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Functional analysis of the NH_2-terminal hydrophobic region and BRICHOS domain of GKN1

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ABSTRACT

Gastrokine 1 (GKN1) protects the gastric antral mucosa and promotes healing by facilitating restitution and proliferation after injury. GKN1 is down-regulated in *Helicobacter pylori*-infected gastric epithelial cells and loss of GKN1 expression is tightly associated with gastric carcinogenesis. However, the underlying mechanisms as a tumor suppressor are largely unknown. Presently, the hydrophobic region and BRICHOS domain of GKN1, $pGKN1^{D13N}$, $pGKN1^{\Delta 68-199}$, and $pGKN1^{\Delta 1-67,165-199}$ were shown to suppress gastric cancer cell growth and recapitulate GKN1 functions. As well, the hydrophobic region and BRICHOS domain of GKN1 had a synergistic anti-cancer effect with 5-FU on tumor cell growth, implying that the NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 are sufficient for tumor suppression, thereby suggesting a therapeutic intervention for gastric cancer. Also, its domain inducing endogenous miR-185 directly targeted the epigenetic effectors DNMT1 and EZH2 in gastric cancer cells. Our results suggest that the NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 are sufficient for its tumor suppressor activities.

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1. Introduction

Recently, gastrokine 1 (GKN1) was isolated from the gastric mucosa cells of several mammalian species, including the rat [1]. GKN1 is a novel autocrine/paracrine protein that is specifically expressed in gastric mucosa [1,2]. GKN1 protects the antral mucosa and promotes healing by facilitating restitution and proliferation after injury [3]. Interestingly, GKN1 is down-regulated in *Helicobacter pylori* infected gastric epithelial cells and the loss of GKN1 expression is detected in gastric cancer tissues and precancerous lesions, such as intestinal metaplasia [4,5]. We also witnessed frequent loss of GKN1 expression in gastric cancers and tumor suppressor activity in a functional analysis [6]. Moreover, GKN1 plays an important role in the epithelial–mesenchymal transition (EMT) and migration of gastric cancer cells by regulating reactive oxygen species (ROS) and the PI3K/Akt pathway [7].

Here, we hypothesized that GKN1 plays an important role in cell cycle progression. This hypothesis is supported by the recent finding that GKN1 inhibits cell growth by inducing G2/M arrest in SGC-7901 cells [8]. Consistent with these findings, GKN1 induces senescence through p16/Rb pathway activation in gastric cancer cells [2]. However, the molecular mechanism by which GKN1 inhibits the cell cycle is still unknown.

GKN1 protein is a member of the BRICHOS superfamily, which includes proteins associated with the development of dementia, respiratory distress, and cancer [9]. The proteins in the BRICHOS superfamily have four distinct regions: hydrophobic, linker, BRICHOS, and C-terminal. The BRICHOS family is a close relative of the cancer associated GKN1 and GKN2 proteins [10]. The BRICHOS region shows the highest degree of conservation near the aspartic acid and first cysteine residues [10]. Recently, an E104T mutation in the BRICHOS region of GKN1 was identified in breast cancer [11]. In addition, two isoforms with a different N-terminal NH_2-acid residue, aspartic acid (D) and asparagine (N), were found in gastric endoscopy biopsy samples and the isoform containing asparagine decreased or disappeared in some *H. pylori*-positive patients [4]. Although mutation and post-translational modifications may alter the expression level or functional properties of the protein, the molecular function of GKN1 remains to be elucidated.

Thus, we focused on the effect of GKN1 domains on cell viability and proliferation, cell cycle and epigenetic alteration of the cell cycle-related proteins in AGS gastric cancer cell lines. First, we generated wild-type GKN1 and six GKN1 mutants (four deletion mutants and two point mutants), and examined their effects on cell viability and cell cycle. We also investigated alterations in epigenetic changes and expression of cell cycle-related proteins caused by a domain of GKN1. Overall, we showed that the NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 are important in the tumor suppressor activity of GKN1.

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2. Materials and methods

2.1. Cell culture

The AGS gastric cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). AGS cells were cultured at 37 °C in 5% CO_2 in RPMI-1640 medium (Lonza, Basel, Switzerland) with 10% heat-inactivated fetal bovine serum.

2.2. Plasmid preparation and transfection of GKN1

Base on sequence annotation information (http://www.uniprot.or/uniprot/Q9NS71), we generated four deletion-formed plasmids, pGKN1$^{\Delta 68-199}$ containing the NH_2-terminal hydrophobic region, pGKN1$^{\Delta 1-67,165-199}$ with the BRICHOS domain, pGKN1$^{\Delta 1-164}$ with the COOH-terminus and pGKN1$^{\Delta 1-67}$ with the RBICHOS and COOH-terminus. In addition, since the NH_2-terminal hydrophobic region acts as transmembrane anchor and/or signal peptide [10] and post-translational modification from D to N of GKN1 has been reported in *H. pylori*-infected gastric mucosa [4], two mutant plasmids with a mutation on codon 13 were also generated using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). All plasmids were verified by sequencing. All cDNAs were subcoloned into pcDNA 3.1-FLAG (Invitrogen, Carlsbad, CA, USA), and transient transfection with seven constructs including wild-type GKN1 and six mutants was performed in AGS cells using Lipofectamine (Invitrogen) following the manufacturer's instructions. Then, we examined the effects of these GKN1 constructs on cell viability and proliferation, and colony formation in AGS cells. The detailed information regarding primers and the others used to generate these constructs are described in Table S1. To further examine whether GKN1 contributes to the chemosensitivity of 5-fluorouracil (5-FU), a viability assay based on the colorimetric conversion of 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) was performed on AGS at 24 and 48 h after simultaneous treatment with each construct including wild-type and six mutant GKN1 constructs, and 5-FU.

2.3. Measurement of cell viability, proliferation, and colony formation

For cell viability analysis, the MTT assay was performed at 24, 48, and 72 h after transient transfection of each construct. Absorbance was measured using a spectrophotometer at 540 nm and cell viability was expressed relative to mock (empty vector + Lipofectamine).

For the cell proliferation assay, a BrdU incorporation assay was performed 24, 48, and 72 h after transient transfection of each construct using the BrdU cell proliferation assay kit (Millipore, Billerica, MA, USA), according to the manufacturer's instructions. Absorbance was measured using a spectrophotometer at 450 nm and proliferation was expressed relative to mock cells.

To measure the proliferative ability of a single cell in vitro, a plate clonogenic assay was performed. Briefly, 1×10^3 AGS cells transfected with each construct were seeded into a 6-well plate and cultured in RPMI 1640 for 2 weeks to allow colony formation. Colonies were fixed in 1% formaldehyde, stained with 0.5% crystal violet solution, and counted by the colono-count program.

2.4. Flow-cytometry analysis of cell cycle

For cell cycle analysis, AGS cells from each experimental group were collected and stained with propidium iodide (PI) for 45 min in the dark before analysis. The percentages of cells in different phases of the cell cycle were determined using a FACSCalibur Flow Cytometer with CellQuest 3.0 software (BD Biosciences, Heidelberg, Germany). Experiments were performed in triplicate.

2.5. Expression of cell cycle regulators

To determine whether each domain of GKN1 is involved in the regulation of cell cycle, the expression of cell cycle regulatory proteins including p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CDK4, cyclin D1, cyclin A, cyclin E, and cyclin B (Cell Signaling Technology, Danvers, MA, USA) was examined in AGS cells 24 h after each domain of GKN1 transfection. Cell lysates were separated on a 10% polyacrylamide gel and transferred onto a Hybond PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After blocking, the membrane was subsequently probed with antibodies against cell cycle regulatory proteins. Protein bands were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.6. Expression of epigenetic regulators

We also analyzed the expression levels of EZH2 and DNMT1, which are involved in epigenetic process, in AGS cells 24 h after each domain of GKN1 transfection. Cell lysates were separated on 10% polyacrylamide gel and blotted onto a Hybond-PVDF transfer membrane (Amersham), which had been subsequently probed with anti-DNMT1 (Abcam, Cambridge, UK), anti-EZH2 (BD bioscience), and then incubated with anti-mouse IgG conjugated with horseradish peroxidase. The protein bands were detected using enhanced chemiluminescence Western blotting detection reagents (Amersham).

2.7. Measurement of DNMT1, EZH2 and miRNA-185 expression

Real-time RT-PCR was performed using SYBR Green Q-PCR Master Mix (Stratagene, La Jolla, CA, USA) according to the manufacture's instructions. RNA input was normalized by human U6 snRNA. *DNMT1, EZH2,* and *CDKN2A* mRNAs were quantified by SYBR Green Q-PCR and normalized to mRNA of the housekeeping gene *GAPDH*. Data are reported as relative quantity according to an internal calibrator using the $2^{-\triangle\triangle CT}$ method [12]. The sequences of the primers are described in Table S1. All PCR procedures were done in triplicate.

2.8. Measurement of DNMT1 activity

Cells were collected and suspended in PBS. After centrifugation, the pellet was lysed in lysis buffer (10 mM Tris–HCl pH 7.5, 10 mM NaCl, 2 mM $MgCl_2$) containing protease inhibitor mixture (Complete; Roche Molecular Biochemicals). Then, 6 μl of 20% NP-40 was added and the mixture was incubated for 10 min at 4 °C and centrifuged for 5 min at 3000 rpm. The supernatant was collected and the pellet containing the nuclei was resuspended in 50 μl of extraction buffer (20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM $Mgcl_2$, 0.2 mM EDTA and 10% glycerol) followed by incubation for 30 min at 4 °C and collection of the nuclear extract by centrifugation. The DNMT1 activity assays were performed using the DNMT1 activity assay kit (abcam) according to manufacturer's instruction. All reactions were carried out in triplicate.

2.9. Statistical analysis

Student's *t*-test was used to analyze the effect of GKN1 on cell viability and proliferation. Data are expressed as means ± SD from at least three independent experiments. A *P*-value less than 0.05 was considered to be the limit of statistical significance. All

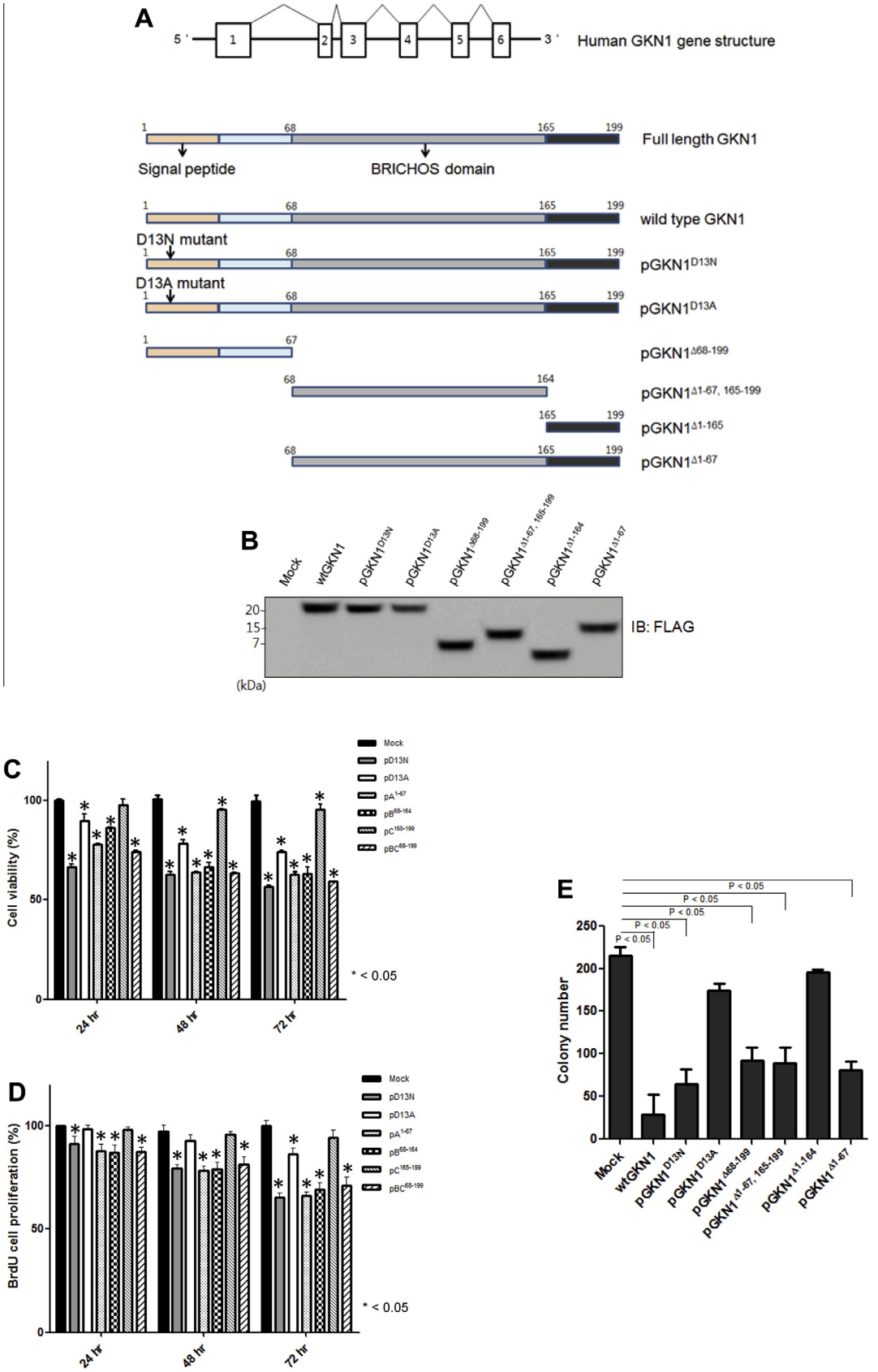

Fig. 1. Wild type, NH_2-terminal hydrophobic region, and BRICHOS domain of GKN1 are required for anti-cancer effect. (A) We generated wild-type GKN1, pGKN1^{D13N}, pGKN1^{D13A}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, pGKN1$^{\Delta 1-164}$, and pGKN1$^{\Delta 1-67}$ constructs. (B) Expression levels of each plasmid of GKN1 and GAPDH were determined by Western blot analysis. (C, D) Transient transfection with pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ induced time-dependent inhibition of cell viability (C) and proliferation (D) in AGS cells, whereas pGKN1^{D13A} and pGKN1$^{\Delta 1-164}$ had no effect on cell viability and proliferation. $^{*}p < 0.05$ compared to mock by Student's *t*-test (E) Tumorigenicity was measured by colony formation assay in AGS cells. Wild-type GKN1, pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ showed significant inhibition of colony formation.

experiments were performed in triplicate to verify the reproducibility of the findings.

3. Results

3.1. NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 reduces cell viability, proliferation, and colony formation

To identify the specific region of GKN1 that regulates cell proliferation, we first generated six mutants of GKN1 (Fig. 1A). Expression of wild-type GKN1, the two mutants, and four deletion variants was confirmed by Western blot analysis of lysates from AGS cells (Fig. 1B). As shown in Fig. 1C and 1D, ectopic expression of wild-type, pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ plasmids significantly reduced cell viability and proliferation in AGS cells. However, the plasmids pGKN1^{D13A} and pGKN1$^{\Delta 1-164}$, which contained only the carboxyl terminus, had minimal effect on cell viability and proliferation (Fig. 1C and 1D).

In the colony formation assay, the ectopic expression of wild-type, pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ markedly reduced the number and size of surviving colonies in AGS gastric cancer cells compared with the empty vector-transfected control cells (mock) (Fig. 1E).

To further examine the effect of GKN1 domains on the chemosensitivity of 5-FU, we used a MTT assay with AGS cells 24 and 48 h after simultaneous treatment with 5-FU, and wild- or mutant-type GKN1. As expected, AGS cells treated with wild-type GKN1, pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ showed synergistic inhibition of cell viability with 5-FU in a time-dependent manner (Fig. 2). However, two mutants, pGKN1^{D13A} and pGKN1$^{\Delta 1-164}$, only marginally affected cell viability (Fig. 2).

3.2. NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 induces G0/G1 and G2/M arrest

As expected, pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ showed increased numbers of sub-G0 cells and a modest effect on the cell cycle G0/G1 and G2/M phase progression. However, pGKN1^{D13A} and pGKN1$^{\Delta 1-164}$ had no effect on cell cycle progression (Fig. 3A). Ectopic expression of pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ down-regulated the expression of CDK4, cyclin D, and cyclin B in AGS cells (Fig. 3B).

3.3. NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 are epigenetic regulators

As shown in Fig. 4A, ectopic expression of pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ reduced the expression of DNA methyltransferases 1 (DNMT1) and Enhancer of zeste homolog 2 (EZH2) proteins (Fig. 4A). When we examined the DNMT1 activity in AGS cells transfected with pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$, decreased DNMT1 activity was observed (Fig. 4B) and NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 inhibited *DNMT1*, *EZH2* mRNA expression or increased *CDKN2A* mRNA expression (Fig. 4C–E).

3.4. NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 induce miR-185 expression

To certify the effect of GKN1 domains on miR-185 expression, we examined whether domains of GKN1 could induce expression of miR-185. Interestingly, a significantly increased level of miR-185 was detected in the AGS cells transfected with pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ (Fig. 4F), whereas pGKN1^{D13A} and pGKN1$^{\Delta 1-164}$ had no effect on miR-185 expression (Fig. 4F).

4. Discussion

The structure of the BRICHOS superfamily consists of four distinct regions: hydrophobic, linker, BRICHOS, and C-terminal [9]. GKN proteins contain the enigmatic BRICHOS domain and a COOH-terminal segment showing considerable divergence between the GKN paralogs, but strong amino acid sequence conservation [13], and hydrophobic NH_2-terminal signal peptide, the processing of which is predicted to generate mature ~160 NH_2 acid proteins with molecular mass ~18 kDa [10]. The NH2-terminal

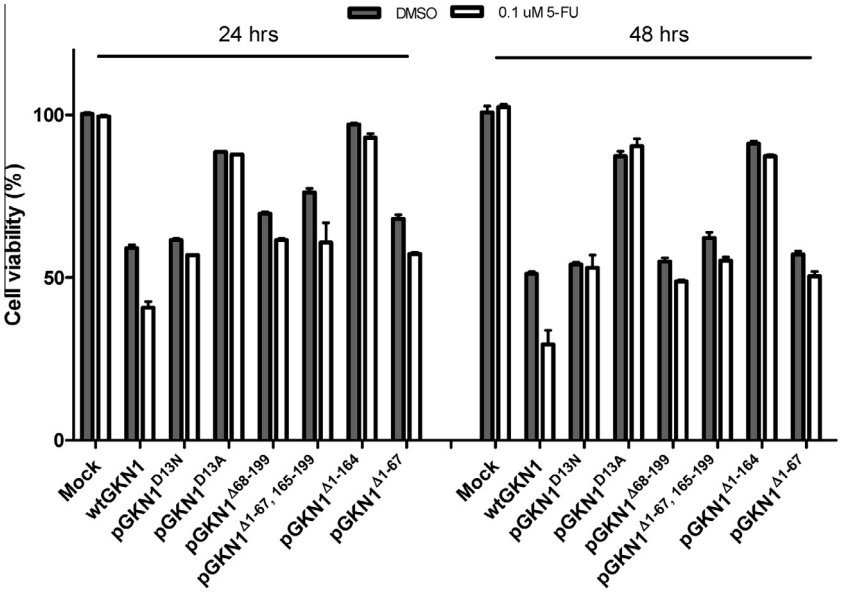

Fig. 2. NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 has a synergistic cytotoxicity with 5-FU. Ectopic expression of wild-type GKN1, pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ showed synergistic inhibition of cell viability with 5-FU.

hydrophobic region acts as a transmembrane anchor and/or signal peptide [10]. Clinically, BRICHOS proteins are associated with dementia, respiratory disease, and cancer [9,10]. Although it has been suggested that BRICHOS domain has a range of possible roles, including intracellular trafficking, propeptide processing, chaperonin function/protein folding, and secretion [9], the biological activities of the each domain of the GKN1 have not been elucidated.

Here, we tried to identify the specific region underlying the tumor suppressor activities of GKN1 in gastric cancers. Finally, we found that the NH_2-terminal hydrophobic region and BRICHOS domain of the GKN1 significantly reduced the cell viability, proliferation and colony formation of AGS cells (Fig. 1). In chemosensitivity assay, ectopic expression of above two domains of GKN1 and 5-FU showed synergistic inhibition on cell viability and proliferation (Fig. 2). The fact that the NH_2-terminal hydrophobic region and BRICHOS domain of the GKN1 suppressed cell proliferation implies that the NH_2-terminal hydrophobic region and BRICHOS domain of the GKN1 may be the key domains to modulate cell cycle-regulating components. Therefore, we further examined the effect of each domain of GKN1 on the cell cycle and the regulatory components of the G1/S and G2/M transition. A concomitant increase of G0/G1 phase and G2/M phase was found in AGS cells transfected with $pGKN1^{D13N}$, $pGKN1^{\Delta 68-199}$, $pGKN1^{\Delta 1-67,165-199}$, and $pGKN1^{\Delta 1-67}$ constructs (Fig. 3A). Down-regulation of CDK4 and cyclin D was also observed when $pGKN1^{D13N}$, $pGKN1^{\Delta 68-199}$, $pGKN1^{\Delta 1-67,165-199}$, and $pGKN1^{\Delta 1-67}$ constructs were ectopically expressed (Fig. 3B). Consistent with these findings, GKN1 induces senescence by activating p16/Rb pathway in gastric cancer cells [2]. These results suggest that the NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 are sufficient for tumor suppressor activity by directly regulating a set of proteins involved in cell cycle control. In contrast, the mutant plasmids $pGKN1^{\Delta 1-164}$ containing only the COOH-terminal segment had minimal effect on cell viability and proliferation (Figs. 1–3), suggesting that COOH-terminal segment is not critical for anti-proliferative function of the GKN1.

All BRICHOS proteins, including GKNs, are secreted or processed to release small secreted peptides with the NH_2-terminal hydro-

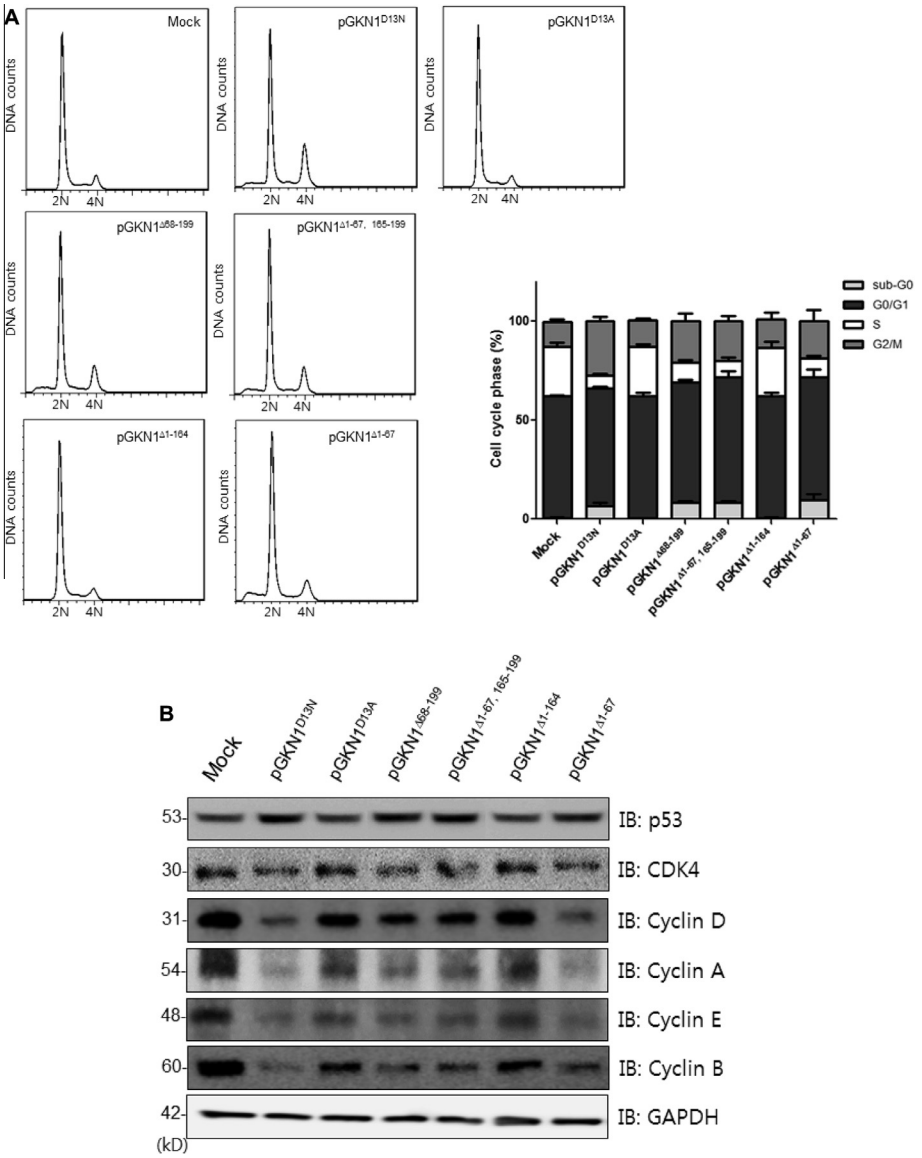

Fig. 3. NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 negatively regulates cell cycle progression. (A) Cell cycle arrest at G0/G1 and G2/M phase was found in AGS cells transfected with each domain of GKN1, including $pGKN1^{D13N}$, $pGKN1^{\Delta 68-199}$, $pGKN1^{\Delta 1-67,165-199}$, and $pGKN1^{\Delta 1-67}$. (B) Western blot analysis following each domain of GKN1 transfection in AGS cells. $pGKN1^{D13N}$, $pGKN1^{\Delta 68-199}$, $pGKN1^{\Delta 1-67,165-199}$, and $pGKN1^{\Delta 1-67}$ transfected AGS cells showed upregulated p53 and downregulated expression of CDK4, cyclin D, cyclin A, cyclin E, and cycline B.

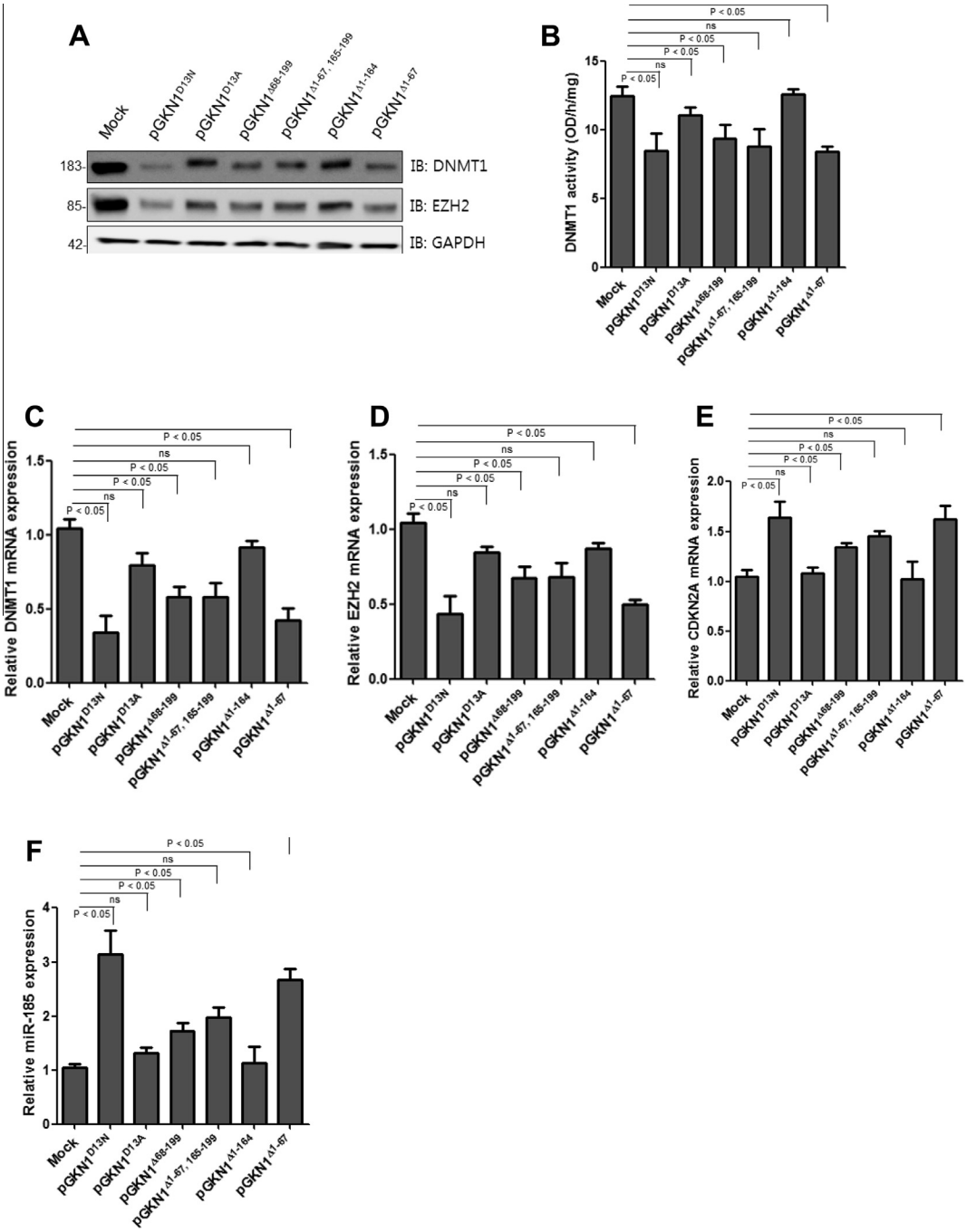

Fig. 4. NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 negatively regulates expression of epigenetic regulators and induces miR-185 expression. (A) pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ transfected AGS cells showed down-regulated expression of epigenetic regulators including DNMT1 and EZH2. (B) pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ inhibited DNMT1 activity. (C–E) pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ significantly down-regulated *DNMT1* and *EZH2* mRNA expression (C, D) and up-regulated *CDKN2A* mRNA expression (E). (F) pGKN1^{D13N}, pGKN1$^{\Delta 68-199}$, pGKN1$^{\Delta 1-67,165-199}$, and pGKN1$^{\Delta 1-67}$ transfected AGS cells significantly induced increased miR-185 expression.

phobic region acting as transmembrane anchor and/or signal peptide [10]. Pre-AMP-18 (GKN1) has a 20-amino acid, hydrophobic NH_2-terminal domain that is apparently a signal peptide [1]. GKN1 and GKN3 are likely subject to posttranslational modification by glycosylation [14,15]. A consensus asparagine (N)-linked glycosylation sequon occurs adjacent to the signal peptide cleavage site in GKN3 and likely forms the NH_2 terminus of the processed mature protein [13]. In this study, two critical amino acid residues (D13 and N13) in NH_2-terminal domain but not the pGKN1^{D13A} mutant were found to be important in anti-proliferative function of GKN1 (Figs. 1 and 3), indicating that an aspartic acid or asparagine variant at codon 13 may play an important role in the growth inhibitory effect of GKN1. Loss of the asparagine variant isoform, but not aspartic acid, has been reported in *H. pylori*-infected individuals [4], implying that these two isoforms might have different roles in the gastric mucosal response to *H. pylori*. Further studies are strongly necessary to clarify the physiological functions of these variants.

In our previous study, we confirmed that GKN1 has an miR-185-dependent and -independent mechanism for chromatic and DNA epigenetic modification [16]. Thus, we set out to determine if the NH_2-terminal hydrophobic region and BRICHOS domain of the

GKN1 contribute to the expression of epigenetic regulators [17]. Consistent with our previous results [16], depletions of DNMT1 and EZH2 were observed in AGS cells transfected with the NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 (Fig. 4A). Furthermore, the NH_2-terminal hydrophobic region and BRICHOS domain of the GKN1 significantly inhibited expression of *DNMT1* and *EZH2* mRNA and DNMT1 activity (Fig. 4B–D), but increased *CDKN2A* mRNA expression (Fig. 4E). DNMT1 is primarily involved in the maintenance of methylation during DNA replication phase, and the overexpression of DNMT1 has been reported in gastric cancer [18]. EZH2, a histone methyltransferase, is involved in epigenetic silencing of large number of genes involved in differentiation and proliferation [19]. EZH2 is considered as a driver oncogene, where it is involved in the aberrant hypermethylation of tumor suppressor genes in multiple human malignancies [20] and its expression promotes the activation of the wnt signaling pathway in gastric carcinogenesis through the down-regulation of CXXC4 expression [21]. Here, we demonstrate that both the NH_2-terminal hydrophobic region and BRICHOS domain of the GKN1 contribute to the regulation of epigenetic modification.

To further confirm the epigenetic regulation of GKN1 by miR-185, we also analyzed the expression levels of miR-185 in AGS cells transfected with each domain of GKN1. Interestingly, ectopic expression of NH_2-terminal hydrophobic region and BRICHOS domain of the GKN1 significantly up-regulated miR-185 expression in AGS cells (Fig. 4F). These findings are consistent with recent reports describing that miR-185 inhibits tumorigenicity, cell growth, migration, and invasion in prostate cancer cells [22], and that miR-185 reduces global DNA methylation and induces the expression of promoter-hypermethylated genes in glioma cells by directly targeting DNMT1 [23]. Consequently, these data indicate that NH_2-terminal hydrophobic region and BRICHOS domain of the GKN1 may be involved in down-regulation of the epigenetic regulators and positive cell cycle components via up-regulation of miR-185.

A previous study that involved a group of gastric cancer patients treated with a cisplatin/5-FU-based neoadjuvant chemotherapy reported the concordant methylation of multiple genes, suggesting an association with a worse response to therapy [24]. In this study, NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 synergistically increased the effect of cell growth inhibition induced by 5-FU (Fig. 2).

Taken together, these data suggest that NH2-terminal hydrophobic region and BRICHOS domain may be the main domains for the GKN1 tumor suppressor function by regulating epigenetic modification. Finally, we provide evidence that modulating the NH_2-terminal hydrophobic region and BRICHOS domain of GKN1 could significantly impact the development of novel gastric cancer treatment, which will ultimately achieve the goal of gastric cancer prevention and remission.

Author contributions

J.H.Y., W.S.P. designed research; J.H.Y. Performed research; J.H.Y., W.S.P. Analyzed data; J.H.Y., Y.J.C., Y.S.C., S.W.N., J.Y.L. Contributed reagents/materials/analysis tools; J.H.Y., W.S.P. Wrote the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.123.

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