 expression and promotes the metabolism of ammonia. The transcription of CPS1 has also been reported to be associated with the glucocorticoid and glucagon signaling pathways (34,35). DNA methylation is one of the critical mechanisms regulating CPS1 expression. Liu *et al* (36) have reported that DNA methylation suppresses CPS1 expression in primary human hepatocellular carcinoma by regulating its promoter activity. Comprehensive and integrative genomic characterization of hepatocellular carcinoma data from TCGA database has also revealed that CPS1 is downregulated by hypermethylation, which results in metabolic reprogramming (37). In the present study, data acquired from the MethSurv database also

demonstrated that in GC, low levels of CPS1 expression may be associated with altered DNA methylation patterns.

In conclusion, the results of the present study demonstrated that CPS1 was a highly specific marker of IM in the gastric mucosa, and CPS1 expression was gradually lost during the progression of intestinal-type GC. This loss of CPS1 expression was significantly associated with tumor invasion and the clinical stage of intestinal-type GC, and low CPS1 levels were associated with a short OS time in the clinic. Downregulation of CPS1 may occur due to hypermethylation in its promoter region. Further studies are warranted to elucidate the underlying mechanisms of the effects of CPS1 in IM, the dynamic changes in the progression of intestinal-type cancer and the role of high CPS1 expression levels in diffuse-type GC.

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Availability of data and materials

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XF, XW, EX, FY and PC performed the data analyses and wrote the manuscript. XF, XW, EX, FY and PC revised the manuscript and provided constructive criticism for the final version. EX and FL contributed materials and performed statistical analysis, as well as interpreted the data. QL and QM analyzed the data and performed the immunohistochemistry staining. PC and FY contributed to the conception and design of the study. XF, FY and PC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of human tissues in the present study was approved by the Ethics Committees of Nantong Tumor Hospital and Ruijin Hospital, and patients provided written consent to use their medical information and biological samples.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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