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Biology of Human Tumors

Clinical Cancer Research

Cyclin-Dependent Kinase 5 Decreases in Gastric Cancer and Its Nuclear Accumulation Suppresses Gastric Tumorigenesis

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Abstract

Purpose: As a cyclin-independent atypical CDK, the role of CDK5 in regulating cell proliferation in gastric cancer remains unknown.

Experimental Design: Expression of CDK5 in gastric tumor and paired adjacent noncancerous tissues from 437 patients was measured by Western blotting, immunohistochemistry, and real-time PCR. The subcellular translocation of CDK5 was monitored during gastric cancer cell proliferation. The role of nuclear CDK5 in gastric cancer tumorigenic proliferation and *ex vivo* xenografts was explored. Furthermore, by screening for compounds in the PubChem database that disrupt CDK5 association with its nuclear export facilitator, we identified a small molecular (NS-0011) that inhibits gastric cancer cell growth.

Results: CDK5 level was significantly decreased in the majority of gastric tumor tissues, and the reduction of CDK5 correlated with the severity of gastric cancer based on tumor and lymph node metastasis and patient 5-year fatality rate. Nuclear localization of CDK5 was found to be significantly decreased in tumor tissues and gastric cancer cell lines, whereas exogenously expression of nucleus-targeted CDK5 inhibited the proliferation and xenograft implantation of gastric cancer cells. Treatment with the small molecule NS-0011, which increases CDK5 accumulation in the nucleus, suppressed both cancer cell proliferation and xenograft tumorigenesis.

Conclusions: Our results suggest that low CDK5 expression is associated with poor overall survival in patients with gastric cancer, and nuclear accumulation of CDK5 inhibits the proliferation and tumorigenicity of human gastric cancer cells. *Clin Cancer Res; 21(6); 1419–28. ©2015 AACR.*

Introduction

Gastric cancer is a major public health concern, with a global incidence of approximately 989,600 in 2012, and is currently the second leading cause of cancer-related death after lung cancer (1–2). Human gastric cancer tumorigenesis is a multistep and multifactorial process related to various genetic and molecular alterations including the activation of various oncogenes, inactivation of tumor suppressor genes, and abnormal expression of cell-cycle–associated proteins (3–6). Cell-cycle progression is a highly ordered and tightly regulated process that requires sequential oscillation of cyclin expression and activation of cyclin-dependent kinases (CDK). Abnormal expression and dysregulation of cyclins and CDKs have recently emerged as an important mechanism underlying the tumorigenesis of certain types of cancers (7, 8). For example, dysregulation of classic CDKs including CDK1, CDK2, CDK3, CDK4, and CDK6 contributes to tumor development by initiating unscheduled cell division (9–18). However, detailed roles of CDKs in gastric tumorigenesis remain unclear.

CDK5 is a unique member of the CDK family. Despite its sequence homology to other CDKs (19), CDK5 can be activated by non-cyclin proteins p35 and p39 (20–22). Although CDK5 is ubiquitously expressed, the high neuronal expression of the p35 and p39 implies that CDK5 exerts its major physiologic function in a specific repertoire of tissues, such as the central nervous system. Indeed, previous studies indicated that CDK5 plays a role in a variety of pathologic and physiologic processes associated with neuronal migration during brain development, synaptic activity in mature neurons, and regulation of neuronal cell survival and death (23–25). Interestingly, these CDK5-dependent functions are unrelated to the regulation of the cell cycle as seen in other CDKs. Indeed, our previous studies have defined CDK5 as

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Translational Relevance

Cyclin-dependent kinases (CDK) contribute to tumor development by initiating unscheduled cell division. As an atypical CDK, CDK5 has been traditionally viewed as a functional component in the central nervous system rather than in cell proliferation. Here for the first time, we identify a dramatic reduction of CDK5 expression in gastric tumors and reveal a significant correlation of reduced CDK5 expression with advanced clinical stage and poor survival in gastric cancer patients. Moreover, we demonstrate that it is the nuclear localization of CDK5, rather than CDK5 activity, that suppress tumorigenic progression in gastric cancer. Our findings provide a basis for future work delineating pharmacologic modulation of CDK5 nuclear accumulation in gastric cancer and suggest that CDK5 may have a role in inhibiting cell-cycle progression.

an important cell-cycle suppressor in the nervous system (26–31): we found that nuclear but not cytoplasmic CDK5 can inhibit neuronal cell-cycle re-entry in a kinase activity–independent manner. We reported that CDK5 contains 2 nuclear export signals (NES) that interact with the nuclear export mediator CRM-1 and thus facilitate the cytoplasmic localization of CDK5. In addition, CDK5 has no intrinsic nuclear localization signal (NLS) and CDK5 nuclear localization relies on its binding between CDK5 N-17 terminal with the CDK inhibitor p27 (29). Upon serum starvation or other growth factor deprivation, the nuclear accumulation of p27 facilitates its binding with CDK5 and induces the nuclear localization of CDK5 (27, 29).

Interestingly, an increasing body of evidence over the past decade has suggested that CDK5 may also play a significant role in the tumorigenesis of multiple organs. For example, CDK5 has been implicated in regulating the motility of prostate cancer cells and glioblastoma cells, apoptosis of leukemia cells, astrocytoma, proliferation of medullary thyroid carcinoma cells, and phosphorylation of PIKE-A in glioblastoma cells. However, these CDK5-dependent tumorigenic processes are mainly based on its kinase activity and appear to be unrelated to the cell-cycle regulation (32–35).

To study the possible role of CDK5 in gastric cancer, we first compared CDK5 expression in gastric tumor tissues and respective adjacent nontumor tissues. We found that the expression of CDK5 was significantly decreased in gastric cancer and that reduced CDK5 expression was directly correlated with decreased survival. Reduced CDK5 in gastric tumor tissues prompted us to examine the nuclear localization of CDK5. Surprisingly, little nuclear CDK5 could be detected in gastric cancer cells, whereas CDK5 was clearly present in both the nucleus and cytoplasm in noncancerous gastric epithelial cells. Moreover, upon exogenously expressing nuclear CDK5 or chemically blocking CDK5 in the nucleus, we were able to inhibit the proliferation of tumor cells and tumorigenic xenografts in nude mice. Together, these results identify a critical role of nuclear CDK5 in the pathogenesis of gastric cancer.

Materials and Methods

Reagents

Antibodies against CDK2(M2), CDK3(Y-20), CDK4(C-22), CDK5(C-8), p35(C-19), p21(C-19), p27(N-20), and p57(C-20) were purchased from Santa Cruz Biotechnology. Anti-GAPDH and anti-GFP antibodies were from Cell Signaling Technology. Anti-CDK1(ab18), p16(EP1551Y), and hnRNP antibodies were from Abcam. Anti-tubulin, Anti-lamin A/C antibodies, and horseradish peroxidase–conjugated goat anti-rabbit IgG and goat anti-mouse IgG were from Sigma. Secondary antibodies (goat anti-mouse Alexa 488 and 594; goat anti-rat Alexa 488 and 594; goat anti-rabbit Alexa 488 and 594) used for immunocytochemistry were from Life Technologies. GFP-C3 was purchased from Clontech. The NLS (sequence: PKKKRKV from simian virus large T-antigen) was added into the GFP-C3 vector between *Nhe*I and *Age*I sites to generate GFP-NLS vector (31). CDK5 was amplified by PCR and inserted into GFP-NLS to generate GFP-CDK5-NLS vector. Site direct mutation of CDK5 on D144N (kinase dead) was performed using QuikChange Site-Directed Mutagenesis kit (Stratagene) to generate GFP-CDK5-KD-NLS. The NLS signal targets control GFP or GFP-CDK5 to the nucleus. Unless specified, all chemicals were obtained from Sigma-Aldrich Co.

Cell culture and transfection

Human gastric cancer cell lines MGC-803, SGC-7901, BGC-823, and the gastric epithelial cell line GES-1 were purchased from Shanghai Cell Bank. All the cell lines have been tested and authenticated by Shanghai Cell Bank. We took *Mycoplasma* tests for the cell line' authentication in our laboratory. The morphology and behavior were consistent with Shanghai Cell Bank descriptions. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in RPMI-1640/10% FBS/Pen/Strep medium. DNA constructs were introduced into cells using TurboFect or Lipofectamine 2000, following the manufacturers' protocols. Cells were refreshed with culture media 6 hours after transfection.

Tumor tissues

Human gastric tumor tissues and respective adjacent nontumor tissues (within a minimum distance of 5 cm from the excised tumor) of 437 patients were obtained from the Fujian Medical University Union Hospital (Fujian, PR China) with detailed clinic pathologic parameters and detailed follow-up information. All patients with gastric cancer were diagnosed and samples were obtained by gastrectomy with lymph node dissection from 2006 to 2012. None of the patients underwent preoperative chemotherapy and radiation therapy. The stage of each tumor was classified and histologically confirmed by pathologists. Samples from 171 patients (collected by gastrectomy from 2011 to 2012) were subjected to Western blotting. Samples from the other 266 patients (collected by gastrectomy from 2006 to 2007) were collected for immunohistochemical staining and RT-qPCR analysis. This study was approved by the ethics committee of Fujian Medical University Union Hospital and written consent was obtained from all patients involved.

Flow cytometric sorting

MGC-803 cells were seeded in 10-cm culture dishes for 24 hours before transfection. GFP-NLS, GFP-CDK5-NLS, or GFP-CDK5-KD-NLS plasmid was transfected into MGC-803 cells. Forty-eight hours after transfection, cells were collected and resuspended in 2 mL of FACS buffer (phosphate-buffered salt solution with 2% FBS) and filtered through a 40-μm cell

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strainer (BD Biosciences). GFP-positive MGC-803 cells were isolated using a Beckman Coulter MoFloTM XDP cell sorter with a 488-nm laser beam to excite GFP (collected in the 530/40 nm channel).

Tumor xenograft study

All animal procedures in this study were approved by the Institutional Animal Care and Use Committee of Xiamen University (Xiamen, PR China). For mouse xenograft experiments, MGC-803 cells (2.0×10^6) expressing GFP-NLS or GFP-CDK5-NLS with nearly 100% GFP transfection efficiency selected through flow cytometry were suspended in 150 μL PBS. Cells were subcutaneously injected into the right flank of 6-week-old male BALB/c immunocompromised mice. The tumor diameters were measured every 5 days using calipers. All mice were sacrificed 30 days after injection.

In some experiments, MGC-803 cells were pretreated with NS-0011 (2 μmol/L) or DMSO for 48 hours. Cells were then harvested and resuspended in 2.5 mL of PBS with NS-0011 (4 μmol/L) or DMSO. The cell number was counted using a MuseTM Cell Analyser (Merck) to approach approximately 2.0×10^7/mL. Two hundred microliters of cell suspension was injected into male BALB/c immunocompromised mice (6 week old) bilaterally (NS-0011 into the right side and DMSO control into the left side). Mice were sacrificed and the size of the tumors was measured 14 days after injection.

Isothermal titration calorimetry measurement

Isothermal titration calorimetry (ITC) experiments were performed at 25°C using an isothermal titration calorimeter (ITC200; MicroCal). The energetics for the binding of NS-0011 to CDK5 was determined in titration buffer containing 50 mmol/L HEPES. The reference chamber was filled with the corresponding degassed buffer. Purified CDK5 protein solution (80 mmol/L) was kept in the sample chamber, whereas the working solution of NS-0011 (1 mmol/L) was filled in a syringe. Before loading, all solutions were degassed. Each experiment was conducted with 20 injections of the working solution. To ensure a homogeneous mixing in the chamber, the stirrer speed was kept constant at 300 rpm. The heat effect per injection was determined by automatic peak integration of the thermal power versus time curve. The resulting data were fitted by using the Origin software from MicroCal. To account for the heat of dilution, the result of background titration was subtracted from each working solution titration.

Identification of NS-0011 by pharmacophore screening of PubChem

We previously developed a novel pharmacophore screening strategy to identify small molecules with a Michael acceptor moiety as the thiol-reactive "warhead" homing at CRM1 (chromosome region maintenance 1; also referred to as exportin1) as well as other "cysteinome" targets (36, 37). Briefly, a few query structures with sterically accessible β-carbon atoms that can react with cysteine residue were used to search PubChem database (209 sub-databases, 41,000,000 molecules in total). The following searching criteria were used during the screening procedure to optimize for positive hits: $250 \leq MW \leq 500$; $RB < 10$; $HBA \leq 10$; $HBA \leq 5$. Screening the entire PubChem database required 3 weeks and yielded more than 200 small molecules. which were then subjected to manual inspection. NS-0011 (PubChem CID: 25104471) was chosen for further experiments.

Computational analysis

The crystal structure of CDK5 was obtained from the PDB bank (PDB code: 1UNL). For clarity, the 2 NES domains of CDK5 were used in the simulation. Molecular docking was performed using our recently developed in-house docking tool FIPSDock (38). First, polar hydrogens and partial charges were assigned to the ligand, which was subsequently saved in PDBQT file format. The protein target files were prepared according to standard protocols. A grid cube with an edge length of about 37 Å (grid size of $96 \times 98 \times 110$ points with a spacing of 0.375 Å) was applied to the protein targets. The Amber99sb force field implemented in the latest Gromacs4.63 package was used throughout the molecular dynamics (MD) simulation. Partial atomic charges were assigned by the CHIMERA analysis package. The starting complex structure was taken from the prior docking simulation with the best ligand-binding affinity. A 10-ns simulation at constant pressure with a target temperature of 300 K and pressure of 1 atm was conducted. Both energies and coordinates were saved every 10 ps for the postproduction energetic analysis. The reference structure used in the root mean square deviation (RMSD) calculation was the energy-minimized structure (relaxed) at the first stage of MD simulation. All the simulations were performed on a high-performance DELL T7500 workstation with 24 core 2.66 GHz processors (32 GB memory) running Linux operating system.

Immunofluorescence staining and BrdUrd incorporation

Staining and bromodeoxyuridine (BrdUrd) incorporation assays have been described previously (29, 35). Detailed methods are available in the Supplementary files.

Cytosolic/Nuclear fractionation assay

The assays were performed as described previously (29, 35). Detailed methods are available in Supplementary files.

Statistical analysis

Clinicopathologic parameters were analyzed by χ^2 test. Univariate survival analysis was performed using the Kaplan–Meier method, and the significance of difference between groups was analyzed using the log-rank test. Stepwise multivariate survival analysis was carried out using the Cox proportional hazards regression model to measure the independent contribution of each variable to overall survival. All other statistical analyses were performed using SPSS 17.0 for Windows (SPSS), by one-way ANOVA (*post hoc*: Bonferroni test) or *t* test, and $P < 0.05$ was considered significant. Values were mean values ± SEM.

Results

Gastric cancer tissues exhibit reduction of CDK5 levels

We examined CDK5 mRNA and protein levels in gastric tumor and paired adjacent noncancerous tissues from 171 patients by RT-PCR and Western blotting, respectively. CDK5 protein expression was apparent in samples from 144 patients and relatively nondetectable in samples from the other 27 patients (Fig. 1A, Supplementary Fig. S1, bracketed, Supplementary Fig. S1B). In samples with detectable CDK5 protein expression, we observed that gastric tumor tissues had

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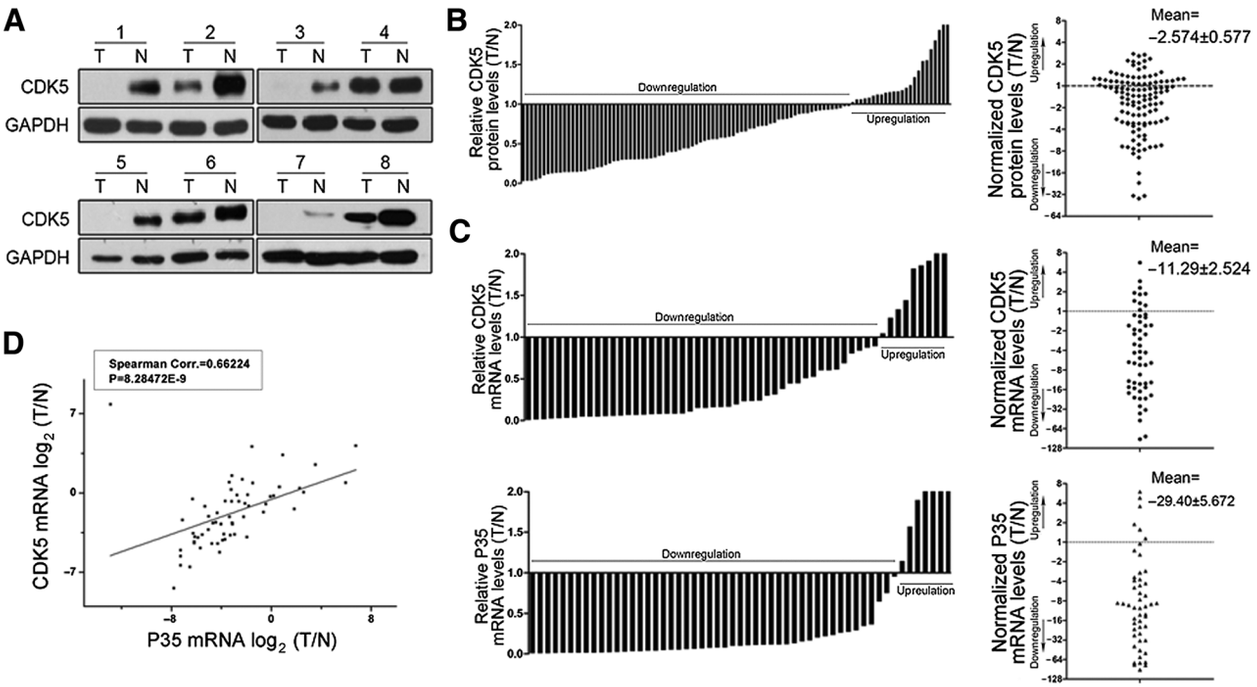

Figure 1.
Decrease of CDK5 expression in gastric cancer. A, CDK5 protein levels were measured in gastric tumor tissues and respective adjacent nontumor tissues from 171 patients. Representative bands are shown. B, left, CDK5 protein levels were quantified by densitometry. The ratios of CDK5 in gastric tumors compared with paired nontumor tissue controls (T/N) from 144 patients are presented. Gastric tumor tissues express significantly lower levels of CDK5 compared with noncancerous gastric tissues in the majority of patients (63%). Right, The reduction ratio average of CDK5 protein levels in T/N. Mean values ± SEM are presented. C, *CDK5* and *p35* transcripts were measured in gastric tumor and corresponding nontumor tissues by real-time qPCR. Ratios from gastric tumor compared with respective nontumor tissue controls (T/N) from 65 patients are presented. *CDK5* (among 77% or 46 of 60 of patient cases) and *P35* (among 85% or 51 of 60 of cases) transcripts were lower in tumor tissues than in noncancerous tissues. The reduction ratio average of *CDK5* or *P35* mRNA levels in T/N. Mean values ± SEM are presented on right. D, correlation comparisons between *CDK5* and *p35* transcripts (T/N) in human gastric cancer were evaluated, and the resulting Spearman correlation was calculated as 0.66 where $P = 8.28E\text{-}9$.

significantly low levels of CDK5 expression when compared with noncancerous gastric tissues in the majority of patients (63%, Fig. 1B), with an average 2-fold reduction calculated from all patients (Fig. 1B). In addition, we found that *CDK5* mRNA levels were much lower in tumor tissues than in noncancerous tissues (among 77% or 46 of 60 of patient cases), with an averaged 11.3-fold reduction. Since the level and activity of CDK5 is closely regulated by P35 (20, 26), we also studied and found that *P35* mRNA levels were lower in tumor tissues than in noncancerous tissues (among 85% or 51 of 60 of cases), with an averaged 29.4-fold reduction (Fig. 1C). Moreover, *p35* mRNA levels were significantly correlated with *CDK5* mRNA levels in patients with gastric cancer ($P < 0.001$, Fig. 1D).

Reduction of CDK5 in tumor is associated with poor prognosis in human gastric cancer

We next analyzed the clinicopathologic characteristics of gastric cancer patients with different levels of CDK5 expression and found that patients with nondetectable levels of CDK5 also exhibited a more advanced tumor–node–metastasis (TNM) stage and lymphatic metastasis (Fig. 2A), suggesting that CDK5 deficiency may exacerbate the pathologic development of gastric cancer.

To further evaluate the clinically prognostic role of CDK5 in human gastric cancer, we examined an additional 206 samples of gastric patients who underwent surgery at least 5 years ago and had paired detailed pathologic scoring record. Immunohistochemistry CDK5 staining was performed and samples were classified into 2 groups according to CDK5 expression: 155 of the 206 (75.2%) cases had lower expression levels of CDK5 in tumor tissues than in noncancerous tissues and were classified as "low CDK5," whereas the remaining 51 (24.8%) cases had a comparable CDK5 level between tumor and noncancerous tissues and were classified as "high CDK5" (Fig. 2B and C). Five-year survival analysis revealed that "low CDK5" group patients had a much shorter survival rate than "high CDK5" group patients (Fig. 2D). In addition, "low CDK5" group patients showed more deleterious clinicopathologic features, namely, the Borrmann type, grade of differentiation, depth of invasion, TNM stage, and lymph node metastasis than "high CDK5" group patients (Supplementary Table S1). Univariate analysis indicated that similar to other factors such as tumor location, tumor size, Borrmann type, grade of differentiation, depth of invasion, TNM stage, lymphatic metastasis, and distal metastasis, CDK5 expression level was also significantly associated with survival rate (Supplementary Table S2). Multivariate analysis indicated that lymphatic metastasis and CDK5 expression level were independent prognostics factors of gastric cancer (Supplementary Table S3).

Nuclear localization of CDK5 is depleted in gastric cancer cells

CDK5 normally localizes both in the nucleus and cytoplasm. Immunohistochemical analyses of samples from gastric cancer

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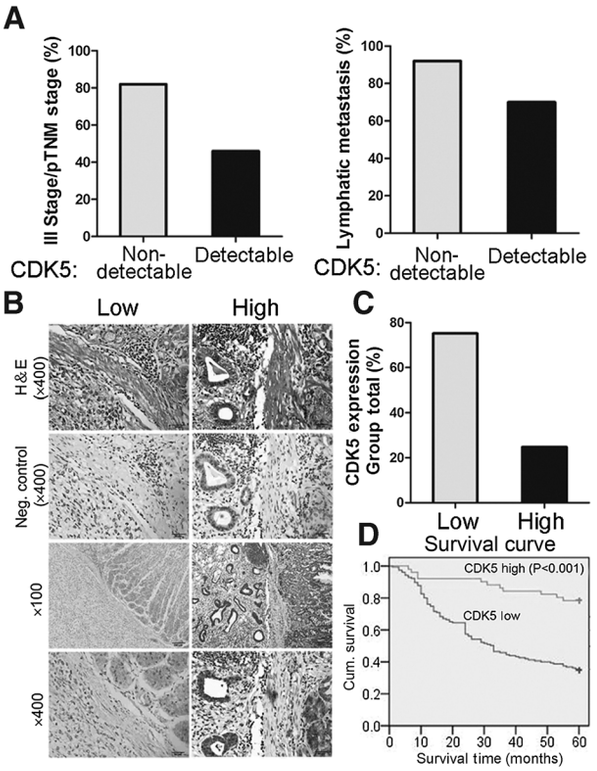

Figure 2.
CDK5 reduction in tumor tissues correlates with the severity of gastric cancer and patient 5-year fatality rate. A, comparison of clinicopathologic outcome with CDK5 expression. CDK5 was undetectable in 27 of 172 gastric cancer patients' samples by Western blotting. Patients with gastric cancer without CDK5 expression had significantly higher TNM stage (stage III or more) and lymphatic metastasis than those with CDK5 expression. *, $P < 0.05$. B, representative immunohistochemical staining of CDK5 in primary gastric tumor and adjacent nontumor tissues (magnification, ×100 and ×400). H&E staining was used to demonstrate gastric tissue characteristics. C, immunohistochemistry CDK5 staining was performed and samples were classified into 2 groups according to CDK5 expression: Low CDK5 group (155 of 206; 75.2%) and High CDK5 group (51 of 206; 24.8%). D, Kaplan–Meier survival curves of "High CDK5" and "Low CDK5" group gastric cancer patients. The survival time of patients of the "low CDK5" group was significantly shorter than that of patients of the "high CDK5" group ($P < 0.001$, log-rank test).

patients also indicated that CDK5 signal is apparent in both the nucleus and cytoplasm in nontumor cells. However, CDK5 staining is almost completely excluded from the nucleus of tumor cells (Fig. 3A). In addition, immunofluorescent studies showed that CDK5 was present in both the nucleus and cytoplasm in the gastric epithelial cell line GES-1 but clearly absent in the nucleus in 3 human gastric cancer cell lines (MGC-803, SGC-7901, and BGC-823; Fig. 3B). We also performed subcellular fractionation experiments and confirmed the observation that CDK5 was mostly depleted in the nucleus in gastric cancer cells (Fig. 3C). Unlike CDK5, classical CDKs such as CDK1, CDK2, CDK3, and CDK4 were all present in both the nucleus and cytoplasm in gastric cancer cell lines and gastric epithelial cell line (Supplementary Fig. S2A–S2D).

As a nucleocytoplasmic protein, CDK5 can shuttle between the nucleus and cytoplasm (29). By using immunofluorescent staining (Supplementary Fig. S3) and Western blotting (Fig. 3D) approaches, we observed that CDK5 largely accumulated in the nucleus in 2 gastric cancer cell lines (MGC-803 and SGC-7901) when cell proliferation was inhibited by serum starvation and serum replenishment re-localized CDK5 into cytoplasm. We previously found that the nuclear localization of CDK5 replies on its N-terminal binding with p27(29); as predicted, CDK5 lacking the N-terminus (N26 or N91) retained in the cytoplasm in the condition of serum starvation in MGC-803 and SGC-7901 cells (Supplementary Fig. S3). These results confirmed that the nucleus localization of CDK5 in gastric cancer cells is also dependent on its association with p27. The cell-cycle phases were measured and confirmed by flow cytometry (Supplementary Fig. S4).

Nuclear CDK5 suppresses gastric cancer cell proliferation and xenograft tumorigenesis

On the basis of our observation, it is conceivable to postulate that nuclear CDK5 is able to suppress gastric cancer cell growth. To prove this hypothesis and to test whether the kinase activity of CDK5 is involved in this process, we overexpressed GFP-tagged CDK5-NLS and kinase-dead CDK5 (CDK5-KD-NLS) in SGC-7901 and MGC-803 gastric cancer cell lines. BrdUrd incorporation assays showed that cell proliferation was significantly inhibited by nuclear CDK5 expression (Fig. 4A). Similarly, nuclear CDK5 or kinase-dead CDK5 expression dramatically reduced colony formation of both gastric cancer cells (Fig. 4B).

We next purified GFP-CDK5-NLS, GFP-CDK5-KD-NLS, and control GFP-NLS cells to homogeneity by FACS (Fig. 4C) and found that cells overexpressing nuclear CDK5 or CDK5-KD cells exhibited a much retarded *in vitro* growth rate than control cells (Fig. 4C and Supplementary Fig. S5). Furthermore, we performed xenograft experiments and found that although subcutaneously injecting cancer cells expressing control GFP-NLS resulted in tumor formation in immunocompromised mice injecting cells expressing GFP-CDK5-NLS could not. CDK inhibitors (CKIs) are key regulators for suppressing cell proliferation. We found that overexpression of nuclear CDK5 or kinase-dead CDK5 significantly increased p16 but had no effects on p21, p27, or p57 (the other 3 members of CKIs) expression in MGC-803 and SGC-7901 cells (Fig. 4D). These results strongly support the notion that CDK5 can suppress gastric tumorigenesis through its increased localization in the nucleus rather than through its activity change.

NS-0011 is identified as a CDK5 translocation inhibitor

CDK5 contains 2 NES which facilitate its interaction with the nuclear export mediator CRM-1 (29). We sought to identify compounds that can disrupt binding of CRM-1 to the NES domains of CDK5 and hence accumulate CDK5 in the nucleus. The small molecule NS-0011 was identified from PubChem database based on molecular docking coupled with MD simulation (Fig. 5A). The details of the computational protocol were described in the experimental methods. Through the *in silico* analysis, we found that NS-0011 was correctly fitted into the groove sandwiched between the 2 NES domains of CDK5 (residue 63-83 and residue 128-147, Fig. 5B and C). Moreover, the backbone residues of CDK5 complexed with NS-0011 exhibited a rather stable RMSD compared with the initial structure (Fig. 5A). A closer analysis of the structural complex derived from the MD

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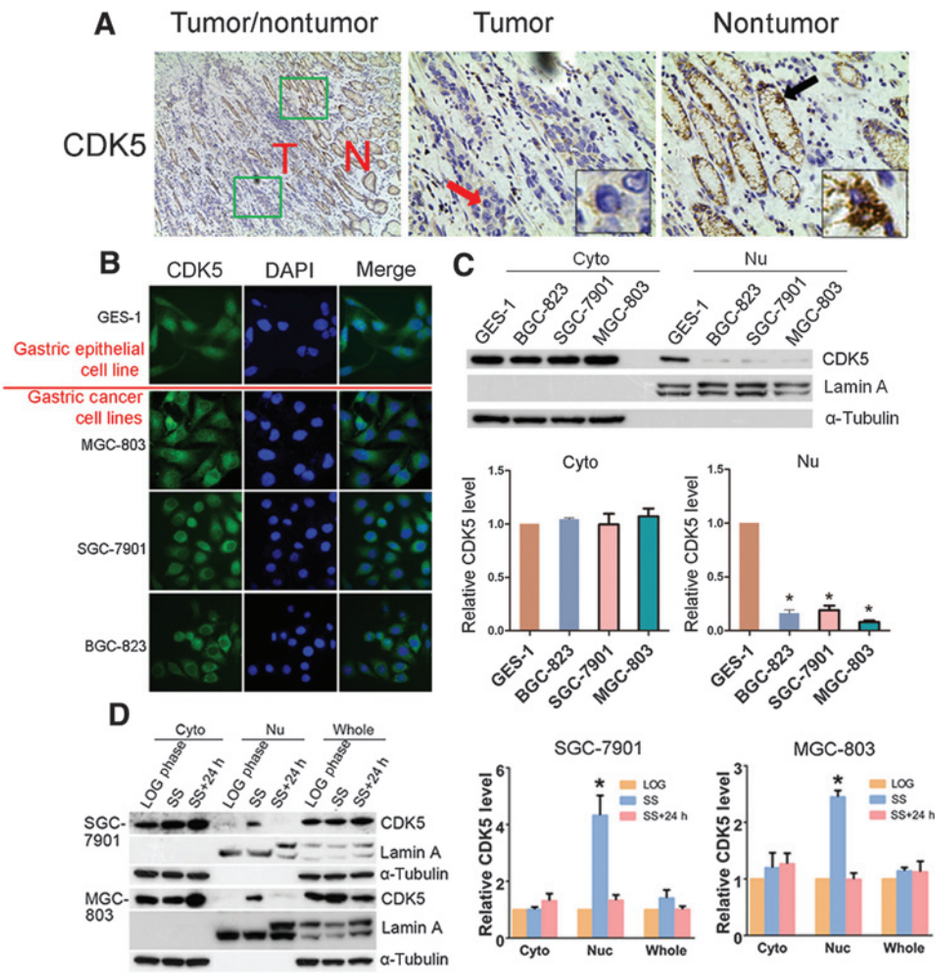

Figure 3.
Diminished nuclear localization of CDK5 in gastric tumor cells. A, representative immunohistochemical staining of CDK5 in gastric tumor tissues and adjacent nontumor tissues. Red arrow indicates negative staining of CDK5 in the nucleus of gastric tumor cells. Black arrow indicates positive staining of CDK5 in the nucleus of nontumor cells. B, immunofluorescent staining of CDK5 in one normal gastric epithelial cell (GES-1) and 3 gastric cancer cell lines (MGC-803, SGC-7901, and BGC-823). 4′,6-diamidino-2-phenylindole (DAPI) was used as a nuclear counterstain. C, cytoplasm (Cyto) and nuclear (Nu) fractions of the above 4 cell lines were generated. CDK5 levels were detected in each fraction by Western blotting. Lamin A and α-tubulin serve as nuclear and cytoplasmic loading controls, respectively. The quantification of CDK5 expression was presented in mean values ± SEM. *, $P < 0.001$ by ANOVA compared with GES cells. D, cells at different stages were harvested. Cytoplasmic (lanes 1–3, from left to right), nuclear (lanes 4–6), and whole-cell lysates (lanes 7–9) were analyzed for CDK5 by Western blotting. α-Tubulin and Lamin A serve as nuclear and cytoplasmic loading controls, respectively. The quantification of CDK5 expression was presented in mean values ± SEM. Ratios of CDK5 normalized to respective loading controls were calculated and compared with ratios obtained from log-phase cells (set to 1; $P < 0.01$, $P < 0.05$, respectively).

simulation revealed that Leu^{133} makes hydrophobic contact with the pyridine moiety of NS-0011 and Asp^{86} forms putative hydrogen bonding interactions with the trifluoromethyl group of NS-0011. Hence, the simulation results demonstrate that NS-0011 binds to the NES domains of CDK5.

To confirm the *in silico* results, ITC was used to measure binding affinity of NS-0011 to recombinant CDK5 protein. Figure 5D shows an ITC-binding isotherm of NS-0011 to recombinant CDK5 at 25°C in which each peak in the top represents a single injection of the drug into protein solution. The bottom shows an integrated plot of the amount of heat liberated per injection as a function of the molar ratio of NS-0011 to CDK5. The ITC data strongly suggest that NS-0011 can bind to CDK5 with a binding constant at about 16 μmol/L.

NS-0011 suppresses gastric cancer cell proliferation and xenograft tumorigenesis by accumulating CDK5 in the nucleus

Immunostaining and Western blotting showed that NS-0011 treatments resulted in a clear accumulation of CDK5 in the nucleus (Fig. 6A); a dose-dependent effect of NS-0011 was observed with an optimal effect at 2 μmol/L of NS-0011 (Supplementary Fig. S6). NS-0011 at 2 μmol/L completely inhibited proliferation of MGC-803 cells (Fig. 6B, left); and this effect was largely reversed when CDK5 was downregulated by siRNA (Fig. 6B, right). Similar to the results of Fig. 4D, NS-0011 also significantly increased p16 but not p12, p27, or p57 levels in MGC-803 or SGC-7901 cells (Fig. 6C). The above results suggested that NS-0011 exerts its

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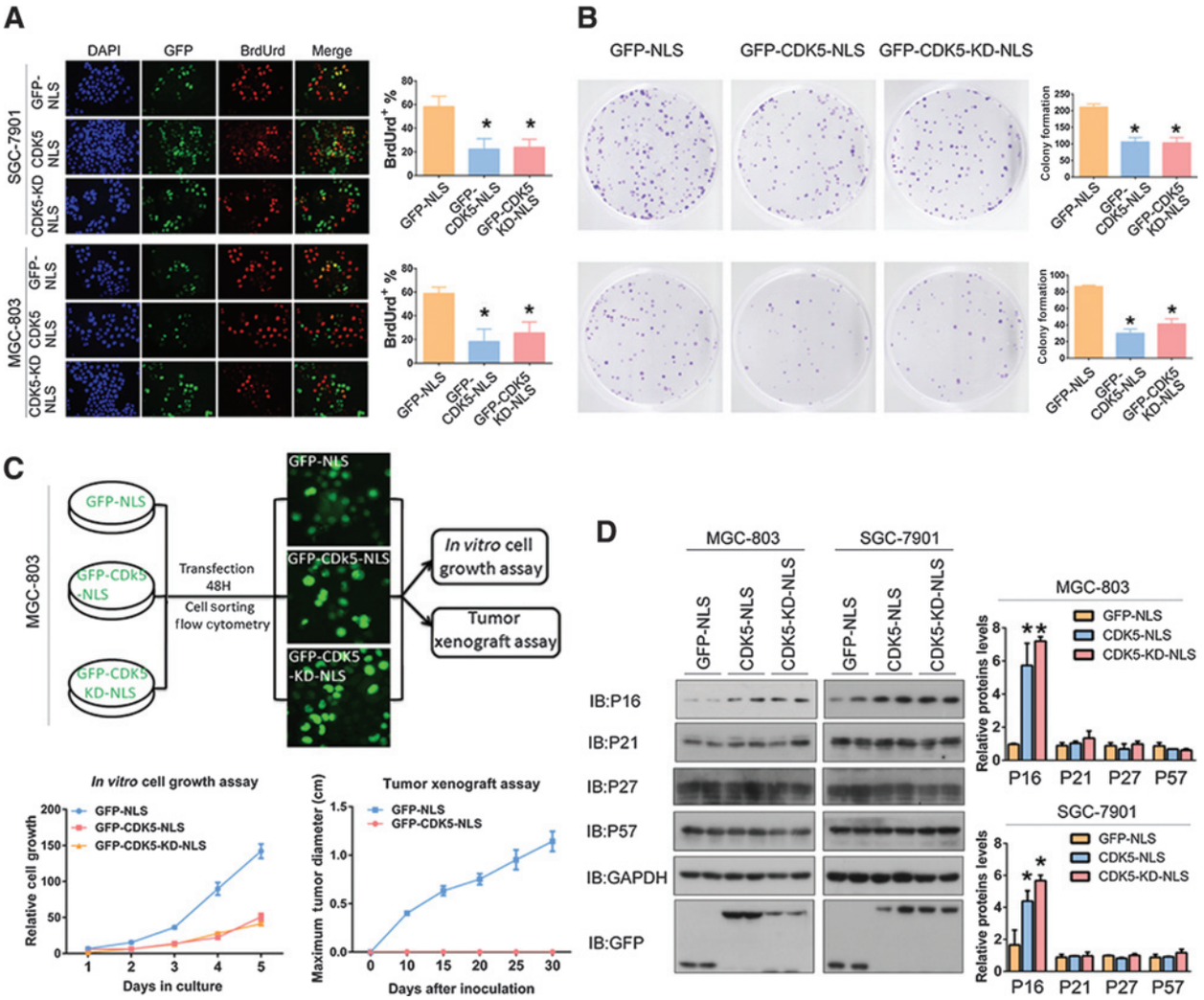

Figure 4.

Suppression of gastric cancer cell tumorigenic growth in xenograft implants by nuclear CDK5 expression. A, SGC-7901 and MGC-803 cells were transfected with GFP-CDK5-NLS, GFP-kinase-dead-CDK5-NLS (GFP-CDK5-KD-NLS), and GFP-C3-NLS constructs, respectively. BrdUrd incorporation was measured to investigate cell division activity. Left, representative immunostaining of BrdUrd-labeled cells transfected with GFP-CDK5-NLS, GFP-CDK5-KD-NLS, or GFP-NLS. DAPI was used to counterstain nuclei. Right, the percentage of GFP-positive cells that were labeled with BrdUrd from A (*, $P < 0.05$). B, representative results of colony formation assay from SGC-7901 (top) and MGC-803 (bottom) cells transfected with GFP-CDK5-NLS, GFP-CDK5-KD-NLS, or GFP-NLS constructs. The number of colonies counted and the quantifications were present on right. *, $P < 0.05$; **, $P < 0.01$. C, strategy for sorting GFP-positive MGC-803 cells transfected with GFP-NLS, GFP-CDK5-NLS, or GFP-CDK5-KD-NLS by flow cytometry. Sorted GFP-positive cells were used for subsequent *in vitro* cell growth and tumor xenograft transplantation assays. Down, left, GFP-positive MGC-803 cells expressing GFP-NLS, GFP-CDK5-NLS, or GFP-CDK5-KD-NLS were isolated by flow cytometry and used to detect cell growth rate. The number of GFP-NLS–positive MGC cells almost tripled after culture for 5 days, whereas GFP-CDK5-NLS–positive cells showed little growth within the same time period. Down, right, sorted MGC-803 cells transfected with GFP-NLS or GFP-CDK5-NLS were subcutaneously injected into BALB/c male nude mice. Tumor growth curve derived from the maximum tumor diameter was measured every 5 days in 30 days. D, MGC-803 or SGC-7901 cells were transfected with GFP-NLS, GFP-CDK5-NLS, or GFP-CDK5-KD-NLS vector. The p16, p21, p27, and p57 levels were measured by Western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as loading control, and the relative expression of GFP-NLS, GFP-CDK5-NLS and GFP-CDK5-KD-NLS was detected by blotting with GFP antibody. The quantifications of p16, p21, p27, and p57 levels are also presented on right. *, $P < 0.05$.

inhibitory effect on cell proliferation by specifically targeting CDK5. Moreover, we carried out xenograft experiments by subdermal transplantation with donor gastric cancer cells (MGC-803) pretreated with NS-0011 (2 μmol/L) or DMSO. Analysis of tumors isolated 14 days following xenograft implantation indicated that NS-0011 pretreatment significantly suppressed xenograft tumorigenesis and reduced the size of tumors (Fig. 6D).

Discussion

Evidence so far has illustrated a key role for cell-cycle dysregulation in carcinogenesis: failure to maintain normal cell proliferation machinery will result in uncontrolled cell growth and eventual tumor development (7, 8). To date, at least 11 structurally related CDKs (CDK1–CDK11) have been identified and shown to regulate cell cycle and proliferation.

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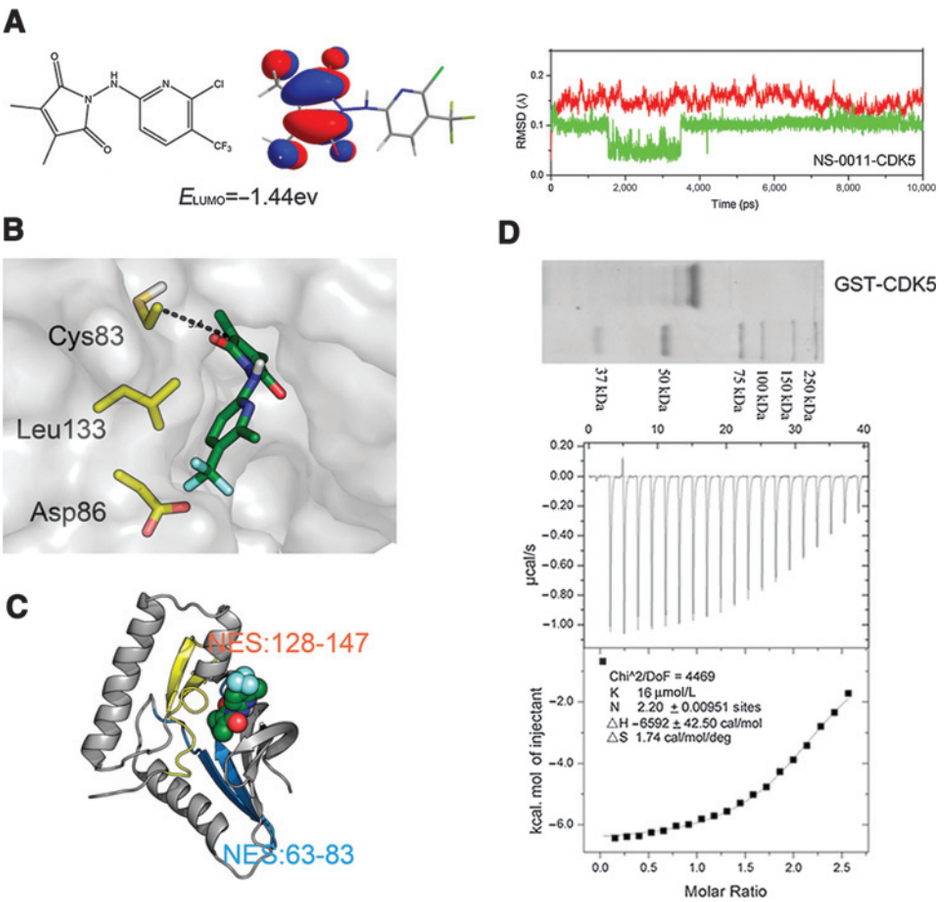

Figure 5.
Binding of NS-0011 with CDK5. A, chemical structure and LUMO orbital of NS-0011. The LUMO of NS-0011 was calculated by AM1 method and the orbital energy of LUMO is −1.44 eV. The alpha orbital is depicted in blue and the beta orbital is depicted in red. Right, 10 ns MD trajectory of NS-0011 binding with CDK5. The values for the backbone atoms of the protein and the ligand are represented in red and green lines, respectively. B, the binding configuration of NS-0011 in the groove between 2 NES signals of CDK5 after 10 ns MD simulation. Residues making possible contact with NS-0011 are depicted using a stick model. C, snug-fit-in model of NS-0011 in the groove between 2 NES signals of CDK5 after 10 ns MD simulation. D, ITC titration profile of NS-0011 with CDK5 binding at pH 7.40. Commassie blue staining of purified recombinant GST-CDK5. The temperature was 25°C and all the solutions contain 50 mmol/L HEPES. Each pulse corresponds to a 2.0 µL injection of 2 mmol/L NS-0011 into the ITC cell (200 µL) containing 80 µmol/L CDK5 protein. The area of each peak was integrated and corrected for the heat of dilution, which was estimated in a separate experiment by injecting NS-0011 into the buffer.

Hyperactivation of these CDKs promotes tumor development by initiating unscheduled cell division (9–15). It has also been documented that different CDKs may contribute specifically to different tumor types in humans (17, 18). CDK5, an atypical CDK that was previously considered to function in processes unrelated to cell-cycle regulation, has been shown to play a fundamental role in several kinds of carcinomas including breast, lung, prostate, multiple myeloma, and pancreatic cancers (39–43). However, the potential role of CDK5 in gastric cancer has never been reported. Here, we discover a striking decrease of CDK5 expression (both mRNA and protein levels) in gastric tumor tissues when compared with respective adjacent nontumor tissues (Fig. 1; Supplementary Fig. S1) and demonstrate that such a decrease significantly correlates with TNM stage in gastric carcinoma. We also demonstrate that overall survival rate after gastrectomy for patients with low CDK5 expression is significantly less than in patients with high CDK5 expression (Fig. 2D).

Since CDKs usually promote cell proliferation and often correlates with elevated expression levels in human tumors (9–18), it is surprising to find that CDK5 levels are dramatically decreased in gastric cancers. This may suggest that CDK5 in fact plays an inhibitory role in gastric tumorigenesis. The kinase activity of CDK5 has been thought to mediate carcinogensis of certain non–gastric cancer types (39–43). Our previous studies indicated that nuclear CDK5 can function as a cell-cycle suppressor in the nervous system (28–30). In the nucleus, CDK5 binds to the cell-cycle–associated transcription factor E2F1 to disrupt its association with its co-transcription factor DP1. This in turn attenuates E2F1 DNA interactions which consequently inhibits cell proliferation (28). Furthermore, we have shown that the antiproliferative function of CDK5 is mediated independently of its protein kinase activity (29–30). Our results indicate that nuclear localization of CDK5 which normally mediates an antiproliferative function, is lost in gastric cancers and that expression of nuclear CDK5 can restore carcinogenic proliferation by kinase activity independent way. This is consistent with the antiproliferative role of nuclear CDK5 previously described in the nervous system. CDK inhibitors (CKI) are inhibitory proteins to suppress cell-cycle progression. CKIs contain 2 families, Kip/Cip family of proteins (p21, p27, and p57) target to inhibit the G_1–S transition; INK4 family (p16, p15, p19, and p18) normally suppress the S–G_2 transition (44, 45). Our current study further identified that nuclear CDK5 and NS-0011 both can specifically upregulate the expression of p16 (INK4 family) but not Kip/Cip family of proteins (p21, p27, and p57; Figs. 4D and 6C), which suggested that nuclear CDK5 may block the S–G_2 transition contributing to the inhibition of gastric cancer cell proliferation

Although attenuation of CDK5 levels in gastric cancers are only correlative, or may alternatively be an unrelated consequence of upstream events in gastric tumorigenesis, our results indicate that exogenous nuclear CDK5 may be a driving factor in suppressing

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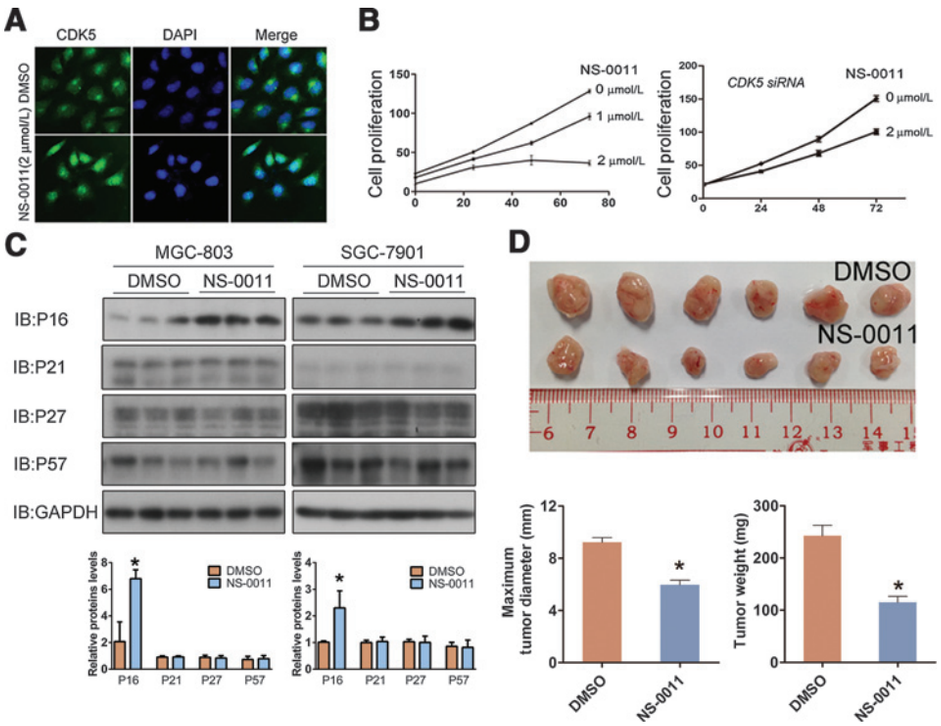

Figure 6.

Suppression of gastric cancer cell proliferation and xenograft tumorigenesis upon accumulation of CDK5 in the nucleus by NS-0011 treatment. A, MGC-803 cells were treated with 2 μmol/L NS-0011 or DMSO control for 24 hours and then subjected to immunofluorescent staining of CDK5. CDK5 was found to be relocated and concentrated in the nucleus upon NS-0011 treatment. Green represents endogenous CDK5 staining. DAPI was used to counterstain the nuclei. B, left, MGC-803 cells were treated with indicated amounts of NS-0011 for indicated time periods. Cell proliferation was analyzed on the basis of cell numbers. The number of untreated cells almost tripled in 80 hours, whereas treatments with 1 or 2 μmol/L NS-0011 significantly inhibited cell growth. Right, CDK5 was depleted by siRNA transfection in MGC-803 cells. Cells were then treated with 2 μmol/L NS-0011. Cell proliferation was analyzed. C, MGC-803 or SGC-7901 cells were treated with 2 μmol/L NS-0011 or DMSO control for 48 hours. The cell lysates were then isolated and subjected to Western blotting of p16, p21, p27, and p57. GAPDH serves as loading control. The quantifications of p16, p21, p27, and p57 levels were also presented. *, $P < 0.05$. D, MGC-803 cells were pretreated with NS-0011 or DMSO and subcutaneously injected into BALB/c male nude mice. After 30 days, mice were sacrificed and tumors were collected to assay the maximum tumor diameter and tumor weight. Significant differences between the maximum tumor diameter (*, $P < 0.05$) and the tumor weight (*, $P < 0.05$) between NS-0011 pretreated and control samples were observed.

tumorigenic proliferation in cell cultures and *ex vivo* xenografts. Furthermore, pharmacologically induced nuclear accumulation of CDK5 using a small-molecule inhibitor (NS-0011) similarly suppressed cell proliferation in cultured cells and xenograft implants. Together, these results provide good evidence that nuclear CDK5 may actively drive antiproliferative tumor suppression mechanisms.

Our current study represents the first comprehensive investigation into the functional relationship between CDK5 and gastric cancer through the survey of tumor tissues from 437 patients with well-documented clinical pathology and history. We have demonstrated that nuclear localization of CDK5 may be part of an antiproliferative mechanism in normal cells, and exogenous expression of nuclear CDK5 suppresses gastric cancer tumorigenesis. This provides a novel mechanism of tumor suppression by an atypical CDK5 family member, and its nuclear-dependent antiproliferative function may be an attractive target for future therapeutic strategies in treating gastric cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Zhou, G. Bu, H. Xu, Y. Yang, C. Huang, J. Zhang
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Cao, J. Zhou, J. Zhang, S. Wu, X. Yang, H. Li, G. Lin, H. Lin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Cao, J. Zhou, S. Wu, X. Yang, G. Lin, X. Hu, G. Bu, Y.-w. Zhang, H. Xu, Y. Yang, J. Zhang
Writing, review, and/or revision of the manuscript: L. Cao, J. Zhou, J. Zhang, X. Yang, X. Hu, G. Bu, Y.-w. Zhang, H. Xu, Y. Yang, C. Huang, J. Zhang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Cao, J. Zhou, X. Yang, H. Li, M. Luo, Q. Yu, J. Xie, P. Li, C. Zheng, C. Huang, J. Zhang
Study supervision: J. Zhou, H. Xu, C. Huang, J. Zhang

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