CDK5 suppresses the metastasis of gastric cancer cells by interacting with and regulating PP2A

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Abstract. Several previous studies have demonstrated that cyclin-dependent kinase (CDK)-5 expression serves an important role in promoting the development of malignant tumours. We have previously reported that CDK5 suppresses gastric tumourigenesis. The aim of the present study was to investigate the mechanistic basis of CDK5. The results of immunoprecipitation and western blot analysis demonstrated that CDK5 could interact with serine/threonine-protein phosphatase 2A (PP2A). The use of an inhibitor of PP2A in CDK5-overexpressing gastric cancer (GC) cell lines antagonized CDK5-mediated suppression in GC cells. Further analysis revealed that PP2A expression was downregulated in GC and patients with low levels of PP2A had worse survival outcomes than those with high levels of PP2A (P=0.035). Therefore, the present study provided a novel mechanism for CDK5-mediated tumour suppression, suggesting that CDK5 may be an attractive target for future therapeutic strategies for treating GC. In addition, low levels of PP2A may indicate a tendency for poor prognosis in patients with GC.

Introduction

Gastric cancer (GC) has the fourth highest incidence among all types of cancer and is the third leading cause of cancer-associated mortalities worldwide (1). Despite a steady decline in the incidence and rate of mortality associated with GC due to improved nutrition and the eradication of H. pylori in recent years, GC remains a major public health concern (1). Although various treatment options are available, GC has a poor prognosis (2). Human GC tumourigenesis is a multistep and multifactorial process that is associated with several genetic and molecular alterations, including the activation of various oncogenes, inactivation of tumour suppressor genes and abnormal expression of cell cycle-associated proteins (3-6).

The abnormal expression and dysregulation of cyclin-dependent kinases (CDKs), including CDK1, CDK2, CDK3, CDK4 and CDK6, have recently emerged as important mechanisms underlying the tumourigenesis of certain types of cancer (7-18). However, the role of CDK5 in GC remains relatively unknown. CDK5 is a proline-directed serine/threonine kinase that was first discovered and reported by Hellmich et al in 1992 (19). Unlike the other CDKs, CDK5 has no known cell cycle or mitotic function and is not activated by cyclins (20). Recently, CDK5 activities beyond the nervous system have emerged, and an increasing body of evidence has indicated that CDK5 may serve a role in cancer tumourigenesis and progression (21-25). Our previous study demonstrated that CDK5 levels decrease in GC and that CDK5 nuclear accumulation suppresses gastric tumourigenesis (26).

Serine/threonine-protein phosphatase 2A (PP2A) is a serine/threonine phosphatase that is comprised of catalytic, scaffold and regulatory subunits. The catalytic and scaffold subunits have 2 isoforms, and the regulatory subunit is derived from 4 different families of isoforms. The regulatory subunit is the most diverse, with temporal and spatial specificity. PP2A dephosphorylates a number of critical cellular molecules, including protein kinase B, mitogen-activated protein kinase kinase (MEK), extracellular signal-regulated kinase (ERK), p53, c-Myc, and β-catenin; it also regulates a variety of cellular processes, including cell proliferation, signal transduction and apoptosis (27). Aberrant expression, mutations and somatic alterations of PP2A have been associated with the development of human lung (28), breast (29), skin (27) and colon cancers (30). Tsuchiya et al (31) reported that the phosphatidylinositol derivative, 1,2-O-bis-[8-[2-(2-pentyl-cyclopropymethyl)-1-cyclopropyl]-octanoyl]-sn-glycero-3-phosphatidyl-D-1-inositol, serves as an enhancer of PP2A to dephosphorylate and inactivate MEK, thereby inducing the caspase-independent apoptosis of MKN28 human GC cells with high MEK activity. However, the role of
PP2A in GC metastasis has not been reported. Based on our previous research, it was hypothesized that a functional association between CDK5 and PP2A may affect GC metastasis.

Materials and methods

Cell culture. The human GC cell line HGC-27 was obtained from the Cell Line Bank, Chinese Academy of Sciences (Shanghai, China). The cell line was verified by polymerase chain reaction (PCR) and cultured without mycoplasma contamination; the species origin was also confirmed by PCR. In addition, the identity of the cell line was authenticated with short tandem repeat profiling. HGC-27 cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Immunoprecipitation (IP). Cells were washed with ice-cold PBS and lysed in Tris-buffered saline (pH 7.4) containing 50 mmol/l Tris, 150 mmol/l NaCl, 1% Nonidet P-40, 1 mmol/l EDTA, 1 mmol/l Na₃VO₄, 10 mmol/l NaF, 2.5 mg/ml aprotinin and leupeptin, 1 mmol/l glycophosphate plus 4-(2-aminophenyl) benzenesulfonyl fluoride hydrochloride and 10 mmol/l iodoacetate, on ice for 15 min prior to removing cellular debris and nuclei by centrifugation at 10,000 x g for 5 min at 4°C. Cell lysates were incubated with the corresponding primary antibody CDK5 (cat. no. 2506; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C. Protein A-Sepharose beads (Amersham; GE Healthcare, Chicago, IL, USA) in a 50:50 mixture in 50 mmol/l Tris buffer, pH 7.0, were added and further incubated for 4 h at 4°C. The immunoprecipitates were washed 4 times with Tris-buffered saline and boiled for 5 min in 40 µl Laemmli buffer containing 0.02% blue bromophenol and 2% mercaptoethanol.

Western blot analysis. The cells were seeded into 60-mm dishes at a concentration of 5x10⁴ cells/well (20-30% confluence) and cultured to 80% confluence. The cells were then scraped and lysed in Radioimmunoprecipitation Assay buffer, and the lysates were centrifuged at 10,000 x g (4°C for 10 min). Protein concentrations were determined using the BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. A total of 40 µg of protein from each sample was denatured, loaded into each well, separated by 10% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA) and purified by Shanghai GeneChem Co., Ltd. (Shanghai, China) and were transfected into the HGC-27 cell line (5x10⁴ cells/well in 6-well plates) with the Xfect Transfection Reagent (cat. nos. 631317 and 631318; Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's protocol. EGFP-CDK5-WT plasmids were then introduced using doxycycline (Sigma-Aldrich; Merck KGaA; cat. no. D9891). Then, once selected using puromycin or G418 (Sigma-Aldrich; Merck KGaA), stable cells were harvested and stored in liquid nitrogen until subsequent use. CDK5 knock-down using Lenti-shCDK5, HGC-27 cells were transfected with shPP2A (lenti-shPP2A) and control plasmid (Shanghai GeneChem Co., Ltd.) according to the manufacturer's protocol using LipofectamineTM 3000 Transfection Reagent (Thermo Fisher Scientific, Inc.). All of the stable cells were then stored in liquid nitrogen for subsequent experimentation within 6 months post-transfection. CDK5-overexpressing GC cells

Plasmids and the construction of stable transfectants for immunofluorescence. EGFP-CDK5-nuclear localisation signal (NLS), EGFP-CDK5-nuclear export signal (NES), EGFP-CDK5-wild-type (WT), pTRE-EGFP, short hairpin (sh)-CDK5 (Lenti-shCDK5) and the corresponding empty vectors Lenti-scramble (Lenti-scr) were constructed, packed and purified by Shanghai GeneChem Co., Ltd. (Shanghai, China) and were transfected into the HGC-27 cell line (5x10⁴ cells/well in 6-well plates) with the Xfect Transfection Reagent (cat. nos. 631317 and 631318; Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's protocol. EGFP-CDK5-WT plasmids were then introduced using doxycycline (Sigma-Aldrich; Merck KGaA; cat. no. D9891). Then, once selected using puromycin or G418 (Sigma-Aldrich; Merck KGaA), stable cells were harvested and stored in liquid nitrogen until subsequent use. CDK5 knock-down using Lenti-shCDK5, HGC-27 cells were transfected with shPP2A (lenti-shPP2A) and control plasmid (Shanghai GeneChem Co., Ltd.) according to the manufacturer's protocol using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, Inc.). All of the stable cells were then stored in liquid nitrogen for subsequent experimentation within 6 months post-transfection. CDK5-overexpressing GC cells
were treated with Okadaic Acid (Cell Signaling Technology, Inc.; cat. no. 5934) at 100 nM for 30 min according to the manufacturer's protocol prior to subsequent experiments.

**Immunofluorescence.** Cells were grown on glass coverslips, washed twice with PBS, fixed with PBS containing 4% formaldehyde at 4°C for 10 min, and permeabilized with 0.2% Triton X-100 in PBS at 4°C for 10 min. Following washing with PBS, cells were blocked with 10% goat serum (Abcam, Cambridge, MA, USA; cat. no. ab7481) at room temperature for 2 h. Then cells were incubated overnight at 4°C with primary antibody against CDK5 (Abcam; cat. no. ab151233; 1:1,000), washed with PBS and incubated with Alexa Fluor 488 IgG (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. A-11008; 1:100; according to the manufacturer's instructions. Cells were incubated with 4 μg/ml PBS containing 0.2% bovine serum albumin for 45 min at room temperature.). Alexa Fluor® 568 phalloidin (Invitrogen; Thermo Fisher Scientific, Inc.) was employed to observe the lamellipodia, and nuclei were stained with DAPI (Sigma-Aldrich; Merck KGaA) at 37°C for 5 min. The coverslips were mounted with the SlowFade® Gold reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and observed under a laser confocal scanning microscope.

**In vitro cell function experiments.** Cell migration (using non-Matrigel coated pore membrane) and invasion assays (using Matrigel coated pore membrane) were performed in Transwell chambers (polycarbonate filters of 8-mm porosity; BD Biosciences, Franklin Lakes, NJ, USA). Cells with serum-free RPMI-1640 were seeded in the upper chamber (5x10^3 cells/well) for 24 h at 37°C, then medium in the lower chamber was replaced with complete growth medium containing 5% FBS (Gibco; Thermo Fisher Scientific, Inc.) and chambers were cultured for 45 h at 37°C. Non-invading cells were removed from the upper surfaces of the invasion membranes and the cells on the lower surface were stained with 0.1% crystal violet for 5 min at room temperature. Cells at the subcellular level of the microporous membrane were observed and photographed under an optical microscope (magnification, x200).

 Colony formation assays were performed to assess the ability of the cells to form colonies. Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and Sulforhodamine B (SRB; Sigma-Aldrich; Merck KGaA; cat. no. S9012) cell proliferation assays were performed to examine cell viability and cell proliferation. Briefly, cells were seeded into 96-well tissue culture plates at 1,000 cells/well. Plates were incubated for 24, 48, 72, 96 or 120 h at 37°C with 5% CO₂ in a humidified incubator. Once cells were treated and following incubation, 20 µl of CCK-8 was added to each well and cells were further incubated for an additional 3 h. Plates were read on a Biorad ELISA plate reader (Biotek Instruments, Inc., Winooski, VT, USA) using a 450-nm filter. Results of at least three independent experiments were analysed in duplicate.

 The relative cell proliferation ratios were plotted along with non-treated controls to determine the 100% level of activity. For the SRB colorimetric assay, cells were seeded into 96-well tissue culture plates at 1,000 cells/well and allowed to adhere for 5 days. Following 24, 48, 72, 96 or 120 h, cells were harvested and subjected to the SRB protocol, as described previously (32).

**Human gastric tumour tissues.** GC tissues and the respective adjacent non-tumour tissues were obtained following R0 gastrectomy procedures from 80 patients admitted to Fujian Medical University Union Hospital (Fujian, China) between February 2013 to February 2014 as well as detailed information regarding the clinicopathological parameters. All of the patients underwent R0 gastrectomy. None of the patients underwent preoperative chemotherapy or radiotherapy. Cases with the following characteristics were also excluded: Remnant GC, treated with palliative surgery and concurrent malignancy in other organs. Postoperative adjuvant chemotherapy was performed using 5-fluorouracil-based drugs plus oxaliplatin in advanced cases. The pathological stage of the tumour was reassessed according to the 2010 International Union Against Cancer Tumour-Node-Metastasis (TNM) classification on GC (7th edition) (33). The present study was approved by the Ethics Committee of Fujian Medical University Union Hospital (no. UH-2015009), and written informed consent was obtained from all patients involved.

**RNA isolation and reverse transcription-quantitative PCR (RT-qPCR).** RNA isolation was performed using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's manual. RT-qPCR was performed with the ReverTra Ace qPCR RT kit (Toyobo Life Science, Osaka, Japan) using oligo-dt-primers and 1 µg of mRNA/reaction as a template. The temperature protocol for RT-qPCR was as follows: 25°C for 5 min, 42°C for 60 min and 70°C for 5 min. RT-qPCR analysis was performed in triplicate on the ABI PRISM 7500 Sequence Detection System (Thermo Fisher Scientific, Inc.) using the Thunderbird SYBR qPCR Mix (Toyobo Life Science) according to the manufacturer's recommendations. The thermocycling conditions for qPCR were as follows: 40 cycles at 95°C for 3 min, 95°C for 15 sec and 60°C for 30 sec. The relative expression of PP2A was normalized to GAPDH expression levels. The primer sequences were as follows: PP2A, 5'-CGAGAGTCATGACACGGAG-3' (forward) and 5'-GAGAAGCTTTCCATTTGCC-3' (reverse); and GAPDH: 5'-GGTCGAGGTCAACGGATTG-3' (forward) and 5'-ATG AGCCGCCAGCTTCTCCAT-3' (reverse). RNA expression levels were obtained using the comparative 2-ΔΔCq method (34).

**Follow-up.** All patients were systematically followed up by trained doctors based on the institutional follow-up protocol using several approaches, including outpatient service, written letters, telephone calls, mail or visitation. Follow-up was conducted every 3 months during the first year and every 6 months from the second year onwards, and all surviving patients were followed for >3 years. The survival time was defined as the time from the date of surgery until the date of last contact or mortality. All 80 patients involved in the immunohistochemical analysis were followed up, and none were lost.

**Statistical analysis.** All statistical analyses were performed using SPSS 18.0 for Windows (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Chi-squared tests were used to evaluate differences in proportions. Differences among groups were assessed by one-way analysis of variance.
CDK5 overexpression in GC cells inhibits the development of GC. To examine the function of CDK5 in different subcellular locations, the present study first overexpressed EGFP-CDK5-WT, EGFP-CDK5-NLS, EGFP-CDK5-NES or p-TRE-EGFP in the HGC-27 GC cell line (Fig. 1A-C). Then, CCK-8 and SRB assays were performed, which...
revealed that cell proliferation and colony formation were significantly inhibited by nuclear CDK5 expression (Fig. 1D and E). Similarly, nuclear CDK5 expression markedly reduced the colony formation of HGC-27 GC cells and the metastasis of HGC-27 cells in Transwell assays. (Fig. 1F and G). Therefore, in vitro, CDK5 overexpression inhibited HGC-27 cell proliferation, colony formation and metastasis, which was consistent with our previous data obtained with the SGC-7901 and MGC-803 GC cell lines (26).

**Downregulation of CDK5 in GC cells promote metastasis.** Furthermore, to examine the effect of CDK5 on GC tumourigenicity, HGC-27 cells with CDK5 stably knocked down and control cells were generated (Fig. 2A). In vitro, it was observed that no significant differences were evident...
CDK5 mediates gastric cancer cell metastasis via PP2A

In terms of proliferation and colony formation between the shCDK5 cells and control cells (Fig. 2B and C). However, the knockdown of CDK5 in HGC-27 GC cells promoted metastasis (Fig. 2D).

Screening and verification of novel protein target of CDK5.
To investigate the functional mechanism of CDK5 in GC, the present study performed IP and LC-MS to identify potential targets of CDK5 in HGC-27 GC cells overexpressing EGFP-tagged CDK5-NLS, EGFP-tagged CDK5-NES or p-TRE-EGFP (Fig. 3A). Seven proteins were expressed in all of the groups (Fig. 3B). Further examination by co-IP and western blot analysis revealed that CDK5 interacted with PP2A (Fig. 3C). Therefore, it was hypothesized that CDK5 in combination with PP2A may serve a crucial role in GC.

Inhibition of PP2A antagonizes the metastasis of CDK5-overexpressing GC cells.
Next, cells were treated with okadaic acid, an inhibitor of PP2A, and alterations in the proliferation, colony formation and metastasis of HGC-27 GC cells overexpressing CDK5 and in the control group were observed. No significant differences were identified between the two groups in terms of proliferation (Fig. 4A), though a reduction in the metastasis of GC cells overexpressing CDK5 was revealed (Fig. 4B). Furthermore, knockdown of PP2A reduced metastasis (Fig. 4C and D) in CKD5 knockdown (shCDK5 #1) HGC-27 cells, but it did not affect proliferation (Fig. 4E). Consequently, it was concluded that CDK5 may influence the metastasis of GC by interacting with PP2A.

PP2A expression is downregulated in GC.
In the present study, it was demonstrated that PP2A interacted with CDK5 and subsequently influenced the metastasis of GC. For further analysis, RT-qPCR was performed to examine the relative expression levels of PP2A in 80 pairs of GC tissues matched with normal tissues (Fig. 5A and B). PP2A expression in the GC tissues was lower than in the corresponding adjacent non-tumour tissues (Fig. 5).
To analyse the association between PP2A expression and clinicopathological features, specimens were collected from 80 patients with GC. The cohort was divided into groups: Low PP2A expression group and a high PP2A expression group, according to the median PP2A value. No significant differences were observed in terms of the clinical and pathological characteristics between the high- and low-PP2A groups (Table I). To investigate whether PP2A could serve as a prognostic marker, overall survival (OS) curves were plotted using the Kaplan-Meier method according to the PP2A expression level. Patients with low levels of PP2A had a poorer OS rate when compared with those with high levels of expression (P=0.035; Fig. 6). In addition, to assess

### Table I. Association analyses between expression levels of serine/threonine-protein phosphatase 2A and clinicopathological features in 80 patients with gastric cancer.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Low expression (n=53)</th>
<th>High expression (n=27)</th>
<th>P-value</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Age, years</td>
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<td></td>
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</tr>
<tr>
<td>&lt;60</td>
<td>27</td>
<td>50.9</td>
<td>7</td>
</tr>
<tr>
<td>≥60</td>
<td>26</td>
<td>49.1</td>
<td>20</td>
</tr>
<tr>
<td>Sex</td>
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</tr>
<tr>
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<td>15</td>
<td>28.3</td>
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</tr>
<tr>
<td>Male</td>
<td>38</td>
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<tr>
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<tr>
<td>III</td>
<td>26</td>
<td>49.1</td>
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CEA, carcinoembryonic antigen; CA 19-9, cancer antigen 19-9; AJCC TNM, American Joint Committee on Cancer Tumour-Node-Metastasis.
whether the use of PP2A to predict OS was independent of the other clinical or pathological factors of the patients with GC, univariate and multivariate Cox proportional hazards analyses were performed. The results revealed that the PP2A levels and TNM stage were independent prognostic factors for OS in patients with GC (Table II). Therefore, low levels of PP2A may be indicative of a poor prognosis in patients with GC.

**Discussion**

Recently, many therapeutic strategies have been developed to improve the functional outcomes of GC (36,37). CDK5, a typical CDK that was previously considered to function in processes unrelated to cell cycle regulation, has been shown to serve a fundamental role in several types of carcinomas, including multiple myeloma, and breast, lung, prostate and pancreatic cancers (22,24,25,38,39).

In the present study, the results demonstrated that nuclear CDK5 overexpression markedly reduced the proliferation,
colony formation and metastasis of HGC-27 GC cells, which was consistent with our previous data obtained with the SGC-7901 and MGC-803 GC cell lines (26). However, CDK5 downregulation was observed to induce metastasis but not proliferation. Zhang et al (40) revealed that CDK5 could function as a cell cycle suppressor when localized to the nucleus of the neuron. To date, the mechanism through which CDK5 regulates the development of GC is unknown. Based on the present IP and liquid chromatography/MS analysis, it is possible that PP2A may be a potential target of CDK5.

PP2A, a serine/threonine phosphatase, comprises catalytic, scaffold and regulatory subunits and is associated with the development of numerous human cancers (27-30). Therefore, the aim of the present study was to understand whether CDK5 functioned with PP2A during the development of GC. When cells were treated with an inhibitor of PP2A, it was demonstrated that the metastasis of CDK5-overexpressing GC cells was restored without affecting proliferation. When PP2A was knocked down in CDK5 knockdown HGC-27 cells, the results were consistent those aforementioned. Therefore, it was proposed that CDK5 may influence the metastasis of GC by interacting with PP2A. In addition, Tsuchiya et al (31) identified a novel PP2A enhancer that induced the caspase-independent apoptosis of MKN28 GC cells, which is consistent with the present results. Further analysis revealed that relative PP2A expression was downregulated in 80 pairs of matched GC and normal tissues. Based on the median PP2A value, the cohort was divided into groups with low and high PP2A expression, and notably, the patients with high levels of PP2A had better survival outcomes than those with low PP2A expression (P=0.035). Furthermore, univariate and multivariate Cox proportional hazards analyses demonstrated that the PP2A level was an independent prognostic factor for OS in patients with GC.

In conclusion, to the best of our knowledge, the present study is the first to report that PP2A may affect metastasis by interacting with CDK5 in GC and that low levels of PP2A may indicate a tendency for poor prognosis in patients with GC.

Acknowledgements

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Availability of data and materials

The datasets analysed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JL conceived and designed the present study. YL and CMH contributed to the design of the present study. JL, YQS and PYZ performed the in vitro experiments. PL, JWX, JBW, and QYC obtained the tumor samples and the clinical data. JXL and LLC analysed the data. JL, PYZ and CHZ wrote and revised the manuscript. CHZ and CMH were also involved in the conception of the study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Fujian Medical University Union Hospital (Fujian, China; no. UH-2013009), and written informed consent was obtained from all patients involved.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

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