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Original Paper

HSP70/HSP90-Organizing Protein Contributes to Gastric Cancer Progression in an Autocrine Fashion and Predicts Poor Survival in Gastric Cancer

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Key Words
Hop • Autocrine • Gastric Cancer • Proliferation • Apoptosis

Abstract
Background/Aims: HSP70/HSP90-organizing protein (HOP) is an adaptor protein that mediates heat shock protein 70 (HSP70) and HSP90 folding. HOP can be secreted by cancer cells and promote malignant cell growth in an autocrine manner. Here, we studied its role in gastric cancer (GC). ***Methods:*** HOP mRNA and protein levels were detected by quantitative real-time PCR and western blotting, respectively, and enzyme-linked immunosorbent assay was used to determine the serum levels. Immunohistochemistry was performed to analyze HOP expression in 117 GC tissues and 32 adjacent normal tissues. The Cell Counting Kit-8 cell viability assay, flow cytometry, and western blotting were used to analyze the effects of HOP on cell proliferation and apoptosis, and the potential underlying mechanisms. ***Results:*** HOP mRNA and protein levels were significantly higher in GC tissues than in normal tissues in our medical center ($P<0.001$) and in The Cancer Genome Atlas database ($P<0.001$). GC patients had higher serum levels of HOP than age-matched healthy controls ($P<0.001$); however, once tumors were removed, serum levels significantly decreased ($P<0.01$). In human GC tissues, increased HOP expression was associated with tumor progression and poor survival. Notably, autocrine HOP promoted cell proliferation through the phospholipase Cγ1-extracellular signal-regulated kinase 1/2-dependent pathway, and inhibited cell apoptosis by regulating the activities of caspase 9, caspase 3, and B-cell lymphoma 2. Blocking extracellular HOP with

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neutralizing antibody reduced proliferation and enhanced fluorouracil-induced apoptosis of GC cells. ***Conclusions:*** Our findings demonstrate that HOP is an important molecular marker and prognostic factor for GC, and functionally contributes to tumor cell growth and survival. These results provide a rationale for considering HOP as a potential therapeutic target and chemosensitizer in GC.

Introduction

Gastric cancer (GC) is among the most threatening carcinomas, with high incidence and cancer-related mortality worldwide [1] and more than 50% of cases occurring in Asia [2]. There are an estimated 680, 000 new cases in China annually, and the incidence is rising steadily [3]. Despite advances in chemotherapy and surgery, the prognosis of patients with advanced GC remains poor [4]. For instance, the 5-year survival rate is only 4% for stage IV GC [5]. Therefore, it is of great significance to further understand the molecular mechanism controlling GC growth and progression and to explore novel targeted drugs for improving the prognosis of GC patients.

HSP70/HSP90-organizing protein (HOP), also referred to as stress-induced protein 1, was initially reported as an auxiliary partner molecule or a scaffold protein that regulates the connection and dimerization of heat shock protein 70 (HSP70) and HSP90, and modulates the function of HSP by changing the dimmer structure [6–8]. Other than serving as an adaptor protein, HOP possesses independent biological activity by regulating the function of several protein complexes, and participating in gene transcription, signal transduction, and cell division [9–13]. HOP lacks a transmembrane domain or signal peptide, and as such, was previously thought to be a cytoplasmic protein [14]. However, some studies have indicated that HOP can be secreted from cells and acts as a cytokine, with the ability to affect cell proliferation and apoptosis [15–17].

Recent studies have indicated that HOP can be overexpressed in human malignant tumors, suggesting that it may be a biomarker in human tumorigenesis. However, the molecular event in the upregulation of HOP in human malignancies remains unknown, and to the best of our knowledge, there have been no reports on the clinical relevance of HOP in GC. To evaluate the potential value of HOP as a novel prognostic biomarker and therapeutic target, and to determine the mechanisms by which HOP promotes human GC tumorigenesis, we analyzed the clinical significance of HOP protein levels in GC patients and studied the effects and mechanisms through which HOP regulates GC cell proliferation and apoptosis *in vitro.*

Materials and Methods

Bioinformatics analysis

We downloaded RNA sequencing gene expression data of GC from the UCSC Cancer Genomics Browser [18], which were collected from The Cancer Genome Atlas (TCGA) [19].

Patient samples

Ethical approval for human subjects was obtained from the Institutional Review Board of the First Affiliated Hospital, Sun Yat-Sen University (FAHSYSU; Guangzhou, China), and written consent was obtained from each patient. Fresh-paired samples from resection specimens were collected from patients with primary GC who were treated with gastric surgery without radiotherapy or chemotherapy before surgical resection at FAHSYSU in 2014 (n=39). All excised tissues were frozen immediately in liquid nitrogen and stored at -80°C. Serum samples were collected from GC patients (n=10) who had not received radiotherapy or chemotherapy before surgical resection at FAHSYSU in 2015. These serum samples were collected just before surgery, and a diagnosis of GC was confirmed via postoperative pathologic examination. The controls were noncancerous healthy volunteers (n=10). Pre- or post-operative serum samples were collected from

5 of the 10 GC patients. GC paraffin-embedded tissues were obtained from the Department of Pathology. Briefly, samples from 117 GC patients, who received surgical treatment at FAHSYSU between 2004 and 2005, were collected and confirmed as GC and then made available for this study. Follow-ups were terminated by December 2013.

Cell culture and reagents

The human GC cell lines AGS, SGC-7901, BGC-823, MGC803, HGC-27, MKN-1, and NCI-N87 and gastric mucosa cell line GES-1 were obtained from the Cell Bank of Chinese Academy of Medical Science (Shanghai, China). These cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS; Invitrogen Life Technology, Carlsbad, CA, USA), penicillin (100 U/mL), and streptomycin (100 mg/mL). Recombinant human HOP (rhHOP) was purchased from R&D Systems (Minneapolis, MN, USA). HOP-neutralization antibody (HOP-NA) was purchased from Abnova (Taiwan, China).

Collection of condition medium

GC cells were grown in 15cm petri dishes until ~80% confluency. The medium was aspirated, and the monolayer was washed three times with phosphate-buffered saline (PBS), once with serum-free RPMI-1640, and then replenished with serum-free RPMI-1640 (10mL). After 48h incubation, the medium was collected, filtered, and stored at -80°C until use. When conditioned medium (CM) was used, 10μL FBS (per 1mL CM) was added to the CM, mixed well, and used in the experiment.

Immunohistochemical staining

Immunohistochemistry (IHC) was conducted as previously described [20]. Briefly, deparaffinized sections were pretreated with 10 mM sodium citrate buffer for antigen unmasking (pH 6.0, boiling temperature, 30 min), blocked in normal serum (Vectastain ABC Kit; Vector Laboratories, Inc., Burlingame, CA, USA), incubated with primary antibodies at 4°C overnight, rinsed, and incubated with secondary antibody (Vectastain ABC Kit). Signals were amplified using the Vectastain ABC Kit as per the manufacturer's instructions. Targeted protein was visualized using diaminobenzidine as the substrate. The results were interpreted by two independent pathologists who were blinded to the specific diagnosis and prognosis for each case, and were scored by a semi-quantitative method in which staining of more than 10% of the tumor cells was considered positive. The staining intensity was scored as "negative," "weak," "moderate," and "strong." Low HOP expression was referred to as negative and weak staining, and high HOP expression was referred to as moderate and strong staining.

Western blot analysis

Cell lysates were obtained as previously described [21]. Briefly, total protein was extracted with cell lysis buffer (KeyGene, Nanjing, China) and the protein concentration was quantified using the Enhanced BCA Protein Assay Kit (KeyGene). Proteins were separated by 8–10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 1 h in 5% BSA in TBS-T and probed with corresponding primary antibodies overnight at 4°C, followed by incubation with rabbit and mouse radish peroxidase-coupled secondary antibodies for 1 h. Specific bands were detected using enhanced chemiluminescence reagent (Millipore) on autoradiographic film. The primary antibodies used were as follows: anti-HOP, anti-phospholipase Cγ1 (PLCγ1), anti-phosphorylated PLCγ1, anti-extracellular signal-regulated kinase 1/2 (ERK1/2), anti-phosphorylated ERK1/2 (Cell Signaling Technology, Danvers, MA, USA), anti-poly (ADP-ribose) polymerase (PARP), anti-caspase 3, anti-caspase 9, anti-B-cell lymphoma 2 (BCL2), and anti-GAPDH (Proteintech, Wuhan, China). The PLCγ1 inhibitor was purchased from Selleck (Shanghai, China).

Quantitative real-time PCR

Total RNA was isolated using RNA plus reagent (TaKaRa, Tokyo, Japan). Complementary DNA was prepared using oligodT primers according to the protocol supplied with the Primer Script TM RT Reagent (TaKaRa). Expression of HOP was determined by quantitative real-time PCR (qRT-PCR) using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA).

Proliferation assay

The Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) assay was used to evaluate cell proliferation. In brief, cells were seeded onto 96-well cell culture cluster plates (KeyGene) at a density of 2×10^3 cells/well in 100μL culture after being treated with CM in the presence or absence of HOP-NA, rhHOP, U73122, and fluorouracil (5-FU) for 48 h. Then, 10μL CCK-8 reagent was added to each well for 2 h incubation at 37°C according to the manufacturer's instructions. The absorbance was read at a wavelength of 450 nm in an automated plate reader (M200 PRO; Tecan, Morrisville, NC, USA).

Flow cytometry analysis of cell apoptosis

Cultured cells were harvested at 24 h after treatment with CM in the presence or absence of HOP-NA, rhHOP, or 5-FU for cell apoptosis analysis by flow cytometry. For the apoptosis assay, the cells were harvested by trypsinization and washed with PBS. The cells were resuspended in 1× Binding Buffer at a concentration of 3×10^6 cells/mL, and then were stained with FITC Annexin V and propidium iodide. The cells were analyzed using an Epics Profile II flow cytometer (Beckman Coulter, Fullerton, CA, USA) and Multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) was used to detect serum levels of HOP in GC patients pre- and post-operatively and in age-matched healthy controls using an ELISA Kit (Lifespan BioSciences, Seattle, WA, USA). Briefly, 100 μL per well of serum or CM and standard solution were added to antibody-coated 96-well plates and incubated for 2 h at room temperature, followed by the addition of biotin-conjugated polyclonal antibody specific for HOP and incubation for 1 h. Then the plate was washed and incubated with avidin conjugated to horseradish peroxidase (Lifespan BioSciences, USA) for 1 h. Color was developed using TMB substrate (eBioscience, San Diego, CA, USA), stopped by adding sulfuric acid, and measured using a plate reader (M200 Pro;Tecan) at a wavelength of 450 nm.

Immunofluorescence

AGS and SGC-7901 cells were plated into 6-well plate with slides. Cells were fixed in 4% paraformaldehyde for 15 min and then washed three times with PBS for 3 min each. The cell membrane was ruptured with 0.2% Triton at room temperature for 15 min, and nonspecific antigen-binding sites were blocked in 5% BSA for 30 min. The cells were incubated overnight at 4°C with HOP and β-actin, and the nuclei were stained with DAPI (1:1000) after incubation with corresponding secondary antibodies for 60 min. The cells were observed with a fluorescence microscope.

Statistical analyses

SPSS version 18.0 (SPSS Inc, Chicago, IL, USA) was used for data analysis. The relationship between HOP expression and features of tumor progression was analyzed using the Chi-square and Fisher's exact tests. Kaplan–Meier survival curves were constructed and the log-rank test and univariate analysis were conducted. Multivariate analysis was performed using the Cox proportional hazards model. A P value of 0.05 was considered statistically significant for all analyses.

Results

HOP was overexpressed in primary GC tissues

To evaluate the expression of HOP in GC, we first analyzed HOP expression in the TCGA GC and Gene Expression Omnibus datasets. For TCGA cohort, the mRNA levels of HOP in GC tumor tissues (n=384) were significantly upregulated as compared to normal gastric tissues (n=37) (P<0.001; Fig. 1a, left panel). The upregulation of HOP in GC was further validated in paired tumor and adjacent normal tissues in the TCGA cohort (n=34, P<0.001; Fig. 1a, right panel). Moreover, HOP mRNA expression was also upregulated in other GC cohorts (Fig. 1b). We detected HOP protein and mRNA expression levels in paired GC and adjacent normal tissues by western blotting (Fig. 1c) and qRT-PCR (Fig. 1d). Scatter plots of the densitometrical data depicted that the protein expression of HOP in tumors was higher

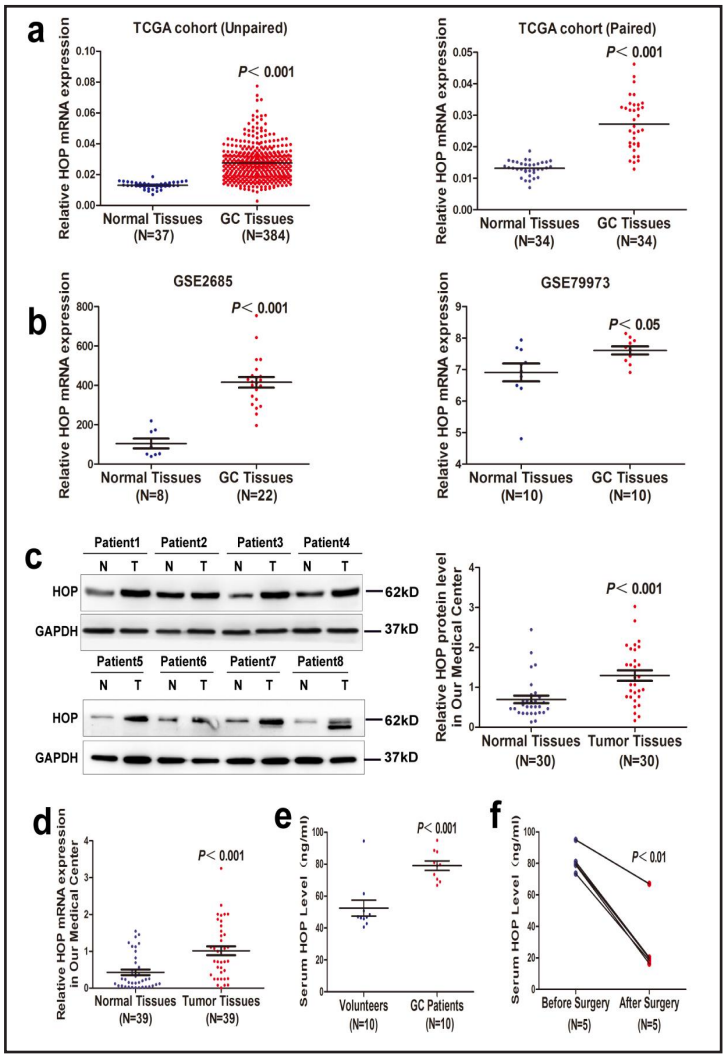

Fig. 1. Increase of HOP expression in GC. (a) Analysis of HOP expression in unpaired GC tissues (n=384) and normal tissues (n=37) in TCGA cohort (P<0.001, left panel); HOP expression in paired normal and GC tumor tissues (n=34) in TCGA cohort (P<0.001, right panel). (b) HOP mRNA expression levels in GC and normal tissues were assessed by analyzing the GSE2685 (left panel) and GSE79973 (right panel). (c) HOP protein expression levels were measured in GC tissues and respective adjacent nontumor tissues in eight patients by western blotting (left panel); Scatter plots of the densitometrical data depict the distribution of HOP in tumor and normal gastric tissues (P<0.001, n=30, right panel). (d) Expression of HOP was analyzed by qRT-PCR in paired tumor and normal gastric tissues (P<0.001, n=39). (e) Serum levels of HOP were analyzed by ELISA in GC patients and controls (P<0.001, n=10). (f) Serum levels of HOP in pre- or post-operative GC patients (P<0.01, n=5).

than that in normal tissues (n=30, *P*<0.001; Fig. 1c, right panel). HOP mRNA expression was examined in a set of 39 GC tissues and matched adjacent corresponding non-tumor tissues, and found to be significantly higher in tumor tissues than in adjacent normal tissues (n=39, *P*<0.001; Fig. 1d).

HOP levels were increased in the peripheral blood of GC patients

Serum levels of HOP were shown to be higher in patients with ovarian cancer or endometriosis than in healthy controls [22–23], and significantly decreased after tumor resection [24]. The increased HOP expression in GC tissues prompted us to investigate blood levels of HOP in GC patients. We detected serum HOP levels by ELISA in 10 GC patients and 10 age-matched controls and found that levels were higher in GC patients than in the age-matched healthy controls (*P*<0.001; Fig. 1e). To determine whether GC tissues were the source of increased serum HOP levels, 5 of the 10 cases of GC were chosen to detect serum HOP protein level pre- or post-tumor resection. Interestingly, serum HOP concentrations in patients with GC significantly decreased after tumor resection (n=5, *P*<0.01; Fig. 1f), suggesting that cancer tissues may be the origin of circulating HOP.

HOP overexpression was associated with a poor prognosis in patients with GC

We evaluated whether HOP expression was associated with clinical characteristics in GC patients. To this end, IHC was performed and HOP expression was scored in 117 GC tissues

and 32 adjacent normal tissues. HOP was mostly localized to the cytoplasm and partially to the nucleus (Fig. 2a, b). The protein expression of HOP was significantly higher in primary GC compared with adjacent normal tissues (Fig. 2c). Of the 117 GC patients, 63 patients (53.85%) had weak HOP staining (IHC score: 1), 30 patients (25.64%) had moderate staining (IHC score: 2), 14 patients (11.96%) had strong staining (IHC score: 3), and 10 patients (8.55%) had negative staining (IHC score: 0). Negative and weak staining were defined as low HOP expression, whereas moderate and strong staining were defined as high HOP expression. The chi-square test was used to explore the association between HOP expression and clinicopathological variables. As shown in Table 1, high expression of HOP in GC was significantly correlated with advanced Bormann classification (P<0.05), differentiation grade (P<0.01), T staging (P<0.05), N staging (P<0.05), M staging (P<0.05), and tumor-node-metastasis (TNM) staging (P<0.05). However, there were no statistically significant relationships between HOP expression and other clinicopathological variables such as age (P=0.43), sex (P=0.44), tumor location (P=0.16), tumor size (P=0.08), and histological type (P=0.27). Next, we investigated the relationship between HOP expression and prognostic outcome in GC patients. For all patients in the study, the follow-up period ranged from 3 to 114 months, with a mean survival time of 47.6 (47.6±3.6) months, and the 5-year overall survival (OS) rates was 25.6%. Survival between patients with low HOP expression (n=73) and high HOP expression (n=44) was compared by Kaplan–Meier survival analysis. We found that high HOP expression predicted a worse outcome in patients with GC (Fig. 2d). The 5-year OS rate was 34.3% in the low HOP expression group and 11.4% in the high HOP expression group (P<0.01; Fig. 2d). Univariate analysis indicated that sex,

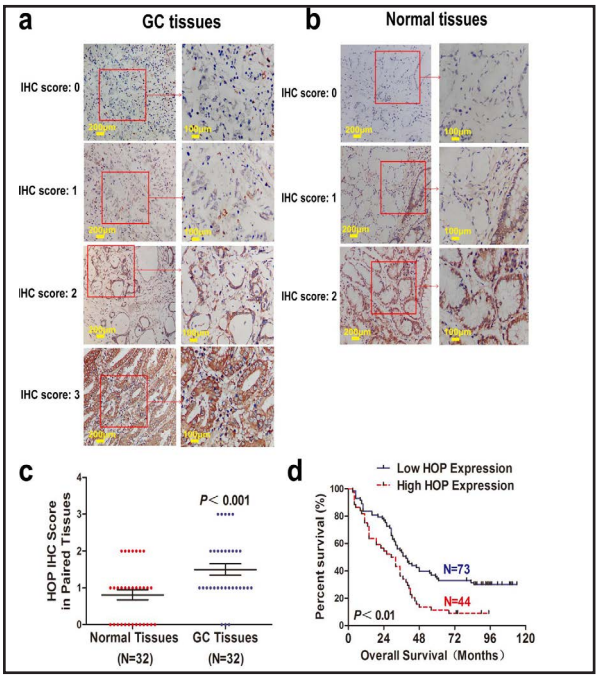

Fig. 2. HOP overexpression in tumors is associated with a poor prognosis in GC patients. (a) IHC staining of HOP protein in GC tissues. (b) IHC staining of HOP protein in normal tissues. (c) HOP protein expression was significantly increased in primary tumor specimens compared with adjacent non-tumor tissues by IHC (P<0.001, n=32). (d) Patients with higher HOP expression had a worse survival than those with lower HOP expression (P<0.01).

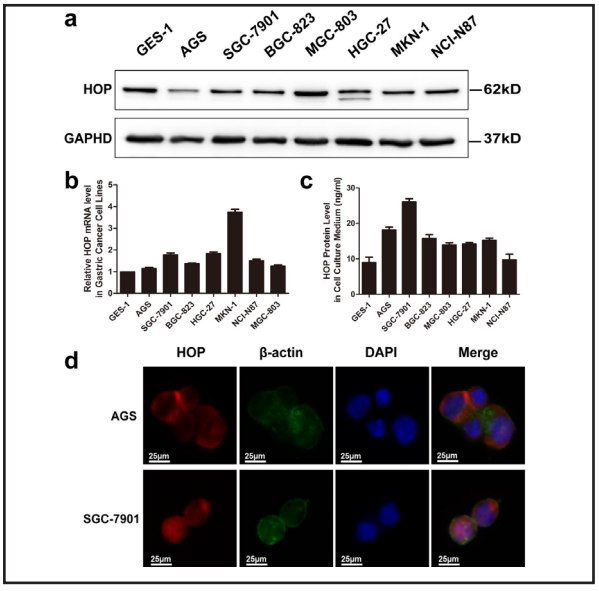

Fig. 3. HOP expression and secreted levels in GC cell lines. (a–b) Expression of HOP protein was analyzed in seven GC cell lines and GES-1 by western blotting (a) and qRT-PCR (b). (c) Secreted HOP in cultured GC cell medium. (d) Localization of HOP protein was analyzed by immunofluorescence in AGS and SGC-7901 cell lines.

age, tumor size, differentiation grade, T staging, N staging, M staging, and HOP expression were significantly associated with an increased risk of cancer-related death (Table 2). Furthermore, multivariate analysis demonstrated that HOP expression was an independent prognostic predictor for GC patients with poor survival (hazard ratio [HR]: 2.1, 95% confidence interval [CI]: 1.3–3.4, $P<0.01$; Table 2).

Table 1. Relationship between HOP expression levels and clinicopathologic characteristics in GC patients

Characteristic	N	HOP expression		X^2 Value	P value
		Low(n=73)	High(n=44)		
Sex					
Male	71	42	29	0.81	0.44
Female	46	31	15		
Age					
≤60 y	74	44	30	0.74	0.43
＞60 y	43	29	14		
Location					
Proximal	14	8	6	5.25	0.16
Middle	18	14	4		
Distal	59	39	20		
More than 2 parts	26	12	14		
Tumor size					
< 5 cm	67	46	21	2.62	0.08
≥5 cm	50	27	23		
Histologic type					
Adenocarcinoma	98	59	39	1.23	0.27
Other	19	14	5		
Bormann classification					
1	6	4	2	8.39	< 0.05
2	24	18	6		
3	69	45	24		
4	18	6	12		
Differentiation grade					
Well	26	16	10	9.46	< 0.01
Moderately	66	35	31		
Poorly	25	22	3		
T staging					
T1-2	38	30	8	6.57	< 0.05
T3-4	79	43	36		
N staging					
N0	21	17	4	3.76	< 0.05
N1-3	96	56	40		
M staging					
M0	101	67	34	4.9	< 0.05
M1	16	6	10		
TNM staging					
I-II	55	40	15	4.72	< 0.05
III-IV	62	33	29		

Table 2. Univariate and multivariate analyses of clinicopathological factors for overall survival in GC patients. CI: confidence interval

Variables	Univariate analysis				Multivariate analysis			
	HR	95% CI		P value	HR	95% CI		P value
		Lower	Upper			Lower	Upper	
Sex	0.5	0.3	0.8	< 0.01	0.5	0.3	0.8	< 0.01
Age	1.9	1.2	3	< 0.01	1.7	1.1	2.7	< 0.05
Tumor location	1.1	0.8	1.4	0.57				
Tumor size	0.6	0.4	1	< 0.05				
Histologic type	0.9	0.7	1.2	0.37				
Bormann classification	1.3	0.9	1.7	0.13				
Differentiation grade	0.6	0.4	1	< 0.05	0.6	0.4	1	< 0.05
T staging	3.3	1.5	7.4	< 0.01	1.9	1.1	3.4	< 0.05
N staging	1.3	1.0	1.7	< 0.05	1.3	1	1.6	< 0.05
M staging	7.3	2.7	19.5	< 0.001	4.1	2	8.2	< 0.001
TNM staging	0.7	0.4	1.3	0.24				
HOP expression	2.1	1.2	3.5	< 0.01	2.1	1.3	3.4	< 0.01

Exogenous HOP signaling promoted cell proliferation in GC cell lines

Because serum levels of HOP were more detectable in GC patients than in age-matched controls (Fig. 1e), we hypothesized that HOP plays a role in gastric carcinogenesis. To test this hypothesis, we first examined HOP expression in GC (AGS, SGC-7901, BGC-823, MGC-803, HGC-27, MKN-1, NCI-N87) and gastric mucosa (GES-1) cell lines by western blotting (Fig. 3a) and qRT-PCR (Fig. 3b). The concentration of HOP in the CM was detected by ELISA in the cell lines (Fig. 3c). The AGS and SGC-7901 GC cells secreted more HOP in the culture medium than the other cell lines. Based on the level of HOP in the CM and HOP protein and mRNA expression in the cell lines, AGS and SGC-7901 were chosen for subsequent experiments. To further confirm the location of HOP in GC cell lines, immunofluorescence was performed in AGS and SGC-7901 GC cell lines. As shown in Fig. 3d, HOP was predominately located in the cytoplasm and partially located in the nucleus, similar to the expression pattern observed in the tissues of patients with GC (Fig. 2a, b). Our previous results indicated that rhHOP promoted hepatocellular carcinoma cell (HCC) proliferation [25], and it was reported that rhHOP increased ovarian cancer cell viability [24] and renal cell cancer metastasis [26]. Thus, we determined the effects of HOP on GC cell growth. After treatment of cells with 0, 25, 50, and 100 ng/mL rhHOP, cell viability was increased in a dose-dependent fashion in AGS (Fig. 4a, left panel) and SGC-7901 (Fig. 4b, left panel) cells. Moreover, treatment with 50ng/mL rhHOP could increase cell viability in a time-dependent fashion in AGS (Fig. 4a, right panel) and SGC-7901 (Fig. 4b, right panel) cells. We also found that treatment with HOP-NA could reduce GC cell proliferation in dose- and time-dependent manners in AGS (Fig. 4c) and SGC-7901 (Fig. 4d) cell lines. Based on these results, the concentrations of rhHOP at 50ng/mL

and of HOP-NA at 30ng/mL were chosen for further experiments. To determine whether the growth promotion effects also existed with physiological concentrations of HOP in GC, AGS, and SGC-7901 cells were treated with their own CM, which contained HOP and other growth factors, in the presence or absence of HOP-NA. The cell proliferation in CM was significantly increased, compared with basal medium for both cells (Fig. 4e). HOP-NA moderately reduced CM-induced cell proliferation in AGS cells (Fig. 4e, left panel), and significantly decreased the viability of SGC-7901 cells (Fig. 4e, right panel), suggesting that functional extracellular autocrine signaling mediated by secreted HOP existed in GC cells.

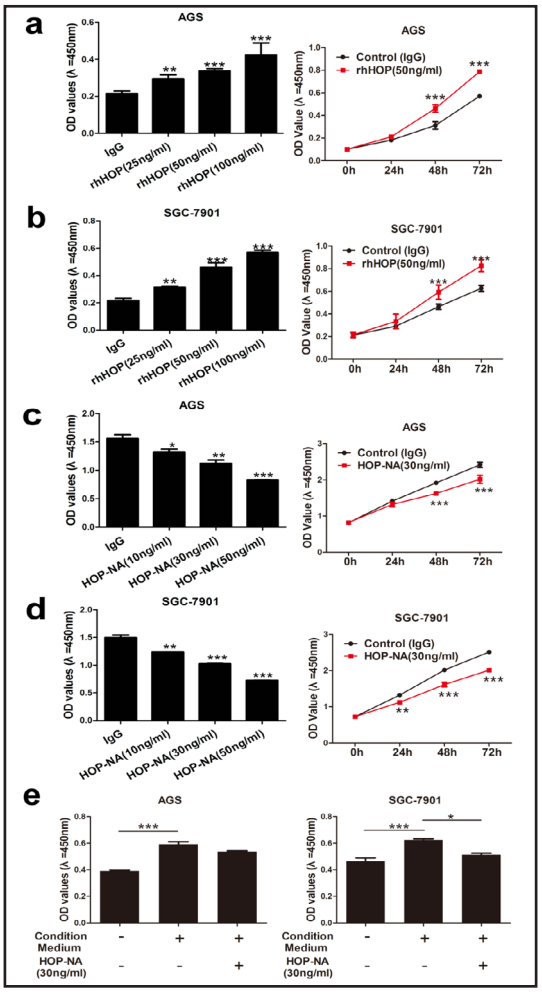

Fig. 4. Autocrine HOP signaling promotes cell proliferation in cultured GC cells. (a–b) Treatment with rhHOP increased the proliferation of AGS (a) and SGC-7901 (b) GC cell lines in dose-dependent (left panel) and time-dependent (right panel) manners. (c–d) HOP-NA treatment decreased the viability of AGS (c) and SGC-7901 (d) GC cell lines in dose-dependent (left panel) and time-dependent (right panel) manners. (e) Proliferation of AGS (left panel) and SGC-7901 (right panel) GC cells in basal medium and CM with or without HOP-NA (30ng/mL). Mean ± standard error of the mean (SEM), t-test, *P<0.05, **P<0.01, ***P<0.001.

Autocrine HOP signaling promoted cell proliferation through the PLCγ1-ERK1/2 pathway

The PLCγ1-ERK1/2 pathway is well recognized as an important pathway that modulates cell proliferation in some malignant tumors [27–28]. To determine whether this pathway was the mechanism underlying HOP regulation of GC cell growth, we evaluated the expression of PLCγ1 and ERK1/2 by western blotting. Western blot analysis showed that rhHOP could increase cell phosphorylated-PLCγ1 and phosphorylated-ERK1/2 expression in a dose-dependent manner (Fig. 5a). Moreover, treatment of GC cells with rhHOP induced phosphorylation of PLCγ1 and ERK1/2 in a time-dependent manner (Fig. 5b). The expression of phosphorylated PLCγ1 and ERK1/2 was increased at 15 min after rhHOP treatment, and peaked at 30 min in AGS cells (Fig. 5b, left panel) and between 30 and 60 min in SGC-7901 cells (Fig. 5b, right panel). To further determine whether autocrine HOP-mediated cell proliferation was promoted through the PLCγ1-ERK1/2 pathway, the cells were treated with their own CM in the presence or absence of HOP-NA. In both cell lines, PLCγ1 and ERK1/2 were activated after treating with CM, and in the presence of HOP-NA, CM did not increase the phosphorylation levels of PLCγ1 and ERK1/2 (Fig. 5c). To further investigate the role of PLCγ1 in GC cell proliferation, we treated GC cells with a PLCγ1 inhibitor, U73122, to determine whether it would affect GC cell viability. In our previous study, we found that 5μM U73122 significantly decreased cell proliferation [20]; thus, that concentration of U73122 was used in this study. To explore whether rhHOP could reverse the inhibition of U73122-induced cell proliferation, we treated cells with U73122 in the presence or absence of rhHOP. In both cell lines, rhHOP did not

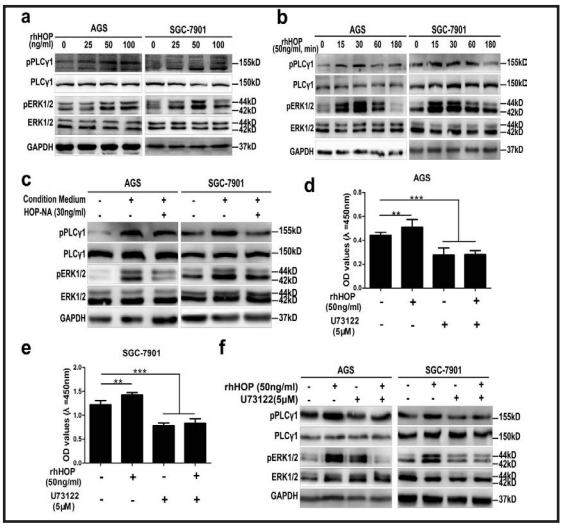

Fig. 5. Autocrine HOP promotes cell proliferation through a PLCγ1-dependent pathway in GC cell lines. (a) AGS (left panel) and SGC-7901 (right panel) cells were treated with rhHOP and harvested after treatment for 30 min. The phosphorylation of PLCγ1 (pPLCγ1) and ERK1/2 (pERK1/2) was detected by western blotting. (b) Western blotting demonstrated that rhHOP induced the phosphorylation of PLCγ1 and ERK1/2 in a time-dependent manner in AGS (left panel) and SGC-7901 (right panel) cells. (c) Western blotting demonstrated that CM activated the phosphorylation of PLCγ1 and ERK1/2, with or without HOP-NA (30ng/mL) in AGS (left panel) and SGC-7901 (right panel) cells. (d) After blocking PLCγ1 with U73122 (5μM), cell proliferation in response to rhHOP (50ng/mL) in AGS cells was assessed. (e) After blocking PLCγ1 with U73122 (5μM), cell proliferation in response to rhHOP (50ng/mL) in SGC-7901 cells was assessed. (f) After treating cells with U73122 (5μM), protein levels in response to rhHOP (50ng/mL) in AGS (left panel) and SGC-7901 (right panel) cells. Mean ± SEM, t-test, *P<0.05, **P<0.01, ***P<0.001.

reverse the inhibitory effect of U73122 (Fig. 5d, e). Then, we confirmed the role of PLCγ1 in autocrine HOP signaling by western blotting. The cells were treated with dimethylformamide or 5μM U73122 overnight followed by 30 min exposure to rhHOP. Again, rhHOP induced the phosphorylation of PLCγ1 and ERK1/2 in both cell lines. Treatment with U73122 diminished the rhHOP-induced phosphorylation of PLCγ1 and ERK1/2 to sub-baseline levels (Fig. 5f). Together, these data showed that autocrine HOP-mediated cell proliferation was at least partially activated through the PLCγ1-ERK1/2 pathway.

Autocrine HOP signaling reduced the apoptosis of GC Cells

To determine whether autocrine HOP affected the apoptosis of GC cells, we analyzed the apoptotic cell population by flow cytometry after rhHOP treatment. As shown in Fig. 6a, rhHOP (50ng/mL) decreased the number of annexin V-positive cells compared with cells treated with IgG control in the GC cells AGS and SGC-7901. To investigate the possible mechanisms by which rhHOP decreased GC cell death, we performed western blotting to investigate the levels of PARP, caspase 9, caspase 3, and BCL2. Compared with the control group, the levels of cleaved PARP, cleaved caspase 9, and cleaved caspase 3 were slightly lower in cells treated with rhHOP, whereas a significant increase in BCL2 was detected in rhHOP-treated AGS (Fig. 6b, left panel) and SGC-7901 (Fig. 6b, right panel) cells. Next, we determined whether autocrine HOP signaling mediated cell apoptosis. By treating AGS and SGC-7901 cells with their own CM in the presence or absence of HOP-NA, the apoptotic mediators (PARP, caspase 9, caspase 3, and BCL2) were assessed by western blotting. As shown in Fig. 6c, we noticed decreased levels of cleaved PARP, cleaved caspase 3, and cleaved caspase 9, as well as the increased expression of BCL2 in cells treated with CM in the absence of HOP-NA. Furthermore, the data also showed that by treating cells with CM in the presence of HOP-NA, the expression levels of cleaved PARP, cleaved caspase 3, and cleaved caspase 9 were higher and the levels of BCL2 were lower than those in cells treated with CM only. These data demonstrate that autocrine HOP signaling reduced cell apoptosis in a BCL2- or caspase 3-dependent manner in GC cells.

Autocrine HOP signaling enhanced the chemoresistance of GC cells exposed to 5-FU in vitro

Because autocrine HOP signaling led to an increase in cell proliferation and decrease in cell apoptosis, we investigated whether it would affect the cellular response to

chemotherapeutic drugs such as 5-FU. We found that 10 μg/mL 5-FU could significantly decrease cell proliferation in AGS and SGC-7901 cells (Fig. 6d). The combination of HOP-NA and 5-FU significantly decreased cell viability and increased cell apoptosis compared with 5-FU alone, and rhHOP treatment reversed the 5-FU-induced decrease in cell viability and increase in apoptosis (Fig. 6e, f). To confirm the potential interaction between 5-FU-induced cell apoptosis and HOP, we compared the activation of apoptotic mediators in SGC-7901 cells treated with 5-FU in the presence or absence of rhHOP and HOP-NA. We observed that, in a dose-dependent manner, rhHOP upregulated BCL2 and downregulated cleaved caspase 9, cleaved caspase 3, and cleaved PARP. In contrast to rhHOP, HOP-NA induced the expression of cleaved caspase 9, cleaved caspase 3, and cleaved PARP and inhibited BCL2 expression (Fig. 6g). These data suggested that autocrine HOP signaling enhanced the chemoresistance of GC cells exposed to 5-FU.

Discussion

This study demonstrated that HOP is a tumor promoter that promotes cell proliferation and inhibits GC cell apoptosis, and represents the first comprehensive functional analysis of HOP in GC. Both HOP mRNA and protein levels were higher in GC tissues than those in matched adjacent noncancerous tissues, which supports the diagnostic value of HOP in GC.

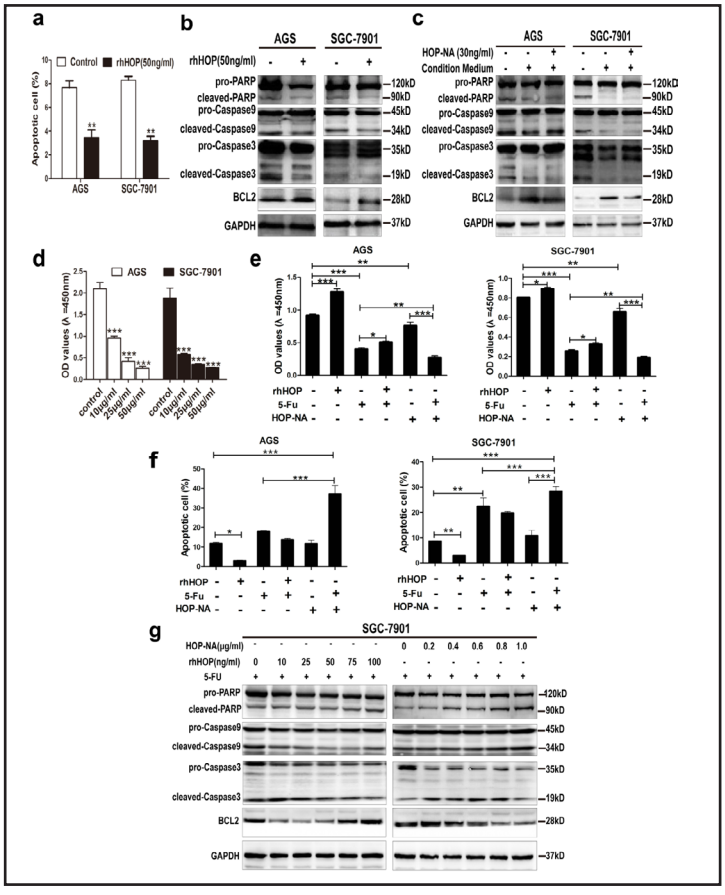

Fig. 6. Autocrine HOP signaling inhibited cell apoptosis and enhanced the chemoresistance of GC cells to 5-FU. (a) Flow cytometry studies showed apoptosis of AGS and SGC-7901 cells treated with rhHOP (50ng/mL) or control reagent. (b) rhHOP decreased the expression of cleaved PARP, cleaved caspase 9, cleaved caspase 3, and increased BCL-2 expression in AGS (left panel) and SGC-7901 (right panel) cells. (c) Western blotting showed that CM decreased the expression of cleaved PARP, cleaved caspase 9, and cleaved caspase 3, and increased BCL-2 expression compared with the control group and CM plus HOP-NA (30ng/mL) in AGS (left panel) and SGC-7901 (right panel) cells. (d) Treatment of 5-FU at different concentrations decreased AGS and SGC-7901 cell viability. (e) Proliferation of AGS (left panel) and SGC-7901 (right panel) in the presence or absence of rhHOP (50ng/mL), 5-FU (25μg/mL), and HOP-NA (30ng/mL). (f) Flow cytometry demonstrated apoptosis of AGS (left panel) and SGC-7901 (right panel) cells in the presence or absence of rhHOP (50ng/mL), 5-FU (25μg/mL), and HOP-NA (30ng/mL). (g) Pro- and activated PARP, caspase 9, caspase 3, and BCL2 in SGC-7901 cells treated with 5-FU in the presence of different concentrations of rhHOP (0–100ng/mL, left panel) or HOP-NA (0–1.0μg/mL, right panel) for 24 h. Mean ± SEM, t-test, *P<0.05, **P<0.01, ***P<0.001.

Furthermore, HOP predicted the survival of GC patients, highlighting the potential value of HOP as a novel prognostic biomarker in human GC. Mechanistically, we demonstrated that secreted HOP promoted cell proliferation through the PLCγ1-ERK1/2 signaling pathway and inhibited GC cell apoptosis by modulating apoptotic effectors in an autocrine fashion. Moreover, the effects of 5-FU on GC apoptosis could be increased by HOP-NA.

Emerging evidence suggests that HOP might serve as a novel biomarker for the prognostic evaluation of patients with malignant tumors. Patients with higher or positive HOP expression showed a worse prognosis and advanced tumor progression in patients with papillary thyroid carcinoma [29], ovarian cancer [30], HCC [25], and epithelial ovarian cancer [31]. In our study, high HOP protein expression in GC tumors was associated with advanced Bormman classification, differentiated degree, T staging, N staging, M staging, and TNM staging (Table 1). Survival analysis demonstrated that patients with high HOP expression had shorter OS than those with low expression (Fig. 2d). These results strongly suggest that HOP is an important molecular marker and prognostic factor for solid tumors.

Wang et al [22]. found that serum levels of HOP in patients with ovarian cancer were higher than those in healthy control volunteers. Similar to Wang's report, our previous study confirmed that HOP concentration in the peripheral blood of HCC patients was significantly higher than that in age-matched controls [25]. In this study, we detected HOP serum levels in GC patients, and found that they were significantly higher in GC patients (79.1±2.9 ng/mL) than in age-matched healthy controls (52.3±5ng/mL). Serum HOP levels in most of the GC patients were higher than those in CM, which were secreted by GC cells (Figs.1e and 3c). Tsai et al [24]. also showed that serum HOP levels decreased after surgical resection in ovarian cancer. In this study, we found that serum HOP levels significantly decreased after surgery in patients with GC (Fig. 1f). These studies and our findings highlight the role of plasma HOP as a tumor marker in solid tumors and suggest that cancer tissues might be the origin of circulating HOP. The limitation of this study is that we detected serum HOP protein levels pre- and post-operatively in only 5 patients; thus, additional studies with larger sample sizes are needed to confirm our findings.

It has been reported that HOP can function as a potential tumor oncogene in different cancers. For example, in pancreatic cancer, knockdown of HOP in pancreatic cancer cells decreased cell invasion via downregulation of matrix metalloproteinase 2 (MMP2) [32]. Cho et al [31]. demonstrated that downregulated HOP in epithelial ovarian cancer cells showed strong inhibition of tumor growth and modulation of ID3 and Smad1/Smad5 expression. Our previous study reported that elevated expression of HOP exhibited metastasis-promoting effects in GC cells through activation of the Wnt/β-catenin signaling pathway [33]. Because HOP harbors multiple structural motifs and interacts with various proteins such as HSP90, HOP70, and JAK2 [6–9, 34], Wang et al [23]. reported that HOP could act as transcription factor to bind to the MMP9 promoter and enhance MMP9 transcriptional expression in endometriosis/adenomyosis. In an autocrine fashion, HOP can trigger different signaling pathways including ERK1/2, PKA, PI3K, ALK2-SMAD, and Wnt/β-catenin pathways in different cancers [24–26, 33, 35, 36], which might explain why exogenous and endogenous HOP acts in an organ-specific fashion. The PLCγ1-ERK1/2 pathway is important for modulating cell proliferation in some malignant tumors [27–28]. However, this study showed that HOP secreted from GC cells could promote GC cell proliferation and induce phosphorylation of PLCγ1 and ERK1/2 in a time- and dose-dependent manner. However, rhHOP and secreted HOP did not increase cell viability after blocking the PLCγ1-ERK1/2 pathway. Thus, autocrine HOP signaling promoted GC cell proliferation via the PLCγ1-ERK1/2 pathway.

Some studies have shown that extracellular HOP signaling can rescue cells from apoptosis [25, 37–39]. The interaction of extracellular HOP protein with prion proteins on the cell surface can inhibit cell apoptosis, enhance neuronal protein synthesis through the mTOR signaling pathway, and trigger neuroprotection [37–39]. Our previous study also demonstrated that rhHOP treatment decreased HCC cell apoptosis and HOP-NA treatment increased the rate of apoptosis [25]. In this study, we found that treatment with rhHOP

significantly decreased GC cell apoptosis, decreased the expression of apoptosis-associated cleaved PARP, cleaved caspase 3, and cleaved caspase 9. Moreover, the expression of BCL2, an anti-apoptosis protein, increased after treating cells with rhHOP. Similar results were found after treatment with CM and HOP-NA. Based on these results, we propose that HOP signaling might inhibit GC cell apoptosis in an autocrine manner.

5-FU is the basic chemotherapeutic drug for GC [40]. Despite its anticancer effects, the clinical use of 5-FU is limited by drug resistance [41, 42]. In this study, we found that rhHOP could successfully rescue cells from 5-FU-induced inhibition of cell proliferation and promotion of cell apoptosis, whereas blocking exogenous or secreted HOP increased the therapeutic effect of 5-FU. We also observed that blocking exogenous or secreted HOP by HOP-NA enhanced 5-FU-induced cell apoptosis. These findings indicate that HOP might protect GC cells from 5-FU chemotherapy in an autocrine manner. Furthermore, blocking exogenous HOP may be a new therapeutic strategy for enhancing the effects of chemotherapy for GC.

In conclusion, GC cells can secrete HOP into the local environment and finally into the blood circulation, and HOP regulates cell growth through the PLCγ1-ERK1/2 signaling pathway and anti-apoptotic pathway in an autocrine fashion. Autocrine HOP signaling may enhance the chemoresistance of GC cells to 5-FU *in vitro*. To the best of our knowledge, this is the first report to demonstrate the correlation between HOP expression and clinicopathological features, and further highlights the importance of autocrine HOP signaling in gastric tumorigenesis.

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Disclosure Statement

The authors declare to have no conflict of interests.

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