Reduced CIAPIN1 expression may contributes to gastric carcinogenesis by up-regulating cell proliferation and accelerating cell cycle progression

Running title: CIAPIN1 in gastric carcinogenesis

Authors's names (omitted)

Authors' affiliations (omitted)

Grant support: This work was supported by grants from the Chinese National Foundation of Natural Sciences (No 30471989).

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Abstract

Purpose: Our previous works have revealed that CIAPIN1 (**c**ytokine **i**nduced **a**poptosis **in**hibitor 1), a newly identified anti-apoptotic molecule playing an essential role in mouse definitive hematopoiesis, is related to the development of multidrug resistance (MDR) in gastric cancer ~~multidrug resistance (MDR) ~~cells. This study aimed to investigate the possible role of CIAPIN1 in gastric carcinogenesis.

Experimental design: ~~We detected h~~Human CIAPIN1 expression in tumor tissues of 83 gastric cancer~~s~~ patients and gastric mucosa of 138 patents with normal mucosa (n=13), chronic gastritis (n=17), gastric atrophy (n=44), intestinal metaplasia (n=43), or dyspepsia (n=21)~~noncancerous gastric mucosal specimens~~ was detected by ~~with~~ immunohistochemistry. ~~We also examined~~Moreover, ~~expression of~~ hCIAPIN1 expression in cancerous and adjacent non-cancerous tissues of 15 gastric cancer cases, ~~by Western blotting in 15 gastric cancers and their adjacent noncancerous specimens as well as~~ and in gastric mucosa of 10 patients with ~~endoscopically normal specimens~~non-ulcer dyspepsia was detected by West blotting. In addition, ~~To further investigate the functional property of CIAPIN1, we observed~~ the influences of hCIAPIN1 on cell proliferation and cell cycle distribution of ~~SGC7901 and MKN28~~ gastric cancer cells were determined *in vitro* by constructing gastric cancer cell sublines of SGC7901 and MKN28 with ectopic CIAPIN1 expression (via plasmid transfection) and CIAPIN1 knockdown (via colony selection).~~.~~

Results*:* The expression of hCIAPIN1 was progressively reduced following the severity of gastric pathology, with the level ~~in gastric cancer~~ was significantly lower in gastric cancer than ~~that~~ in normal gastric mucosa. Western blotting confirmed the ~~downregulated~~ expression of hCIAPIN1 was downregulated in cancerous tissues,

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compared with that in the -non-cancerous tissues and the normal gastric mucosa of non-ulcer dyspepsia patients. In addition, in patients with gastric cancer, t~~T~~he expression level of hCIAPIN1 ~~in TNM stage III+IV~~ was lower in patients with TNM stage III+IV than in those with~~that in~~ stage ~~I + II~~ I+II~~gastric cancers~~. ~~In noncancerous gastric mucosa, the expression of CIAPIN1 in dysplastic gastric mucosa was lower than that in normal gastric mucosa, gastric mucosa with superficial, atrophic gastritis and intestinal metaplasia, whereas the latter four expressed similar levels of CIAPIN1.~~ *In vitro*, ~~Upregulation~~ upregulation of hCIAPIN1 suppressed cell proliferation and inhibited cell cycle progression ~~in SGC7901 and MKN28 gastric cancer cells~~ while knockdown of hCIAPIN1 ~~conferred~~ increased cell proliferation and accelerated cell cycle progression in SGC7901 and MKN28 gastric cancer cells~~opposite effects to the above~~.

Conclusion: ~~Our data suggests that~~ CIAPIN1 expression is reduced progressively in the development of gastric cancer. ~~is a negative regulator in gastric carcinogenesis. Downregulation of hCIAPIN1 might contribute to the development of gastric cancer.~~Reduced CIAPIN1 expression may contribute to gastric carcinogenesis by up-regulating cell proliferation and accelerating cell cycle progression.

Key words: CIAPIN1; gastric cancer; ~~immunohistochemistry; expression~~cell~~;~~ proliferation; cell cycle

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~~INTRODUCTION~~Introduction

Gastric cancer is one of the most common malignancies throughout the world, especially in East Asian countries such as China, Japan, and Korea (1). It is widely accepted that the development of gastric cancer is a multistep process from normal mucosa to chronic gastritis, then to precancerous lesions including gastric atrophy, intestinal metaplasia, and dysplasia, and finally to invasive cancer (Correa P. Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. Cancer Res 1992;52(24):6735-40). Moreover, ~~gastric carcinogenesis is a multi-step process involving multiple genetic and epigenetic events, and morphologically, progresses follow an intestinal metaplasia-dysplasia-invasive carcinoma sequence.~~ i~~I~~t has been established now that multiple and cumulative series of structural and functional genetic alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators, cell adhesion molecules, and growth factors/receptors system are implicated in the onset and progression of gastric carcinogenesis (2-5). ~~But~~ However, the precise molecular mechanisms of this progress remain~~s~~ largely unknown. Seeking for molecules associated with the onset and/or progression of gastric cancer is still a hotspot in the research field of gastric cancer.

CIAPIN1 (**c**ytokine **i**nduced **a**poptosis **in**hibitor 1, initially anamorsin) is a newly identified anti-apoptotic molecule with no structural relation to apoptosis regulatory molecules of the Bcl-2 or caspase families (6). CIAPIN1 was approved to be a mediator of RAS signaling pathway at least in Ba/F3 cells. The expression of CIAPIN1 is upregulated by RAS activity by a possibly indirect process. ~~And,~~ CIAPIN1 exerts an

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anti-apoptotic function probably by transcriptionally up-regulating the expression of Bcl-xL and Jak2. Mouse CIAPIN1 (mCIAPIN1) shares 82% amino acid identity to human CIAPIN1 (hCIAPIN1, Genbank No. **BC024196**). In previous studies~~studying gastric MDR~~, we ~~also~~ found that the expression of CIAPIN1 was upregulared at both mRNA and protein levels in ~~multidrug resistance~~ gastric cancer cell lines SGC7901/VCR and SGC7901/ADR, which are multidrug resistant, compared with their non-resistant parental cell line SGC7901 (7, 8). Moreover, ~~E~~exogenous expression of ~~human~~ hCIAPIN1 in SGC7901 leads to multidrug resistance (MDR) whereas downregulation of ~~human~~ hCIAPIN1 by small interference RNA sensitize~~d~~ SGC7901/ADR to anti-tumor chemotherapeutic drugs, indicating that CIAPIN1 is a mediator of gastric cancer MDR (9).

Recently, ~~Our immunohistochemical study also~~we demonstrated that CIAPIN1 was ubiquitously expressed in normal fetal and adult human tissues, with~~, G~~ gastric mucosa ~~was~~ being one of the tissues with high expression of CIAPIN1, suggesting CIAPIN1 might exert some important function~~(s)~~, such as anti-neoplastic effect, in gastric mucosal cells (10).

In ~~the~~ a study carried out by Shibayama *et al*, oncogenic H-Ras (H-RasG12V) ~~could~~ induced the expression of ~~mouse~~ mCIAPIN1 (6). In our ~~immunohistochemical~~ study (10) ~~to survey the expression of CIAPIN1 in normal fetal and adult human tissues~~, ~~the results~~we ~~disclosed~~ observed that the expression pattern of CIAPIN1 in adult human tissues was similar to that of Ras as previously reported (5, 11-13). Ras signaling pathway is responsible for a wide-range of cellular behaviors such as proliferation, senescence, apoptosis and differentiation, and thus, is involved in tumorigenesis (14, 15).

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批注 [x6]: You may describe more results of this study, and introduce the relation of Ras and CIAPIN1 (a downstream effecter of receptor tyrosine kinase-Ras signaling pathway)?

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Ras is the most frequently mutated proto-oncogene in human tumors. Therefore, we postulated that CIAPIN1 may also play an important role in tumorigenesis, including gastric carcinogenesis, ~~As~~ as a downstream effecter of receptor tyrosine kinase-Ras signaling pathway~~., the expression and role of CIAPIN1 in tumors allured us to examine the expression and possible role in the development of gastric cancer.~~ In this study, we firstly examined the expression of CIAPIN1 in human normal, precancerous ~~lesions~~ and cancerous gastric tissues, and then, we investigated ~~the~~ *in vitro* influences of CIAPIN1 on ~~the~~ cell proliferation and cell cycle distribution of MKN28 and SGC7901 gastric cancer cells. We found that the expression of CIAPIN1 was progressively downregulated in ~~dysplastic and cancerous gastric mucosa compared with normal gastric mucosa, gastric mucosa with superficial gastritis or atrophic gastritis~~the gastric carcinogenesis process, and CIAPIN1 expression conferred ~~suppression~~ suppressive effects on cell proliferation and cell cycle progression of gastric carcinoma cells. These results ~~suggest~~ indicate that CIAPIN1 is a candidate tumor suppressor gene.

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Materials and Methods

Patients and tissue specimen preparation~~s~~

Two sets of gastric specimens were used for the detection of CIAPIN1 protein expression. ~~Approval to conduct this study was obtained from the Fourth Military Medical University and Xi'an Jiaotong University Review Boards.~~ For immunohistochemical evaluation of CIAPIN1 expression, ~~human~~ cancerous and adjacent non-cancerous (~~defined as tissue taken from~~i.e. at least 2.0 cm away from the tumor mass)

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gastric specimens were obtained by surgical resection from 83 patients (M/F, X/Y, average age of XXX, age range, X-X years old) who were admitted to Xijing Hospital, The Fourth Military Medical University. ~~The cancers were histologically classified according to the International Histological Classification of Tumors published by the World Health Organization (WHO) (16). Tumor Node Metastasis (TNM) classification was applied with regard to depth of tumor invasion (17). For purpose of statistical analysis, stage Ⅰ and Ⅱ tumors were combined, as were stage Ⅲ and Ⅳ tumors.~~ In addition, ~~non-malignant~~ gastric mucosa specimens were taken from the gastric antrum ~~endoscopically~~ from 55 patients (M/F, X/Y, average age of XXX, age range, X-X years old) during~~underwent~~ upper ~~endoscopic~~ endoscopy ~~examination~~ for non-ulcer dyspepsia in the same hospital. All the tissues were fixed in paraformaldehyde in 0.1 M PBS, pH 7.4, at room temperature for at least 3 hours. The fixed tissues were dehydrated in ethanol for paraffin embedding. Five-μm thick sections were cut and mounted for hematoxylin and eosin (H&E) staining and immunostaining staining. ~~The histological diagnoses of the specimens were confirmed by Hematoxylin and Eosin (H & E) staining.~~ The adjacent non-cancerous tissues (n=83) and biopsied gastric mucosa ((~~totally~~ n=55~~138 samples~~) were histologically ~~divided~~ classified ~~into~~ as normal, superficial gastritis (or chronic gastritis), atrophic gastritis (or gastric atrophy), intestinal metaplasia (IM) and dysplasia based on criteria proposed by the Chinese Association of Gastric Cancer (18), or Updated Sydney system (Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am J Surg Pathol. 1996 Oct;20(10):1161-81.). Gastric cancer was histologically diagnosed and classified according to the International

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Histological Classification of Tumors published by the World Health Organization (WHO) (16). Tumor-node- metastasis (TNM) classification was applied with regard to depth of tumor invasion (17). For purpose of statistical analysis, stage I and II tumors were combined, as were stage III and IV tumors.

For Western blotting analysis of CIAPIN1 expression ~~in human gastric cancer and benign gastric mucosa~~, ~~an~~other ~~set of 40~~ 25 patients (15 patients with gastric cancer and 10 with non-ulcer dyspepsia) were recruited in the First Affiliated Hospital, School of Medicine, Xi'an Jiaotong University. Gastric ~~endoscopically biopsied~~ specimens ~~(~~including ~~10 grossly normal gastric mucosa and 15~~ cancerous ~~as well as~~and their adjacent non-cancerous gastric tissues (i.e. at least 2.0 cm away from the tumor mass) from the 15 gastric cancer patients and antral mucoma from the 10 non-ulcer dyspepsia patients,were obtained ~~from Department of Gastroenterology, 1st Affiliated Hospital, School of Medicine, Xi'an Jiaotong University~~. Fresh gastric tissues were snap frozen in liquid nitrogen immediately and stored at –80°C~~C~~.

Approval for conducting this study was obtained from the Institutional Review Boards of Fourth Military Medical University and Xi'an Jiaotong University, Xi'an, China.

Immunohistochemistry

Immunohistochemistry was performed using the mouse monoclonal antibody against CIAPIN1 (clone 3C6) produced by our laboratory as described elsewhere (3) and Histostain™-Plus SP kit (ZYMED, USA.). Briefly, the sections were deparaffinized with

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xylane and rehydrated through gradient ethanol emmersion. Endogenous peroxidase activity was quenched by 0.3% (vol/vol) hydrogen peroxide in methanol for 20 min, followed by three 5-min washes in phosphate buffered saline (PBS). The sections were was then blocked with 10% (vol/vol) normal goat serum in PBS for 1 h and subsequently incubated with the anti-CIAPIN1 antibody diluted (1:200) in PBS containing 0.1% (wt/vol) overnight at 4℃. Negative control was performed with replacing the primary antibody with pre-immune mouse serum. After three 5-min washes with PBS containing 0.02% (vol/vol) Tween-20 (PBST), the sections were treated with biotinized goat anti-mouse antibody for 20 min at room temperature, followed by another three 5-min washes with PBST. Then, the specimens were incubated with streptavidin–horse radish peroxidase (S-A/HRP) for 20 min at room temperature, followed by washes as above. Reaction product was visualized with diaminobenzidine (DAB) at room temperature for 5 min. Counterstaining was performed with hematoxylin for 30 seconds and washed with tap water. The sections were immediately dehydrated by sequential immersion in gradient ethanol and xylene, and mounted with Permount permount and cover slips. Images were obtained from an Olympus BX51 microscope (Olympus, Japan) equipped with a DP70 digital camera. H & E staining was also performed to verify the histological diagnosis.

Expression of CIAPIN1 was evaluated according to the ratio of positive cells per specimen and staining intensity as described previously (19). The intensity of the staining was estimated on a scale from 0 to 3 ([0, (absent);, 1, (weak),; 2, (moderate;), and 3, (strong)] and the area of positivity by on a value from 1 to 3s (1, (focal or <10%;), 2, (10–49%;), and 3, (≥50%)], both in consensus of three investigators. Intensity The

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overall intensity score of the immunoreactivity was ~~obtained~~ calculated by multiplying the intensity scale ~~and~~ with the area value~~s~~. Cases with ~~A~~an overall ~~total~~ score ~~of~~ between 6~~–~~ and 9, bwtween 2-4 and between 0-1 ~~was~~were ~~considered~~ defined as having strong, ~~2–4~~ weak, and ~~0–1~~ negative immunoreactivity, respectively.

Western blotting

Tissue lysis was performed with homogenization in RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.02% sodium azide, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% nonidet P-40 and 0.5% sodium deoxycholate) on ice-bath followed by centrifugation at 12,000 rpm for 10 min at 4°C~~℃~~. ~~The supernatants were collected for Western blotting analysis.~~ The concentration of total cell protein~~s~~ in the supernatants was determined by Bradford assay. Equal amounts (50 μg) of total proteins were loaded in 12% SDS-PAGE and electroblotted to a nitrocellulose (NC) membrane (Milipore, Bradford, CA), as previously described (reference). Non-specific binding was blocked with 5% nonfat milk in PBST for 1 h~~r~~ at room temperature. Then, the membrane was incubated with anti-CIAPIN1 Mab (1: 400 in blocking solution) overnight at 4°C~~℃~~, rinsed with PBST ~~for~~ three times, followed by incubation with HRP-labeled goat anti-mouse IgG for 1 h. After ~~three times~~ wash~~ing~~ with PBST three times, the bands were developed with enhanced chemiluminescence (ECL) reagent for 5 min.

CIAPIN1 ectopic expression and siRNA plasmid vector~~s~~ construction (9)

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–A plasmid expressing human CIAPIN1 (pCDNA3.1-hCIAPIN1) was constructed by inserting hCIAPIN1 CDS into the *EcoR*I/*Xho*I sites of pCDNA3.1(+) (Invitrogen, city, country). Two plasmids expressing hCIAPIN1 small interference RNA (siRNA) were constructed. The designed hCIAPIN1 siRNA sequences are: 5'-G GATCCGCAGATGAGCTCCCAACCAATTCAAGAGATTGGTTGGGAGCTCATCTG TTTT

TTGGAAAAGCTT-3' (siRNA1) and 5'-GGATCCGCCAAAGTCAGCTTGTGGAATTCAAGAGAT

_TCCACAAGCTGACTTTGGTTTTTTGGAAAAGCTT-3' (siRNA2). The forward and reverse sequences were synthesized, ~~anealled~~annealed and cloned into the *EcoR*I/*BamH*I sites of pSilencer 3.1-Neo (Ambion, Austin, TX), yielding recombinant plasmids pSihCIAPIN1-1 and pSihCIAPIN1-2. The inserted sequences were confirmed by DNA sequencing. Insert-free pcDNA3.1(+) and pSilencer 3.1-Neo were used in transfection as controls.

Cell culture, transfection and colony selection

—SGC7901 and MKN28 gastric adenocarcinoma cells were cultured in DMEM supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO). The cells were maintained in a humidified chamber with 5% CO2 at 37 ℃.

—Transfection was performed with Lipofectamine™ 2000 following the manufacturer's instruction. Briefly, SGC7901 cells were seeded into a 24-well plate one day before transfection. Upon transfection, 0.8 μg of the plasmid and 2 μl of

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Lipofectamine™ 2000 were mixed in 0.2 ml DMEM and added into the wells. ~~Twenty four hours later, the~~ The cells were tryptinized and ~~1:5~~ splitted at the ratio of 1:5 24 h later. After another 24 h~~ours~~, G418 (geneticin, Gibco BRL) was added to reach a final concentration of 400 μg/ml to select the resistant colonies. After about 8 wks, when the cell colonies were visible, individual colonies were picked up and cultured firstly in 24-well plates and then in 6-well plates. At about 80% confluence, the cell layer was washed with ice-cold PBS~~,~~ (pH 7.4), and scrapped off. Cells were harvested by centrifugation and lysed in RIPA lysis buffer in ice bath for 30 min. After 12,000 rpm centrifugation for 10 min at 4 ℃ ~~℃~~, the supernatant was collected for Western blotting to select colonies with upregulated and downregulated CIAPIN1, respectively. Protein concentration of the supernatant was determined by Bradford assay.

Cell proliferation

~~For cell proliferation experiment,~~ C~~c~~ells were seeded into 96-well plates at 400 cells/well and cultured at X ℃. Then, 20 μl ~~Twenty milliliter~~ of Cell Counting Kit (CCK)-8 ~~reagent~~ solution (source, XXXXX) was added to each well ~~at~~ on days 1, 2, 3, 4, 5, 6 and 7 ~~days~~ after seeding. Two hours after addition of the CCK-8 reagent, optical density (OD) ~~at 495 nm~~ was determined at 495 nm. For each experiment, the CCK-8 assay was conducted in quadruplicate and the experiment was repeated for three times.

Determination of cell cycle distribution~~.~~

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批注 [x19]: The Cell Counting Kit (CCK)-8 assay? You should provide the reference or the company who produces it.

批注 [x20]: Same wells or different wells on different days? I guess different wells, i..e. the wells on different days were all different.

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~~For cell cycle measurement,~~ C~~c~~ells were seeded into 6-well plates and cultured at X ℃ for 24 h. ~~Twenty four hrs later~~Then, cells were harvested by tryptinizing and single cell suspensions were prepared in suspension buffer (PBS + 2% FBS). The cells were washed ~~two times~~twice with suspension buffer, resuspended at 1-2×10^6 cells/ml, aliquoted into 1 ml/tube and fixed by adding 3 ml cold absolute ethanol and storing at 4℃ ~~℃~~for 1 h~~our~~. After being washed two times with PBS, cells were stained with propidium iodide (PI) staining solution (3.8 mM sodium citrate, 50 ug/ml PI in PBS) and RNA was removed by RNase A digestion at 4℃~~℃~~ for 3 h~~rs~~. Flow cytometry was ~~done~~ performed on a FACScan (Becton Dickinson, Heidelberg, Germany) equipped with Cellquest software, and cellular DNA content was determined for 1 × 10^4 cells.

Statistical analysis

Statistical analysis was performed with Mann-Whitney U test and student's t test were used to calculate the P value and to compare the differences of groups for immunohistochemistry. Statistical SPSS software package (SPSS Inc, Chicago) was used to analyze data. Differences were considered statistically significant at P <0.05.

R~~ESULTS~~esults

hCIAPIN1 expression is down-regulated in dysplastic gastric mucosa and gastric cancer tissues

CIAPIN1 was expressed uniformly in all normal gastric mucosa specimens detected,

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with 69.2% of the cases exhibit strong and 30.8% of the cases exhibit weak immunostaining (Fig. 1A and Table 1). In NUD patients with normal gastric mucosa, ~~S~~strong CIAPIN1 immunostaining ~~localizes~~ localized in the lower two thirds of the antral glands while the upper one third of the glands and the apical epithelia showed weak staining. In the gastric body glands, parietal cells exhibited strong positive staining while other cells were weakly stained.

CIAPIN1 expression in gastric atrophy and IM was slightly stronger to that in normal/chronic inflammatory gastric mucosa, but without a statistical significance (Fig 1, A-C). However, in cases with dysplasia, expression level of CIAPIN1 was significantly lower compared with that in histologically normal/ chronic inflammatory gastric mucosa; in some sections where normal, atrophic, IM and dysplastic gastric glands were all present, there was a sharp contrast in CIAPIN1 expression between normal glands with intense immunostaining and dysplastic glands with weak or negative immunostaining (Fig. D). ~~These sections provided reliable evidences of the downregulated expression of CIAPIN1 in dysplastic gastric glands. These results indicated that marked downregulation of CIAPIN1 expression occurs at dysplastic stage of gastric carcinogenesis.~~

Moreover, ~~C~~compared with that in the normal gastric mucosa, expression of CIAPIN1 was markedly decreased in gastric adenocarcinoma tissues, in which 47% was absent of CIAPIN1 immunostaining and only 20.5% showed strong immunostaining (Figures 1E, ~~–~~ and 1F and Table 1). Strong evidence~~s~~ for the difference in CIAPIN1 expression between cancerous and noncancerous gastric tissues came from some sections across the border of the cancer crater where there was a sharp contrast ~~was discerned~~ in

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批注 [x24]: I have stated this in NUD patients in the Methods.

批注 [x25]: This makes scenario more complicated, as you didn't mention you took biopsies from both antrum and body. Oherwise, you have to define and describe the antral, body and global expression of CIAPIN1. Obviously, the location of gastric cancers of the 83 patients should be presented. Were all them in the antral area?

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CIAPIN1 specific immunostaining between cancerous and coexisting non-cancerous tissues (Fig 1E).

FTo furthermore, confirm above results, Western blotting analysis of CIAPIN1 expression were then performed in a second set of 40 biopsy samples including 10 normal/inflammatory gastric mucosa, 15 gastric adenocarcinoma as well as their adjacent noncancerous counterparts. As shown in Fig. confirmed that2, relatively high level of CIAPIN1 was uniformly expressed at relatively high levels in normal/chronic inflammatory gastric mucosa of all the 10 NUD cases, at normal/inflammatory cases and various levels of CIAPIN1 were detected in all the 15 adjacent non-cancerous specimens, and at significantly low while expression of CIAPIN1 was obviously lower in gastric cancerous tissues of the 15 patients with gastric cancer (Figure 2).than their adjacent noncancerous tissues and normal/inflammatory gastric mucosa.

It is commonly considered that most of gastric cancers arise from dysplasia following atrophy and metaplasia. To search for further evidences of CIAPIN1 playing some role in gastric carcinogenesis and at which stage CIAPIN1 exerts its anti-carcinogenesis effect, the expression of hCIAPIN1 in normal gastric mucosa, gastric mucosa with superficial gastritis, atrophic gastritis, intestinal metaplasia and dysplasia was examined immunohistochemically. The results showed that CIAPIN1 expression in atrophic and IM gastric mucosa was similar to that in normal/inflammatory gastric mucosa (Fig 1, A-C), but in dysplastic gastric epithelium, expression level of CIAPIN1 was significantly lower compared with that in histologically normal/inflammatory gastric mucosa. As represented in Fig 1D, in some sections which included both normal/atrophic/IM and dysplastic gastric glands, a sharp contrast between intense immunostaining of normal glands and

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~~weak or negative CIAPIN1 immunostaining could be discerned. These sections provided reliable evidences of the downregulated expression of CIAPIN1 in dysplastic gastric glands. These results indicated that marked downregulation of CIAPIN1 expression occurs at dysplastic stage of gastric carcinogenesis.~~

~~Relationship~~ Association between CIAPIN1 expression and clinicopathological features of gastric carcinoma

Then, we analyzed the relationship between CIAPIN1 expression and the clinicopathological characteristics of the patients. Table 2 presents the association between CIAPIN1 expression with clinicopathological variables. TNM stage I ~~I~~ + ~~II~~II gastric cancers expressed higher levels of CIAPIN1 than stage ~~III+IV~~ III+IV cancers whereas the expression level of CIAPIN1 did not correlate with differentiation grade, metastasis, patients' age and gender (Table 2). ~~These results provide evidence that decreased CIAPIN1 expression level is associated with the progressiveness of gastric cancer but does not correlate with the differentiation of the cancer.~~

CIAPIN1 inhibits proliferation and cell cycle progression of gastric cancer cells in vitro

~~–~~To investigate the influence of CIAPIN1 expression on cell proliferation and cell cycle progression of gastric cancer cells, we constructed gastric cancer cell sublines with ectopic CIAPIN1 expression and CIAPIN1 knockdown via plasmid transfection and colony selection. Cells transfected with pSihCIAPIN1-1 gradually died during G418

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selection and no stable G418-resistent colony was obtained for further analysis, whereas colonies with ~~upregualted~~unregulated CIAPIN1 expression by phCAIPIN1 transfection, colonies with hCIAPIN1 knockdown by pSihCIAPIN1-2 transfection as well as their ~~respective~~ corrsponding controls (transfected with insert-free vectors) were successfully obtained after G418 selection and Western blotting confirmation. Therefore, we used them (SGC7901-phCIAPIN1, MKN28-phCIAPIN1, SGC7901-pSihCIAPIN1-2 and MK28-pSihCIAPIN1-2 for the subsequent experiments.

CCK-8 assay demonstrated that cells with ectopic hCIAPIN1 expression proliferated significantly slower than empty vector transfected control cells, whereas cells with hCIAPIN1 knockdown proliferated faster than the control cells, both for SGC7901 and MKN28 cell lines (Figure 3).

–Subsequently, we analyzed the cell cycle distribution of SGC7901 transfectants and, the results revealed that the population of cells in the S phase was remarkably decreased by phCIAPIN1 expression (15.6% of asynchronous SGC7901-phCIAPIN1 cells in the S phase of the cell cycle compared with 24.2% of control cells) but increased by hCIAPIN1 knockdown with pSihCIAPIN1-2 (28.8% of SGC7901-pSihCIAPIN1-2 cells compared with 23.9% of controls~~;~~ (Table 3). Cell cycle distribution of transfectants derived from MKN-28 yielded similar results (data not shown).

~~DISCUSSION~~Discussion

This study describes the aberrant expression of a newly identified gene, CIAPIN1, in gastric cancer and investigated the potential roles of CIAPIN1 ~~in gastric carcinogenesis~~ as well as the possible mechanisms of CIAPIN1 in gastric carcinogenesis~~played in gastric~~

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~~cancer cells~~. To our knowledge, this is the first report of CIAPIN1 expression in malignant tissues. In this study, we found that CIAPIN1 expression was remarkably downregulated in gastric cancerous tissues, compared with in normal gastric mucosa. This was demonstrated ~~firstly~~ by both immunostaining and ~~then confirmed by~~ Western blotting. ~~In addition, we also noted a drastically decreased expression of CIAPIN1 immunohistochemically in biopsied hepatic carcinoma (22 cases), esophageal carcinoma (6 cases) and surgically resected uterus cervical cancer (5 cases) and their respective adjacent non cancerous counterparts.~~ Furthermore, immunohistochemical staining of CIAPIN1 demonstrated that the expression of CIAPIN1 in gastric mucosa with ~~superficial~~ chronic gastritis, gastric atroph~~yic~~ ~~gastritis~~ and intestinal metaplasia was ~~similar~~ not significantly different from~~to~~ that in normal gastric mucosa, whereas the expression of CIAPIN1 was markedly downregulated in gastric mucosa with dysplasia. In addition, *in vitro* e~~E~~ctopic expression of CIAPIN1 suppressed cell proliferation and cell cycle progression of SGC7901 and MKN28 gastric cancer cells while knockdown of CIAPIN1 exerted opposite effects. These data collectively suggest that CIAPIN1 ~~might be~~is a negative regulator in gastric carcinogenesis.

Gastric carcinogenesis involves a slow but continuous stepwise evolution from ~~superficial~~ chronic gastritis, glandular atrophy to metaplasia, dysplasia and, finally, to adenocarcinoma (Correa P. Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. Cancer Res 1992;52(24):6735-40). Atrophy, metaplasia and dysplasia ~~are~~ have generally considered to be precancerous lesions and closely related to the onset of gastric adenocarcinoma (20, 21). In precancerous lesions, CIAPIN1 expression was

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~~similar~~ slightly reduced ~~in normal/inflammatory,~~in atrophic and metaplastic mucosa, but drastically downregulated in dysplastic mucosal tissues, suggesting that ~~reduced~~ expression of CIAPIN1 is progressively reduced in the multistep process and may~~might~~ participate in the early onset of gastric cancer, and thus CIAPIN1 might be a candidate tumor suppressor in gastric carcinogenesis. Analysis of the ~~relationship~~ association between CIAPIN1 expression and clinicopathological characteristics of the patients revealed that the expression level of CIAPIN1 was inversely correlated with TNM staging of gastric carcinoma but unrelated ~~with~~ to differentiation and metastasis, patients' age and gender, suggesting that downregulation of CIAPIN1 ~~persistent~~ is associated with the progression of gastric cancer.

The development of malignancies is currently regarded as the results of activation of oncogenes and/or inactivation of tumor suppressor genes. The principal function of some ~~of the~~ oncogenes and tumor suppressors is modulation of~~ng~~ ~~the~~ cellular proliferation. ~~The~~ In the present study, ~~demonstrated that~~ CIAPIN1 ~~could~~ inhibited ~~*in vitro*~~ cellular proliferation *in vitro* in two established gastric cancer cell lines, SGC7901 and MKN28. This finding was further strengthened by our recent experiments, which ~~about~~ aimed to determine the influence of CIAPIN1 on cell proliferation with recombinant adenovirus expressing CIAPIN1 and CIAPIN1-specific siRNA, also demonstrated that CIAPIN1 ~~suppresses~~ suppressed cell proliferation in various gastric and hepatic cancer cell lines (our unpublished data). ~~Because cellular proliferation is an important component of the malignant phenotype, the results of cell proliferation test are consistent with our results obtained from immunohistochemical staining of CIAPIN1 expression profile in gastric cancer~~. Furthermore, ~~Cell~~ the present study~~cycle analysis of the SGC7901 and MKN28~~

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~~transfectants~~ showed that CIAPIN1 ~~could~~ induced cell cycle arresting at G1/S phase, indicating that CIAPIN1 could suppress cell proliferation of gastric cancer cells by, at least partly, inhibiting cell cycle progression.

A number of ~~reports~~ studies have indicated that *in vitro* established MDR cancer cell sublines ~~had~~ have lower ~~proliferate~~ proliferative rates or increased doubling times, compared with their parental cell lines (22-30). ~~In o~~Our previous studies ~~it was~~ shown~~n~~ed that MDR gastric cancer cell line SGC7901/ADR and SGC7901/VCR proliferated slower than their parental cell line SGC7901, and~~,~~ the expression of CIAPIN1 was upregulated in MDR gastric cancer cell sublines SGC7901/ADR and SGC7901/VCR compared with their parental cell line SGC7901, ~~and~~ suggesting that CIAPIN1 is a mediator of gastric cancer MDR (7, 8, 10). Our present observation of the alteration in proliferation of transfectants with up- and down-regulated CIAPIN1 was consistent with our former findings.

As a newly identified gene, the function(s) and the regulation mechanism(s) of CIAPIN1 are largely unknown. According to the report by Shibayama et al, CIAPIN1 is a mediator of cytokine-Ras-MAPK signal transduction pathway (6). The fact that this gene is highly conserved in mammals and is ubiquitously expressed in human fetal and adult tissues and the similarity between the expression pattern of CIAPIN1 in normal human fetal and adult tissues and that of p21ras also support the above finding (9). Ras-MAPK pathway is considered to play~~ing~~ a critical role in the regulation of proliferation as well as carcinogenesis. Mutated Ras is confirmed to be a classical oncogene and is closely related to the development of malignancies originated from various tissues. However, the role of wild-type Ras in carcinogenesis is still far from elucidated, and~~,~~ recent studies

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have indicated that wild-type Ras ~~could~~ inhibits the proliferation of cancer cells *in vitro*, suggesting that wild-type Ras might serve as a tumor suppressor. (14, 15, 31-41). Although the overall expression level of p21ras is elevated in gastric adenocarcinoma, RAS mutation is rare (42-47). The reports on the expression level and activity of MAPK in gastric adenocarcinoma have been controversial (48-50). We postulated that XXXXX although ~~Hence,~~ the relationship between Ras and CIAPIN1 in malignancies is left to be further investigated.

The above observations collectively support the hypothesis that CIAPIN1 is a negative regulator of cellular proliferation. Because cellular proliferation is an important component of the malignant phenotype, our present findings suggest that CIAPIN1 might be used as an important target in the design of novel cancer therapies. ~~We think that these results~~The findings in the present study may ~~will~~ form the basis for further studies of this interesting gene, and the possible translation of the discovery of this molecule into potential use in cancer diagnosis and/or treatment.

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References

1. Alberts SR, Cervantes A, van de Velde CJ. Gastric cancer**:** epidemiology, pathology and treatment. Ann Oncol 2003; 14 (Suppl 2): ii31-36. Parkin DM, Bray F, Ferlay J, Pisani P.Global cancer statistics, 2002. CA Cancer J Clin. 2005 Mar-Apr;55(2):74-108. Parkin DM. The global health burden of infection-associated cancers in the year 2002. Int J Cancer. 2006 Jun 15;118(12):3030-44.
2. Tahara E. Molecular mechanism of stomach carcinogenesis. J Cancer Res Clin Oncol 1993; 119: 265–272.
3. Wu MS, Shun CT, Wang HP, et al. Genetic alterations in gastric cancer: relation to histological subtypes, tumor stage, and Helicobacter pylori infection. Gastroenterology 1997; 112: 1457–1465.
4. Yasui W, Yokozaki H, Fujimoto J, Naka K, Kuniyasu H, Tahara E. Genetic and epigenetic alterations in multistep carcinogenesis of the stomach. J Gastroenterol 2000; 35: 111–115.
5. Werner M, Becker KF, Keller G, Hofler H. Gastric adenocarcinoma: pathomorphology and molecular pathology. J Cancer Res Clin Oncol 2001; 127: 207–216.
6. Shibayama H, Takai E, Matsumura I, Kouno M, Morii E, Kitamura Y, Takeda J, Kanakura Y. Identification of a cytokine-induced antiapoptosis molecule anamorsin essential for definitive hematopoiesis. J Exp Med 2004; 199: 581-592.
7. Zhao Y, Han Y, Liu F, An H, Shi Y, Yu Q, Fan D. Differentially expressed gene profiles between multidrug resistant gastric adenocarcinoma cells and their parental cells. Cancer Lett 2002; 185:211–218.

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8. Hao Z, Qiao T, Jin X, Li X, Gao J, Fan D. Preparation and characterization of a specific monoclonal antibody against CIAPIN1. Hybridoma 2005; 24: 141-145.
9. Hao Z, Li X, Qiao T, Du R, Hong L, Fan D. CIAPIN1 confers multidrug resistance by upregulating the expression of MDR-1 and MRP-1 in gastric cancer cells. Cancer Biol Ther 2006; 5: 261-266.
10. Hao Z, Li X, Qiao T, Zhang J, Shao X, Fan D. Distribution of CIAPIN1 in normal fetal and adult human tissues. J Histochem Cytochem 2006; 54: 417-426.
11. Radosevich JA, Combs SG, Ma Y, Lee I, Gould VE, Thor A, Schlom J, Carney WP, Rosen ST. Expression of Ras oncogene p21 during human fetal development as determined by monoclonal antibodies RAP-5, Y13-259, and DWP. Virchows arch B Cell Pathol Incl Mol Pathol 1989; 56: 337-344
12. Chesa PG, Rettig WJ, Melamed MR, Old LJ, Niman HL. Expression of p21ras in normal and malignant human tissues: Lack of association with proliferation and malignancy. Proc Natl Acad Sci USA 1987; 84: 3234-3238
13. Furth ME, Aldrich TH, Cordon-Cardo C. Expression of Ras proto-oncogene proteins in normal human tissues. Oncogene 1987; 1: 47-58
14. Singh A, Sowjanya AP, Ramaskrishna G. The wild-type Ras: road ahead. FASEB J 2005; 19: 161-169.
15. Campbell SL, Khosravi-Far R, Rossman KL, Clark GJ, Der CJ. Increasing complexity of Ras signaling. Oncogene 1998; 17: 1395-1413
16. Kloeppel G, Solcia, E, Longnecker DS, Capella C, Sobin LH: et al. World Health Organization international histological classification of tumors. In: Histological typing of tumours of the exocrine pancreas, 1−61 edn 2 Berlin: Springer-Verlag,

1996.

17. Hermanek, P, Sobin, LH. (eds.) UICC TNM Classification of Malignant Tumours, Ed. 4. 2nd revision. Berlin: Springer-Verlag, 1992.
18. You WC, Blot WJ, Li JY Chang YS, Jin ML, Kneller R, Zhang L, Han ZX, Zeng XR, Liu WD. Precancerous gastric lesions in a population at high risk of stomach cancer. Cancer Res 1993; 53: 1317-1321.
19. Saukkonen K, Nieminen O, van Rees B, Vilkki S, Härkönen M, Juhola M, Mecklin JP, Sipponen P, Ristimäki A. Expression of Cyclooxygenase-2 in Dysplasia of the Stomach and in Intestinal-type Gastric Adenocarcinoma. Clin Cancer Res 2001; 7: 1923–1931.
20. Correa P. Human gastric carcinogenesis: a multistep and multifactorial process—First American Cancer Society award lecture on cancer epidemiology and prevention. Cancer Res 1992; 52: 6735–6740.
21. Lansdown M, Quiorke P, Dixon MF. High grade of dysplasia of the gastric mucosa: a marker for gastric carcinoma. Gut 1990; 31: 977-983.
22. Martin-Orozco E, Ferragut JA, Garcia-Penarrubia P, Ferrer-Montiel A. Acquisition of multidrug resistance by L1210 leukemia cells decreases their tumorigenicity and enhances their susceptibility to the host immune response. Cancer Immnol Immunother, 2005; 54(4): 328-336.
23. Yu DS, Ma CP, Chang SY. Establishment and characterization of renal cell carcinoma cell lines with multidrug resistance. Urol Res, 2000; 28(2): 86-92.
24. Yang LY, Trujillo JM. Biological characterization of multidrug-resistant human colon carcinoma sublines induced/selected by two methods. Cancer Res, 1990; 50(11):

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25. Yu DS, Chang SY, Ma CP. Characterization and modulation of transitional cell carcinoma cell lines with acquired multidrug resistance. Br J Urol, 1998; 81(2): 234-240.
26. Mimnaugh EG, Fairchild CR, Fruehauf JP, Sinha BK. Biochemical and pharmacological characterization of MCF-7 drug-sensitive and AdrR multidrug-resistant human breast tumor xenografts in athymic nude mice. Biochem Pharmacol, 1991; 42(2): 391-402.
27. Serra M, Scotlandi K, Manara MC, Maurici D, Lollini PL, De Giovanni C, Toffoli G, Baldini N. Establishment and characterization of multidrug-resistant human osteosarcoma cell lines. Anticancer Res, 1993; 13(2): 323-329.
28. Fertitta AM, Comin U, Terruzzi V, Minoli G, Zambelli A. Clinical significance of gastric dysplasia: A multicenter follow-up study. Endoscopy 1993; 25: 265
29. Klijn JG, Berns EM, Bontenbal M, Foekens J. Cell biological factors associated with the response of breast cancer to systemic treatment. Cancer Treat Rev, 1993; 19 (Suppl B): 45-63.
30. Lee WP. The role of reduced growth rate in the development of drug resistance of HOB1 lymphoma cells to vincristine.Cancer Lett, 1993; 73(2-3): 105-111.
31. Zhang Z, Wang Y, Vikis HG, Johnson L, Liu G, Li J, Anderson MW,. Sills RC, Hong HL, Devereux TR, Jacks T, Guan KL, You M. Wildtype Kras2 can inhibit lung carcinogenesis in mice. Nat genetics, 2001; 29: 25-33.
32. Diaz R, Lopez-Barcons L, Ahn D, Garcia-Espana A, Yoon A, Matthews J, Mangues R, Perez-Soler R, Pellicer A. Complex effects of Ras proto-oncogenes in

tumorigenesis. Carcinogenesis, 2004; 25: 535-539.

33. Passioura T, Dolnikov A, Shen S, Symonds G. N-ras-induced growth suppression of myeloid cells is mediated by IRF-1. Cancer Res, 2005; 65:797-804.
34. Singh A, Sowjanya AP, Ramakrishna G. The wild-type Ras: road ahead. FASEB J, 2005; 19: 161-169.
35. Fisher GH, Wellen SL, Klimstra D, Lenczowski JM, Tichelaar JW, Lizak MJ, Whitsett JA, Koretsky A, Varmus HE. Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. Genes Dev, 2001; 15: 3249–3262.
36. Kitayama H, Sugimoto Y, Matsuzaki T, Ikawa Y, Noda M. A ras-related gene with transformation suppressor activity. Cell, 1989; 56: 77–84.
37. Zhang Z, Wang Y, Vikis HG, Johnson L, Liu G, Li J, Anderson MW, Sills RC, Hong HL, Devereux TR, et al. Wild-type Kras2 can inhibit lung carcinogenesis in mice. Nat Genet, 2001; 29: 25–33.
38. Delgado MD, Vaque JP, Arozarena I, Lopez-Ilasaca MA, Martinez C, Crespo P, Leon J. H-, K- and N-Ras inhibit myeloid leukemia cell proliferation by a p21WAF1-dependent mechanism. Oncogene, 2000; 19: 783–790.
39. Guerra C, Mijimolle N, Dhawahir A, Dubus P, Barradas M, Serrano M, Campuzano V, Barbacid M. Tumor induction by an endogenous K-ras oncogene is highly dependent on cellular context. Cancer Cell, 2003; 4: 111–120.
40. Kitayama H, Sugimoto Y, Matsuzaki T, Ikawa Y, Noda M. A ras-related gene with transformation suppressor activity. Cell, 1989; 56: 77–84.
41. Spandidos DA, Frame M, Wilkie NM. Expression of the normal H-ras1 gene can

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suppress the transformed and tumorigenic phenotypes induced by mutant ras genes. Anticancer Res, 1990; 10: 1543–1554.

42. Ohuchi N, Hand PH, Merlo G, Fujita J, Mariani-Costantini R, Thor A, Nose M, Callahan R, Schlom J. Enhanced expression of c-Ha-ras p21 in human stomach adenocarcinomas defined by immunoassays using monoclonal antibodies and in situ hybridization. Cancer Res, 1987: 47:1413-1420.
43. Czerniak B, Herz F, Gorczyca W, Koss LG. Expression of ras oncogene p21 protein in early gastric carcinoma and adjacent gastric epithelia. Cancer, 1989; 64:1467-1473.
44. Kasper HU, Schneider-Stock R, Mellin W, Roessner A. P21 protein expression and ras-oncogene mutations in gastric carcinoma: correlation with clinical data. Int J Oncol 1998; 12: 69-74.
45. Yoo J, Park SY, Robinson RA, Kang SJ, Ahn WS, Kang CS. ras gene mutations and expression of Ras signal transduction mediators in gastric adenocarcinomas. Arch Pathol Lab Med, 2002; 126: 1096-1100.
46. Lee SH, Lee JW, Soung YH, Kim SH, Park WS, Kim SY, Lee JH, Park JY, Cha YG, Kim CJ, Nam SW, Kim SH, Lee JY, Yoo NJ. BRAF and KRAS mutations in stamoch cancer. Oncogene, 2003; 22: 6942-6945.
47. Craanen ME, Blok P, Top B, Boerrigter L, Dekker W, O fferhaus GJ, Tytgat GN, Rodenhuis S. Absence of ras gene mutations in early gastric carcinomas. Gut, 1995; 37:758-762.
48. Atten MJ, Attar BM, Holian O. Decreased MAP kinase activity in human gastric adenocarcinoma. Biochem Biophys Res Commun 1995; 212: 1001-1006.

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49. Bang YJ, Kwon JH, Kang SH, Kim JW, Yang YC. Increased MAPK activity and MKP-1 overexpression in human gastric adenocarcinoma. Biochem Biophys Res Commun 1998;250: 43-47.

50. Kuno Y, Kondo K, Iwata H, Senga T, Akiyama S, Ito K, Takagi H, Hamaguuchi M. Tumor-specific activation of mitogen-activated protein kinase in human colorectal and gastric carcinoma tissues. Jpn J Cancer Res 1998; 89: 903-909.

Tables

Table 1. Expression of CIAPIN1 in cancerous and their adjacent non-cancerous tissues of patients with gastric cancer and gastric mucosa of patients with non-ulcer dyspepsia according to gastric histological changes (i.e. normal ~~gastric~~ mucosa, ~~gastric mucosa with superficial~~chronic gastritis, gastric atrophy~~ic gastritis~~, intestinal metaplasia, dysplasia and gastric cancer)

	CIAPIN1 immunostaining		
	—	+	++
Cancer cases			
Adjacent tissue (n=83)			
Normal mucosa (n=)	0 (0%)	4 (30.8%)	9 (69.2%)
~~Superficial~~ Chronic gastritis (n=)[1]	0 (0%)	7 (41.2%)	10 (58.8%)
~~Atrophic~~ Gastric atrophy~~gastritis~~ (n=)[2]	8 (18.2%)	14 (31.8%)	22 (50%)
~~IM~~Intestinal metaplasia (n=) [3]	5 (11.6%)	18 (41.9%)	20 (46.5%)
Dysplasia (n=)[4]	9 (42.9%)	6 (28.6%)	6 (28.6%)
Cancerous tissue (n=83) [5]	39 (47%)	27 (32.5%)	17 (20.5%)
Patients with non-ulcer-dyspepsia (n=55)			
Normal mucosa (n=)	0 (0%)	7 (41.2%)	10 (58.8%)
Chronic gastritis (n=)	8 (18.2%)	14	22 (50%)

批注 [x45]: You cannot combine the readings of adjacent non-cancerous tissues and the specimens from NUD patients. First, the CIAPIN1 expression may be lower in adjacent tissues of cancer patients than the specimens of NUD patients. Second, statistically, the method to test the difference in CIAPIN expression between adjacent tissue and cancer tissues (same individuals, paired sample test) is different from that to test difference in CIAPIN expression between NUD patients and cancer patients (two different groups, two-sample test). Using the Table style I suggest, you may produce more meaningful results.

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		(31.8%)	
Gastric atrophy (n=)	5 (11.6%)	18 (41.9%)	20 (46.5%)
Intestinal metaplasia (n=)	9 (42.9%)	6 (28.6%)	6 (28.6%)
Dysplasia (n=)	39 (47%)	27 (32.5%)	17 (20.5%)

vs Normal, [1] $P > 0.05$; [2] $P = 0.132$; [3] $P = 0.111$; [4] $P = 0.008$, [5] $P < 0.001$; Mann-Whitney U test.

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Table 2. Correlation between CIAPIN1 expression and clinicopathological features in gastric carcinoma cases (n=83).

Variables	n	CIAPIN1 expression			*P*
		-	+	++	
Age†					
≥65	26	13	9	4	
＜65	57	26	18	13	0.559
Gender†					
Male	62	30	18	14	

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Female	21	9	9	3	1.00
Differentiation †					
Well	20	9	7	4	
Moderately	26	12	8	6	
Poorly	37	18	12	7	0.947
TNM Classification‡					
~~I +II~~I+II	44	17	14	13	
III~~III~~+~~IV~~IV	39	22	13	4	0.039
Metastasis†					
With	52	22	22	8	
Without	31	17	5	9	0.823

† *P* >0.05; ‡ *P* <0.05. Mann-Whitney U test.

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Table 3. Cell cycle distribution of SGC7901 transfectants

Cell	G1 (%)	S (%)	G2 (%)
SGC7901	68.1 ± 1.7	23.5 ± 2.3	8.4 ± 0.7
SGC7901/pcDNA3.1	64.6 ± 2.2 [1]	24.2 ± 1.4 [3]	11.2 ± 0.8
SGC7901/pCIAPIN1	71.5 ± 2.7	15.6 ± 2.4	12.9 ± 1.4
SGC7901/pSilencer	67.1 ± 2.1 [2]	23.9 ± 1.9 [4]	9.0 ± 2.0
SGC7901/pSihCIAPIN1-2	60.2 ± 1.8	28.8 ± 1.6	11.0 ± 1.5

Each number represents mean ± SD of three different experiments. [1] vs SGC7901/pcDNA3.1, t = 3.433, $P < 0.05$; vs SGC7901, $P > 0.05$. [2] vs SGC7901/pSihCIAPIN1-2, t = 4.41, $P < 0.05$. [3] vs SGC7901/pCIAPIN1, $P < 0.01$. [4] vs SGC7901/pSihCIAPIN1-2, t = 3.417, $P < 0.05$. Student's t test.

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Figure legends

Fig. 1. Representative ~~I~~immunohistochemical ~~detection~~ staining of CIAPIN1 in normal, intestinal metaplastic, dysplastic and cancerous gastric mucosa. (A), Uniform, strong CIAPIN1 staining in normal gastric glands~~;~~ (B), Strong CIAPIN1 expression in gastric atrophic ~~gastric~~ mucosa~~;~~; (C), Strong CIAPIN1 immunostaining in i~~I~~ntestinal metaplastic gastric mucosa~~; with strong CIAPIN1 immunostaining~~. (D), Downregulated expression of CIAPIN1 in dysplastic glands (~~. Note~~ arrow indicates that ~~expression of CIAPIN1 in dysplastic gastric mucosa in~~ a sharp contrast in the expression of CIAPIN1 between dysplastic gastric mucosa and~~to~~ normal gastric mucosa)~~;~~ And (E) & (F), Weak or undetectable CIAPIN1 immunostaining ~~Representative~~ in gastric adenocarcinoma ~~(specimens showing weak or absence of CIAPIN1 immunostaining.~~arrow indicates ~~Note~~ that in ~~specimen~~ Fig.1 E, which ~~stretched~~ covers ~~across~~ the border of the tumor and non-cancerous tissue, there was a sharp contrast in the expression of CIAPIN1 between cancerous gastric tissue (negative CIAPIN1 immunostaining) and adjacent noncancerous gastric mucosa ~~in cancerous gastric tissue was in sharp contrast to~~ (strong CIAPIN1 ~~immunostaining~~ immunostaining).~~in adjacent noncancerous gastric mucosa.~~ Bars = 100 μm.

Fig. 2. Western blotting ~~detection~~ of the expression of CIAPIN1 in gastric ~~–~~tissues. CIAPIN1 expression in ~~15~~ endoscopically biopsied cancerous specimens (T) and their adjacent noncancerous counterparts (P) of 15 gastric cancer patients as well as ~~10 grossly~~ normal gastric mucosal specimens (N) of 10 patients with non-ulcer dyspepsia were detected. β-actin in these specimens was also ~~detected~~ used as an internal control~~s~~.

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Fig. 3. Role of CIAPIN1 in regulating ~~the~~ cell proliferation of gastric cancer cells. (A, B), Western blotting ~~analysis~~ of CIAPIN1 expression in transfected SGC7901 (A) & MKN28 (B) cells,~~.~~ (C, D), CCK-8 assay of ~~M~~monolayer growth rates of transfected SGC7901 (C) & MKN28 (D) cells ~~were determined by CCK-8 assays~~. Values represent the mean ~~±~~standard error of the means (SEM) from at least three separate experiments.

批注 [x46]: This information may be added in the Methods (*Statistical analysis*).

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