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Gastrokine 1 inhibits the carcinogenic potentials of *Helicobacter pylori* CagA

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***Helicobacter pylori* CagA directly injected by the bacterium into epithelial cells via a type IV secretion system, leads to cellular changes such as morphology, apoptosis, proliferation and cell motility, and stimulates gastric carcinogenesis. We investigated the effects of cytotoxin-associated gene A (CagA) and gastrokine 1 (GKN1) on cell proliferation, apoptosis, reactive oxygen species (ROS) production, epithelial–mesenchymal transition (EMT) and cell migration in *CagA*- or *GKN1*-transfected gastric epithelial cells and mucosal tissues from humans and mice infected with *H.pylori*. On the molecular level, *H.pylori CagA* induced increased cell proliferation, ROS production, antiapoptotic activity, cell migration and invasion. Moreover, CagA induced activation of NF-κB and PI3K/Akt signaling pathways and EMT-related proteins. In addition, *H.pylori* CagA reduced *GKN1* gene copy number and expression in gastric cells and mucosal tissues of humans and mice. However, GKN1 overexpression successfully suppressed the carcinogenic effects of CagA through binding to CagA. These results suggest that GKN1 might be a target to inhibit the effects from *H.pylori* CagA.**

Introduction

Helicobacter pylori (*H.pylori*) is a gram-negative pathogen that colonizes approximately 50% of the world's population (1). *Helicobacter pylori* has been identified as the causative agent of chronic gastric inflammation, such as atrophic gastritis and metaplastic gastritis, which can progress to a variety of other diseases, including peptic ulcers, mucosa-associated lymphoid tissue lymphoma or even gastric cancer (1–3). *Helicobacter pylori* translocates virulence factors into host target cells by multi-subunit transport apparatuses known as type-IV secretion systems (4,5). The *H.pylori* cag pathogenicity island is a strain-specific locus that encodes a type IV secretion system, which, in turn, mediates the translocation of bacterial virulence factor CagA (cytotoxin-associated gene A), and injects the CagA oncoprotein, as well as peptidoglycan into host epithelial cells, resulting in activation of NF-κB and induction of potent pro-inflammatory chemokines, such as interleukin (IL)-8 (2,3,5–8). Translocated CagA undergoes tyrosine phosphorylation by Src, leading to actin-cytoskeletal rearrangements, scattering and elongation of infected host cells in cell culture (2,3,5–8). These phenotypic changes resemble those of malignant cellular transformation and have been the subject of intensive studies (2,5–8). However, little is known about the regulation of CagA in the gastric mucosa and the molecular mechanisms underlying the contribution of CagA to gastric carcinogenesis.

Gastrokine 1 (GKN1) protects the antral mucosa and promotes healing by facilitating restitution and proliferation after injury (9). Interestingly, GKN1 is downregulated in *H.pylori*-infected gastric epithelial cells and loss of GKN1 expression is detected in gastric cancer tissues and precancerous lesions, such as intestinal metaplasia (10,11). Previously, we observed frequent loss of GKN1 expression in gastric cancers and tumor suppressor activities of GKN1 by inhibiting cell proliferation, epithelial–mesenchymal transition (EMT) and cell migration (12,13). Moreover, GKN1 inhibited the proliferation of gastric epithelial cells by regulating NF-κB and Cox-2 signaling pathways and cytokine expression (14). Those findings led us to hypothesize that GKN1 may play important roles in maintaining gastric mucosa integrity and may also regulate the gastric cancer risk associated with CagA. Here, we investigated the relationship between expression of GKN1 and CagA status, how *H.pylori* reduces expression of GKN1, and the effects of GKN1 on carcinogenic potential of CagA in gastric epithelium.

Abbreviations: CagA, cytotoxin-associated gene A; EMT, epithelial–mesenchymal transition; GKN1, gastrokine 1; HA, hemagglutinin; IL, interleukin; PBS, phosphate-buffered saline; ROC, receiver operating characteristic; ROS, reactive oxygen species; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl.

Materials and methods

Generation of CagA gene deleted H.pylori strains

The isogenic mutant *H.pylori* 26695 (ΔcagA::aphA), in which most of *CagA* gene were replaced by a aphA (kanamycin resistant gene from pIP1433) cassette, was made using PCR products generated with primers 'kanF' (5′-GATAAACCCAGCGAACCAT-3′) and 'aphAR' (5′-GTACTAAAACAATTCATCCAGTAA-3′) (1402 bp; *aphA* kanamycin resistance cassette); 'CagA F1' (5′-ATCGTTGATAAGAACGATAGGG-3′) and 'CagA R2' (5′-ATGGTTCGCTGGGTTTATCATTGATTGCTTCTTTGACA TCGGTACCAAGCGACCCAAATAG-3′) (552 bp, upstream of deleted cagA segment); 'CagA F5' (5′-TTACTGGATGAAT TGTTTTAGTACATCAAATAGCAAGTGGTTTGGGAATGACCTACT TAACAAAATCATG-3′) and 'CagA R6' (5′-ATTGCTATTAATGCGT GTGTGG-3′) (425 bp; downstream of deleted cagA segment). Natural transformation was carried out by adding 7 µl of purified PCR product containing this Δ*CagA*::*aphA* allele to a lawn of cells (wild-type *H.pylori* 26695) growing exponentially on non-selective medium, and restreaking the population on selective medium (containing 15 µg/ml of kanamycin) after 6–8 h or overnight incubation to obtain transformant colonies. PCR tests and sequencing of representative kanamycin resistant transformants demonstrated the expected replacement of CagA by aphA in each case.

Bacterial strain and animal infection

The bacterial strains used for this study are described in Supplementary Table S1, available at *Carcinogenesis* Online. For the construction of the *CagA* knockout mutant, *H.pylori* 26695 (reference strain, *CagA*$^+$, *vacA*$^+$) was used, as described in the Supplementary Materials and methods, Supplementary Figure 1, available at *Carcinogenesis* Online (15–17). *Helicobacter pylori* was cultured at 37°C in a standard microaerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2) in brain–heart infusion medium (Difco, Detroit, MI) with 7% laked horse blood (Oxoid, Cambridge, UK), 0.4% IsoVitalex™ (BBL, Sparks, MD), vancomycin (6 µg/ml), amphotericin B (8 µg/ml) and trimethoprim (5 µg/ml). Five C57BL/6 female mice aged 5 weeks were purchased from *Qu*-BEST (Seongnam, Korea). Three mice were then inoculated three times by oral gavage with 0.4 ml of the suspension containing *H.pylori* SS1 (2×10^9 c.f.u./ml). Four weeks postinoculation 2 control and 3 mice were killed, and their gastric mucosal tissues were used for molecular studies and determination of colonization.

Cell culture and H.pylori stimulation

AGS human gastric cancer cells were grown as described previously (12). *Helicobacter pylori* was harvested, washed with phosphate-buffered saline (PBS), and then resuspended into antibiotic-free cell culture medium. *Helicobacter pylori* bacteria were co-cultured with AGS cells at a bacterium/cell ratio of 150:1 or 300:1 and the *H.pylori* colony numbers were counted. Cells were collected at 6 h after *H.pylori* infection.

Cell culture and transfection with GKN1

AGS, MKN1 and MKN28 gastric cancer cells without GKN1 expression and HFE-145 immortalized non-neoplastic gastric mucosal cells expressing GKN1 were cultured as described previously (14,18). The *CagA* gene of *H.pylori* was cloned into a pSP65SRalpha vector containing a hemagglutinin (HA) tag. Dr Hatakeyama (Tokyo University, Tokyo, Japan) kindly provided the *CagA* construct. AGS, MKN1, MKN28 and HFE-145 cells were transfected with *GKN1* and *CagA* genes as described previously (14).

Effect of CagA on GKN1 copy number and expression

To examine DNA copy number change of the *GKN1* gene after *CagA* transfection, the forward primers were designed in exon 1 and the reverse primers in intron 2. The copy number and expression of *GKN1* were examined in AGS, MKN1, MKN28 and HFE-145 cells at 24 h after transfection with *CagA* as described previously (12). The primer sequences are shown in Supplementary Table S2, available at *Carcinogenesis* Online. We also measured *GKN1* copy number variation after treatment with *CagA*, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL, Sigma–Aldrich), or H_2O_2 in AGS, MKN1 and MKN28 cells by real-time QPCR. The expression of CagA and GKN1 protein was examined by western blot as described previously (12).

Measurement of cell viability, proliferation and colony formation

3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide and 5-bromo-2-deoxyuridine incorporation assays were performed at 24, 48 and 72 h after transfection with *CagA* and *GKN1* plasmids, as described previously (12). A 96-well plate clonogenic assay was also performed for 2 weeks in order to allow colony formation in AGS, MKN1 and MKN28 cells transfected with *CagA* and/or *GKN1*. Colonies were fixed in 1% formaldehyde, stained with 0.5% crystal violet solution and counted by the colony-count program.

Co-immunoprecipitation

GKN1- or *CagA*-transfected AGS cells were washed with PBS and lysed at 4°C with PBS, pH 7.2 containing 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10mM NaF, 1.0mM $NaVO_4$ and 1.0% protease inhibitor cocktail (Sigma, St Louis, MO) as described previously (19). Equal protein aliquots (1.0 mg) were immunoprecipitated with 2.0 μg of antibodies to GKN1 (Sigma), HA (Sigma) and E-cadherin (Cell Signaling, Danvers, MA) plus protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) as manufacture's recommendation described. Immunoprecipitated proteins were resolved on 12% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene difluoride membranes (BioRad, Richmond, CA). The membranes were blocked for 1 h in PBS containing 0.1% Tween 20 (PBS-T) and 5% non-fat dry milk (Sigma) and reacted with antibodies against HA, GKN1 or E-cadherin, each diluted 1:1000. The membranes were washed with PBS-T, then incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse immunoglobulin G antibody (Sigma), diluted 1:5000, and developed with enhanced chemiluminescence plus western blotting detection system (Amersham Biosciences). Immunoreactive bands were identified by co-migration of prestained protein size markers (Fermentas, Glen Burnie, MD). To confirm equivalent protein loading and transfer, the blots were stripped and re-probed for glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology).

Immunofluorescence

To determine the localization of GKN1 and CagA, cells were fixed in 3% paraformaldehyde for 20 min at room temperature and permeabilized in 0.5% Triton X-100 on ice for 7 min. The cells were washed in PBS plus 0.5% normal goat serum and incubated with one of the following primary antibodies: HA (1:200) and GKN1 (1:100). AlexaFluor-488- or AlexaFluor-555-conjugated secondary antibodies were used as required. Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole. Cells were analyzed using a fluorescent microscope (Zeiss) to visualize the endogenous level of proteins under the study.

Reactive oxygen species analysis

To examine the effect of CagA and GKN1 on reactive oxygen species (ROS) production, we measured ROS levels using 2′,7′-dichlorofluorescin diacetate in mock, *CagA*- or *GKN1*-transfected AGS, MKN1 and MKN28 cells as described previously (13).

Apoptosis and Caspase 3/7 activity assays

For apoptosis assessment, the annexin V-binding assay was performed as described previously (12). To confirm whether CagA inhibits the GKN1-induced apoptosis by caspase activation, we examined caspase-3 and -7 activity using an Apo-One Homogeneous Caspase 3/7 assay kit (Promega Corporation, Madison, WI) as described previously (20). Next, the apoptosis-related proteins were examined by western blot after transfection with *CagA* or *GKN1* into AGS cells and gastric mucosal tissue of the mice infected with *H.pylori*.

Analysis of cell cycle by flow cytometry

For cell cycle analysis, AGS, MKN1 and MKN28 cells from each experimental group were stained with propidium iodide, and 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.

Cell migration and invasion assay

Migration and motility of AGS, MKN1 and MKN28 cells were assessed using the *in vitro* scratch wound-healing assay and 48-well microchemotaxis chambers, respectively, as described previously (13). Invasion was also assayed in BD Biocoat MatrigelTM invasion chambers (BD Bioscience, San Jose, CA), as described previously (13).

Western blot analysis

The expression of CagA and GKN1 was also examined in 54 human and 5 mice gastric mucosal tissues as described previously (12). A total of 54 patients with sporadic gastric cancer who underwent a gastrectomy were included. Only non-neoplastic mucosae remote (≥5 cm) from the tumor were used in this study. The Institutional Review Board of The Catholic University of Korea, College of Medicine approved this study (CUMC09U089).

The expression of cell cycle regulatory proteins, including p53 and p21 and NF-κB-related proteins, including NF-κBp-p65, NF-κBp65, NF-κBp105/50 and RelB, was examined in *CagA*- or *GKN1*-transfected AGS and HFE-145 cells and gastric mucosae of mice infected with *H.pylori*. We also analyzed the expression of apoptosis-related proteins, including poly (ADP ribose) polymerase, Bcl-2, Bcl-xL, BAX, Mcl-1 and caspase-3, and EMT-related proteins, including p-Akt, E-cadherin, β-catenin, slug, snail and vimentin. Antibodies used in this study are listed in Supplementary Table S3, available at *Carcinogenesis* Online.

Real-time RT–PCR

Next, mRNA expression of superoxide dismutase (*MnSOD*), *catalase, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)* and *COX-2* genes was also analyzed, as described previously (13). The primer sequences of each gene are described in Supplementary Table S2, available at *Carcinogenesis* Online.

Statistical analysis

Student's *t*-test was used to analyze the effect of CagA and GKN1 on cell growth, migration and invasion and ROS production. The Pearson correlation test was employed to assess the degree of relationship between the DNA, RNA and protein expression of GKN1. To further evaluate the diagnostic usefulness of the markers based on dichotomous classification, we considered receiver operating characteristic (ROC) curve analysis. A ROC curve is a plot of the true-positive fraction versus the false-positive fraction, evaluated for all possible cutoff point values. Data are expressed as means ± SD from at least three independent experiments.

Results

Effects of H.pylori SS1 infection in C57BL/6 mice

Since *H.pylori* SS1 has been found to consistently infect mice at a high level and to establish infections that survive over many months, SS1 strain is usually proposed as the standard for experimental infection studies (21). The effects of *H.pylori* infection in mice were analyzed in three *H.pylori*-infected C57BL/6 and two non-infected C57BL/6 female mice. *Helicobacter pylori*-infected mice showed increased expression of antiapoptotic proteins and reduced expression of pro-apoptotic protein BAX (Supplementary Figure 2A, available at *Carcinogenesis* Online), as well as decreased expression of p53, p21 and p16, and induced expression of cyclin A, cyclin D1 and cyclin E in gastric mucosal tissues of the mice (Supplementary Figure 2B, available at *Carcinogenesis* Online). Furthermore, *H.pylori* enhanced the expression of NF-κB-related proteins (Supplementary Figure 2C, available at *Carcinogenesis* Online) and mRNA transcript expression of *PI3K* and *COX-2* genes (Supplementary Figure 2D, available at *Carcinogenesis* Online). Moreover, *H.pylori* induced increased expression of p-Akt, β-catenin, snail, slug and vimentin proteins (Supplementary Figure 2E, available at *Carcinogenesis* Online). In addition, the reduced mRNA and protein expression of oxidative stress-responsive genes, *MnSOD* and *catalase*, were found in *H. pylori*-infected mice (Supplementary Figure 2F and G, available at *Carcinogenesis* Online). These results suggest that *H.pylori* infection may increase the ROS levels through downregulation of the

antioxidant genes, and induce cell proliferation and EMT via upregulation of antiapoptotic molecules, NF-κB signaling pathway and EMT-related proteins.

GKN1 inhibits H.pylori CagA function through binding to CagA

The majority of the CagA proteins form complexes with SHP-2, and CagA is also capable of binding to C-terminal Src kinase (22–26). Upon interacting with C-terminal Src kinase, CagA stimulates the kinase activity and, thereby, inactivates Src family kinases. Src family kinases are responsible for CagA phosphorylation, which is an essential prerequisite for CagA·SHP-2 complex formation and subsequent induction of the hummingbird phenotype (27–29). We investigated how GKN1 inhibits the CagA activity in AGS cells. Interestingly, GKN1 bound to CagA protein (Figure 1A), and the co-localization of GKN1 and CagA at the membrane of AGS cells was observed (Figure 1C). The GKN1 binding activity to CagA was also confirmed in human gastric mucosae (Figure 1B). Furthermore, we found that GKN1 inhibits the CagA–SHP-2 complex formation in AGS cells and human gastric mucosae (Figure 1D and E). Additionally, CagA induced increased expression of phospho-c-Met, Ras, Raf family and Src, but the presence of GKN1 reduced the expression of these proteins in gastric cells and human gastric mucosae (Figure 1F and G). These data suggest that GKN1 may inhibit CagA activity by binding to the CagA protein in host cells.

GKN1 is involved in inhibition of the CagA-induced cell growth and colony formation

Helicobacter pylori CagA is a crucial factor for cellular changes such as apoptosis, proliferation, and cell mortality and stimulates gastric carcinogenesis (30,31). There was a significant time-dependent increase in cell growth and proliferation in *CagA*-transfected AGS, MKN1, MKN28 gastric cancer cells and HFE-145 non-neoplastic gastric epithelial cells. In contrast, gastric cancer cells transfected with *GKN1* showed a time-dependent decrease in cell growth. Interestingly, co-transfection with *CagA* and *GKN1* significantly abrogated the proliferative effect of CagA (Figure 2A and B).

In the colony formation assays, ectopically expressed CagA dramatically increased the number of surviving colonies, but GKN1 markedly reduced the number and size of surviving colonies in AGS, MKN1 and MKN28 cells (Figure 2C). These results suggest that GKN1 may inhibit cell proliferation by suppressing CagA.

GKN1 is involved in inhibition of the CagA-induced antiapoptotic activity

Next, we investigated the effects of GKN1 on antiapoptotic activity caused by CagA. Apoptotic cell death was significantly increased in *GKN1*- or *CagA* plus *GKN1*-transfected cells by 30.21% and 17.29%, respectively (Figure 2D).

Expression of apoptosis-related proteins and the activity of caspase 3/7 were examined by western blot and an Apo-One Homogeneous Caspase 3/7 assay kit in *CagA*- or *GKN1*-transfected cells. Although CagA had no effect on apoptosis, ectopic expression of GKN1 induced a time-dependent increase in caspase 3/7 activity and expression of cleaved poly (ADP ribose) polymerase and caspase-3, and downregulated the expression of Mcl-1, Bcl-2 and Bcl-xL (Figure 2E and F). In addition, CagA-positive human gastric mucosae also demonstrated increased expression of antiapoptotic proteins and reduced expression of pro-apoptotic protein BAX (Figure 2G). These results indicate that GKN1 induces apoptosis and inhibits the antiapoptotic activity of CagA.

GKN1 downregulates CagA-induced cell proliferation via inactivation of cell cycle progression, NF-κB and PI3K pathways

Ectopic expression of CagA resulted in increased S phase population, whereas the transfection with *GKN1* and *CagA* plus *GKN1* showed the increase in number of cells in G2/M phase (Figure 3A). CagA decreased the expression of p53, p21 and p16, and induced the expression of E2F1, cyclin D1 and cyclin E, but GKN1 dramatically increased the expression of negative cell cycle regulators and reduced the expression of positive cell cycle regulators in both AGS and HFE-145 cells (Figure 3B). These results were also confirmed in CagA-positive human gastric mucosae (Figure 3C). In addition, CagA enhanced the expression of NF-κB-related proteins in both cell lines and CagA-positive human gastric mucosae, but the presence of GKN1 significantly inactivated NF-κB signaling pathway by induction of p-IκB expression (Figure 3D and E). In RT–qPCR, CagA increased the mRNA transcript expression of *PI3K* and *COX-2* genes in both cells and CagA-positive human gastric mucosae; however, GKN1 considerably suppressed the expression of these genes (Figure 3F and G). Furthermore, CagA increased *IL-1β, -6, -8, -10* and *TNF-α* mRNA expression in both cells, but GKN1 significantly inhibited the expression of genes listed above (Figure 3H). These findings indicate that GKN1 may suppress cell proliferation through inhibiting the cell cycle progression, NF-κB and PI3K pathways.

GKN1 is involved in inhibition of the CagA-induced cell migration and invasion in vitro

CagA may enhance the bacterial adhesion to gastric cancer cells (AGS) via the recruitment of scaffolding protein zonula occludens-1 and junctional adhesion molecule to the sites of bacterial attachment, causing an ectopic assembly of tight-junction components and dysplastic alterations in epithelial cell morphology (6). CagA also alters Madin Darby canine kidney cells from a polarized to an invasive phenotype that resembles an EMT and may be an early event in the carcinogenesis of *H.pylori* infection (32). Comparing the morphology of mock-, *CagA*- and *GKN1*-transfected AGS and HFE-145 cells, CagA induced cell elongation in both cell lines. However, ectopically expressed GKN1 dramatically inhibited the CagA-induced cell elongation (Figure 4A).

In wound healing assays, GKN1 significantly inhibited cell migration into the wound area (Figure 4B). In microchemotaxis assay, cell migration to the lower chamber was increased by 123.5% in *CagA*-transfected AGS cells, but GKN1 markedly decreased cell migration by 67.9%, even in *CagA* plus *GKN1*-transfected AGS cells (Figure 4C). In *CagA*-transfected MKN1 and MKN28 cells, cell migration to the lower chamber was 121.8% and 126.3%, respectively. However, cell migration was considerably decreased by 72.4% and 78.8% in *GKN1*-transfected MKN1 and MKN28 cells, respectively (Figure 4C).

In a Matrigel assay, invasiveness was significantly increased in *CagA*-transfected AGS, MKN1 and MKN28 cells, but it was inhibited in those transfected with *GKN1* (Figure 4D). In sum, these results suggest that GKN1 may inhibit the CagA-induced migration and invasion of gastric cancer cells.

GKN1 is involved in inhibition of the CagA-induced EMT

To further investigate the effect of GKN1 on CagA-induced EMT regulation, expression of EMT-related proteins, including Akt, E-cadherin, β-catenin, snail and slug, was examined in lysates from AGS and HFE-145 cells transfected with *CagA* or *GKN1*. Expectedly, CagA induced increased expression of p-Akt, β-catenin, snail, slug and vimentin proteins in both AGS and HFE-145 cells, and CagA-positive human mucosae (Figure 4E and F). However, in *GKN1*- and *CagA* plus *GKN1*-transfected cells, re-expression of E-cadherin and reduced expression of p-Akt, β-catenin, slug, snail and vimentin proteins were detected (Figure 4E and F). In addition, we found that CagA binds to E-cadherin, thereby, activating β-catenin, but GKN1 inhibits the CagA/E-cadherin complex, resulting in formation of the E-cadherin/β-catenin complex (Figure 4G). These data suggest that GKN1 may revert the CagA-induced EMT in gastric epithelial cells.

GKN1 is involved in inhibition of the H.pylori CagA-induced ROS production

Since *H.pylori* is known to induce frequent chromosomal aberrations, production of ROS and DNA damage in gastric epithelial cells (33,34), we investigated whether CagA-induced ROS is involved in the regulation of *GKN1* copy number. We found the increased

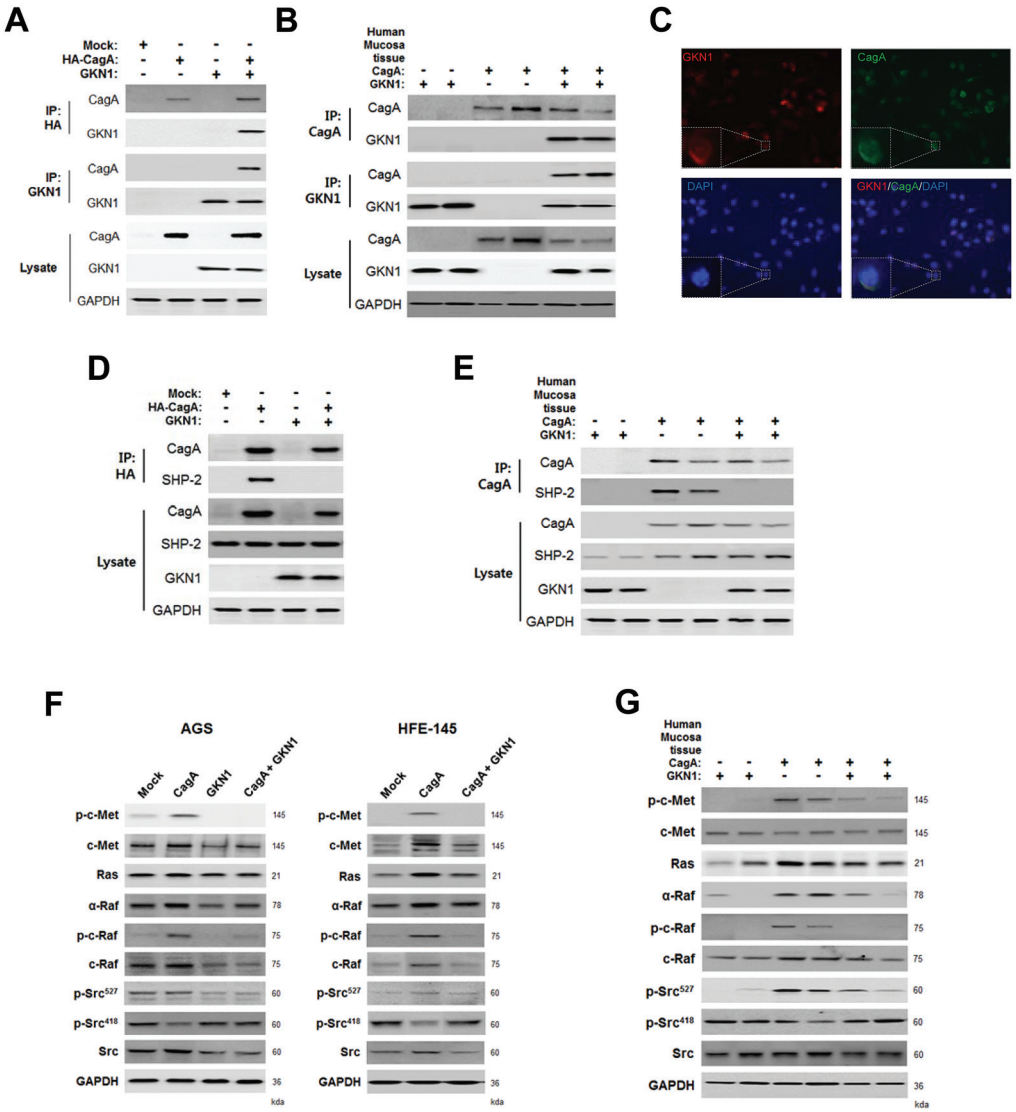


Fig. 1. GKN1 inhibits *Helicobacter pylori* CagA phosphorylation-dependent and -independent pathways via binding to CagA protein. (**A** and **B**) In *GKN1*- or *CagA*-transfected AGS cells (A) and human gastric mucosae (B), GKN1 bound to CagA protein. Immuno precipitated proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and processed for immunoblotting with the indicated antibodies. (**C**) Co-localization of GKN1 and CagA: GKN1 staining (red), CagA staining (green) and merge of GKN1 and CagA (yellow color shows the co-localization of two proteins). (**D** and **E**) In *CagA* plus *GKN1*-transfected AGS cells (D) and human gastric mucosae (E), GKN1 inhibited the CagA/SHP-2 complex formation. Immunoprecipitated proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes and processed for immunoblotting with the indicated antibodies. (**F** and **G**) In *CagA* plus *GKN1*-transfected AGS cells (F) and human gastric mucosae (G), the presence of GKN1 inhibited CagA phosphorylation-dependent and independent pathways. The data are representative of three separate experiments.

ROS levels in *CagA*-transfected cells, although GKN1 significantly decreased CagA-induced ROS production in AGS, MKN1, MKN28 and HFE-145 cells (Figure 5A), indicating that CagA-induced ROS play a role in *GKN1* copy number variation.

Additionally, CagA dramatically reduced *MnSOD* and *catalase* mRNA and protein expression in gastric cells and CagA-positive human gastric mucosae (Figure 5B–E). However, presence of GKN1 induced increased expression of *MnSOD* and *catalase* in cell lines and human gastric mucosae (Figure 5B–E). Thus, we conclude that GKN1 may inhibit CagA-induced ROS by upregulating antioxidant genes.

GKN1 inhibits the CagA-induced genetic alteration

Since GKN1 is downregulated in *H.pylori*-infected gastric epithelial cells (10,11), we constructed *in vitro* CagA-positive and -negative *H.pylori* infection models using AGS gastric cancer cells. The level of *GKN1* mRNA expression was reduced in CagA-positive *H. pylori*-infected AGS cells after *GKN1* transfection, compared with that in CagA-negative *H.pylori* (Figure 6A). Furthermore, ectopic expression of CagA induced the decrease of GKN1 protein expression (Figure 6B). In addition, the gastric mucosal tissues of *H.pylori*-infected C57BL/6 mice also showed decreased DNA copy number, mRNA transcript and protein expression of GKN1 (Figure 6C). In humans, the CagA protein expression was detected in 26 (48.1%) of 54 non-cancerous gastric mucosae, and *GKN1* copy number, mRNA expression, and protein levels were significantly lower in CagA-positive gastric mucosa than in CagA-negative cases ($P = 0.0013$) (Figure 6D). Statistically, there was a strong correlation among DNA, mRNA and protein expression of GKN1 ($P < 0.01$). These results indicate that CagA may be involved in regulation of the GKN1 expression.

Next, we analyzed the effects of CagA on DNA copy number, mRNA and protein expression of GKN1 in AGS, MKN1, MKN28 and HFE-145 cells. As expected, ectopic expression of CagA decreased DNA copy number, mRNA transcript and protein expression of GKN1

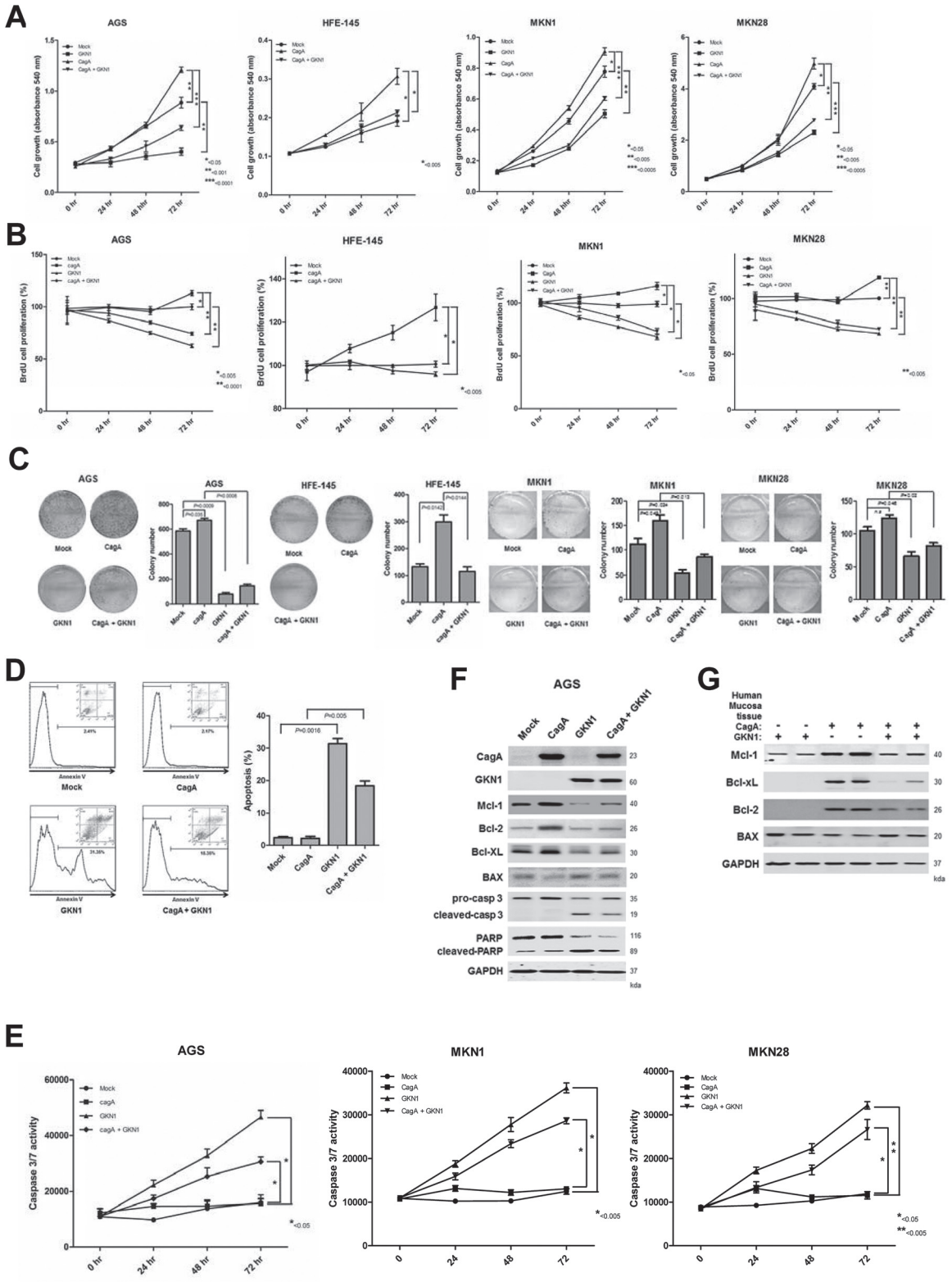


Fig. 2. GKN1 inhibits the *Helicobacter pylori* CagA-induced cell proliferation. (**A** and **B**) CagA induced a time-dependent increase in cell viability (A) and proliferation (B), but the ectopic expression of GKN1 inhibited the CagA-induced increase in cell viability and proliferation. (**C**) CagA induced the colony formation in gastric cancer and HFE-145 cells, whereas GKN1 markedly suppressed the CagA effect on colony formation. (**D** and **E**) Cell death assay revealed a significant increase of apoptotic cells in *GKN1*-transfected AGS cells compared with mock-transfected cells. However, CagA inhibited the GKN1-induced apoptotic cell death (D). There was a significant time-dependent increase of caspase 3/7 activity in *GKN1*-transfected cells, and CagA partially ablated the apoptotic activity of GKN1 (E). (**F**) Western blot analysis showed increased expression of antiapoptotic proteins, including Mcl-1, Bcl-2 and Bcl-xL, in CagA-transfected cells, and decreased expression of these proteins, as well as cleaved caspase-3 and PARP in *GKN1*-transfected cells. (**G**) Human gastric mucosae infected with *H.pylori* also demonstrated increased expression of antiapoptotic proteins. The data are representative of three separate experiments. Data are presented as the mean ± SEM of triplicate experiments.

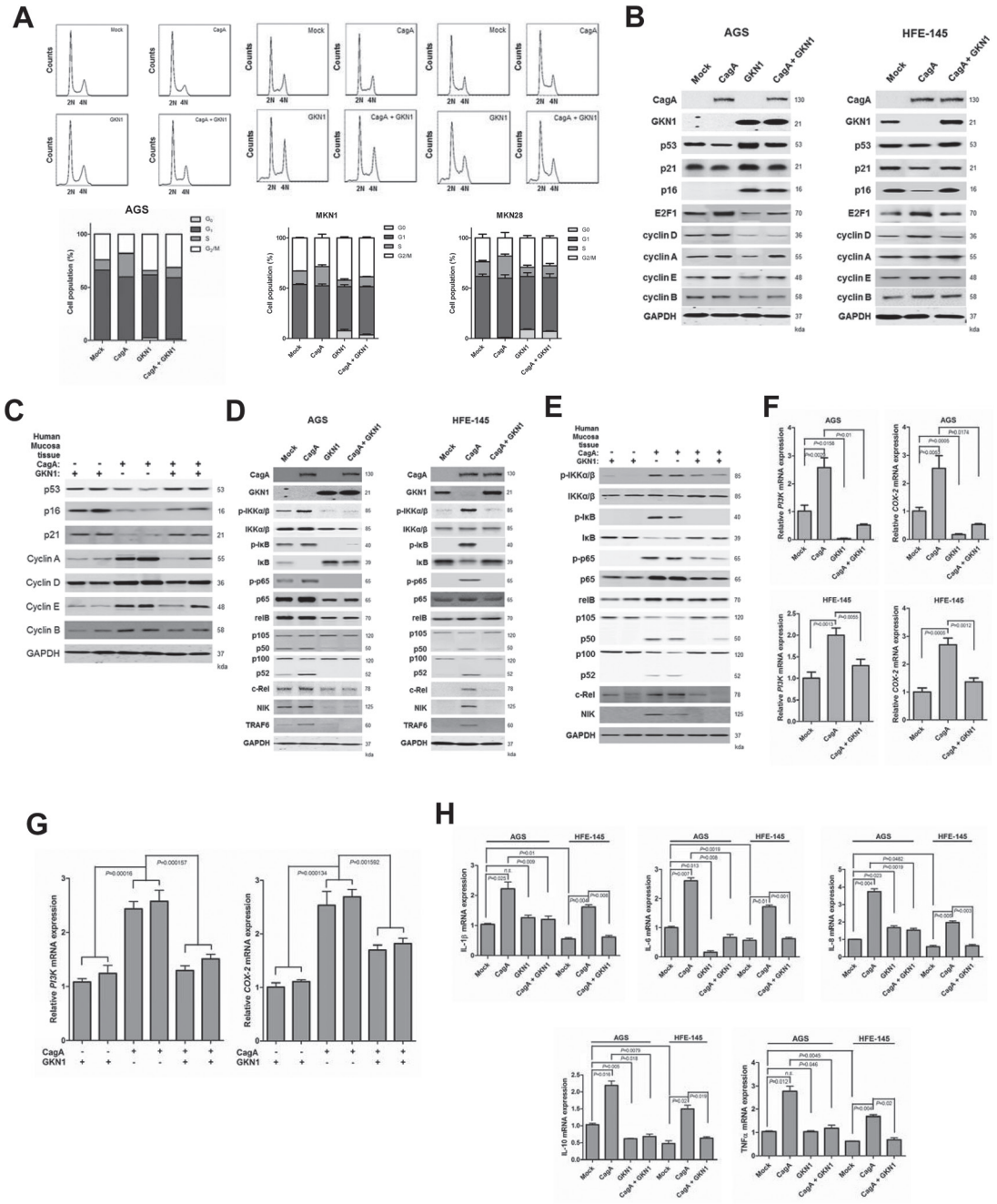


Fig. 3. GKN1 suppresses the CagA-induced cell cycle progression and activation of NF-kB signaling pathway. (**A**) CagA increased the population in the S phase with a corresponding decrease of cells in the G1 and G2/M phases, whereas GKN1 suppressed these effects of CagA. (**B** and **C**) CagA inhibited the expression of p53, p21 and p16 in AGS and HFE-145 cells (B), and human mucosae (C), but GKN1 reverted the expression of these proteins. (**D** and **E**) CagA increased the expression of p-IKKα/β, p-IkB, p-NF-κB p65, p105/50, p100/52 and NIK in AGS and HFE-145 cells (D), and human mucosae (E) However, GKN1 inhibited the expression of these proteins. (**F** and **G**) *Helicobacter pylori* increased the mRNA expression of *PI3K* and *COX-2* in AGS and HFE-145 cells (F), and human mucosae (G). (**H**) CagA increased IL-1β, -6, -8, -10 and TNFα mRNA expression, but GKN1 inhibited the expression of these genes in AGS and HFE-145 cells. The data are representative of three separate experiments. Data are presented as the mean ± SEM of triplicate experiments.

in these cells (Figure 6E and F). However, ectopically expressed GKN1 reverted the CagA effect on *GKN1* DNA copy number and mRNA transcript expression (Figure 6E and F). We also investigated whether CagA-induced ROS production led to the reduction of *GKN1* DNA copy number. Expectedly, CagA and H_2O_2 reduced *GKN1* copy number, whereas the treatment with TEMPOL significantly restored *GKN1* copy number (Figure 6G).

To examine the effect of GKN1 on *H.pylori* colonization, we treated *GKN1* plasmid and recombinant GKN1 protein in *H.pylori*-infected AGS cells. Interestingly, *GKN1*-transfection and recombinant GKN1 treatment considerably decreased the *H.pylori* colony number (Figure 6H), suggesting that GKN1 may contribute to the eradication of *H.pylori*.

In addition, the ability of the *GKN1* to discriminate the patients with CagA infection and gastric cancer from negative controls was analyzed using ROC curve. GKN1 predicted the CagA expression and the risk of gastric cancer with an area under the ROC curve value of 0.866 and 0.910, respectively (Figure 6I). These results demonstrate that GKN1 may inhibit the CagA-induced genetic alterations. Although further studies and clinical trials would be critical to verify

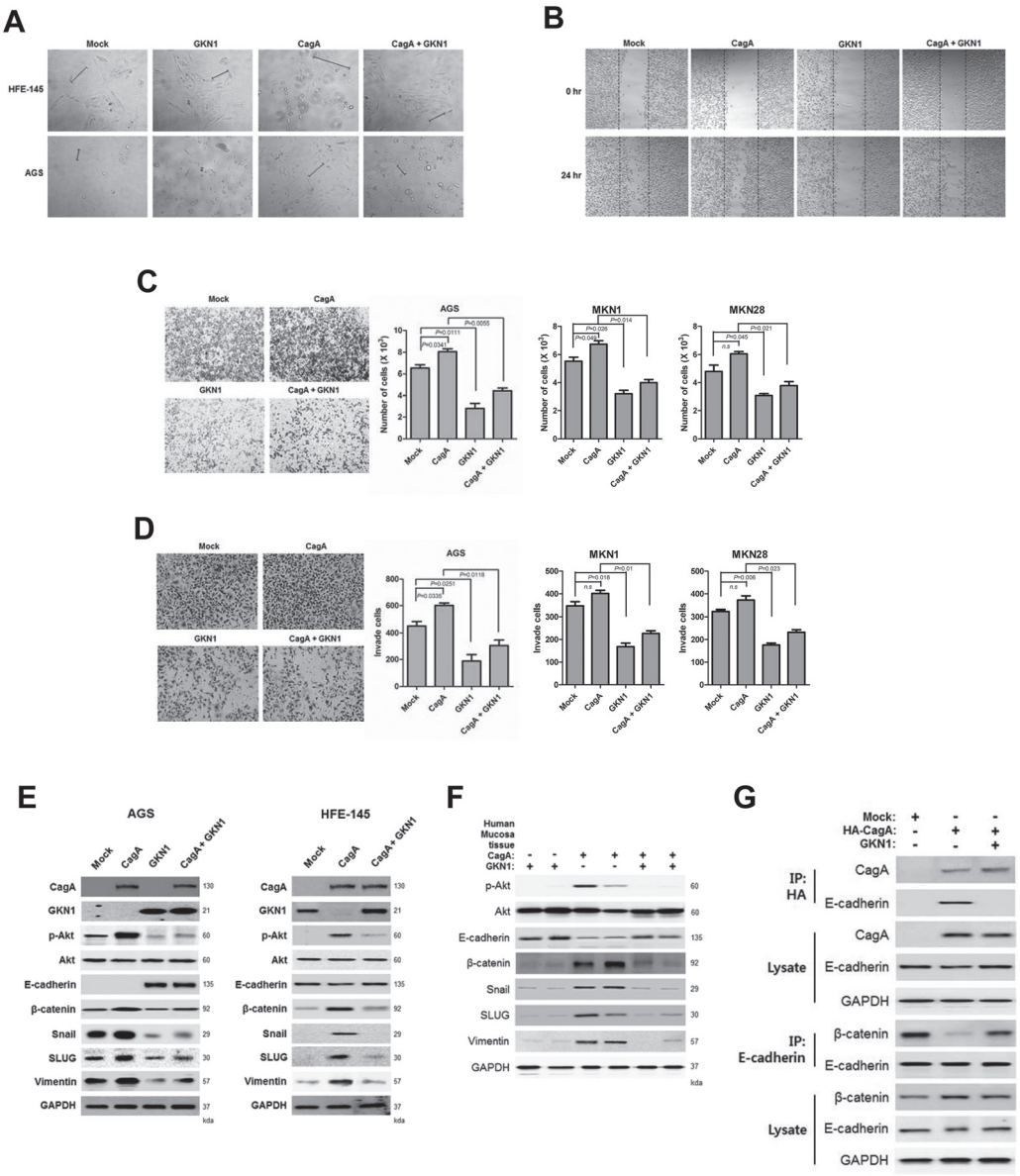


Fig. 4. GKN1 inhibits the *Helicobacter pylori* CagA-induced cell migration, invasion and EMT-related protein expression. (**A**) *CagA* transfection displayed elongated spindle shaped cell morphology, but GKN1 suppressed CagA-induced cell elongation in both cells. (**B**) A scratch wound healing assay demonstrated that cell migration was significantly induced in *CagA*-transfected AGS cells and dramatically inhibited in *GKN1*- or *GKN1* plus *CagA*-transfected cells. The dotted lines indicate the original edges of the scratch defect. (**C**) Cell migration was significantly inhibited by 43%, 28% and 22% in GKN1-overexpressing AGS, MKN1 and MKN28 cells, even in *GKN1* plus *CagA*-transfected AGS, MKN1 and, MKN28 cells, respectively. (**D**) In the Matrigel assay, the cells that invaded through the matrix were photographed 24 h after transfection. AGS, MKN1 and MKN28 cells transfected with *CagA* showed increased cell invasion, whereas ectopic GKN1 expression significantly inhibited cell invasiveness. Data are presented as the mean ± SEM of triplicate experiments. (**E** and **F**) CagA increased the expression of p-Akt, β-catenin, snail, slug and vimentin in both gastric cells (E), and human mucosae (F). Conversely, GKN1 treatment induced re-expression of E-cadherin and suppressed β-catenin, slug, snail and vimentin expression in both cells. (**G**) In *GKN1* or *CagA*-transfected HFE-145 cells, CagA bound to E-cadherin protein, but GKN1 inhibited the CagA/E-cadherin complex formation, resulting in inactivation of β-catenin. Immunoprecipitated proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes and processed for immunoblotting with the indicated antibodies. The data are representative of three separate experiments.

the applicability of GKN1 as a diagnostic marker for patients, it is possible that GKN1 may be a potential diagnostic biomarker for *H. pylori* CagA status.

Discussion

The CagA protein and vacuolating cytotoxin (VacA) protein are the main virulence factors of *H.pylori* infection. *Helicobacter pylori* strains possessing the *CagA* gene are more harmful to the gastric mucosa and are associated with an increased risk of gastric cancer, suggesting that the CagA protein may have carcinogenic potential (35). Virulent *H.pylori* isolates harbor the cag pathogenicity island, a 40 kb stretch of DNA, which encodes CagA and components of the sophisticated type IV secretion system (2,3). Here, we have provided direct molecular evidences that GKN1 can suppress the carcinogenic potential of CagA, resulting in downregulation of the CagA-induced cell proliferation, ROS production and EMT.

GKN1 has a mucosal barrier function to prevent the gastric mucosa from systemic exposure to foreign antigens, bacteria and gastric acid (11,36). Previously, we found that GKN1 inhibited cell proliferation and EMT and induced cell death (12,13). In this study, we investigated the effect of GKN1 on CagA carcinogenic potential.

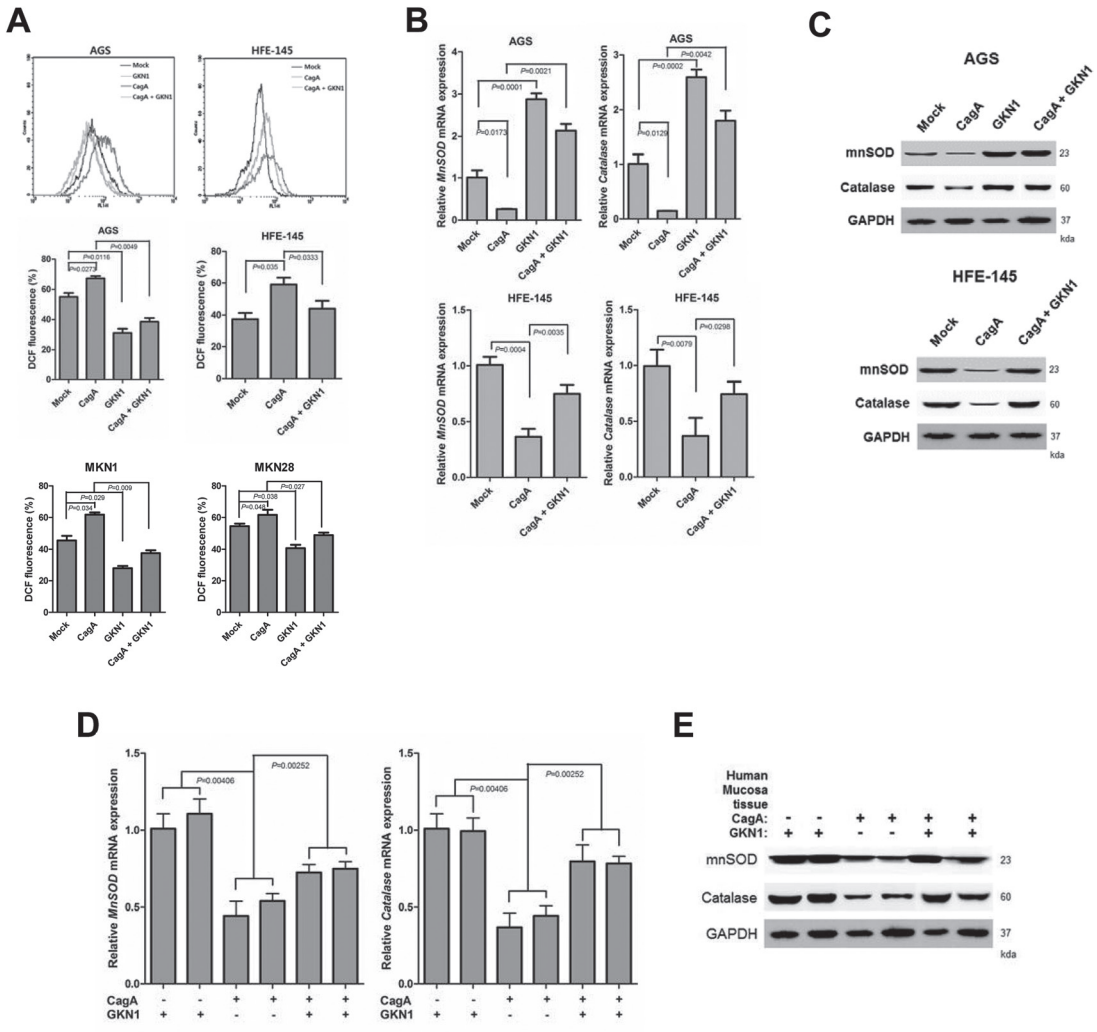
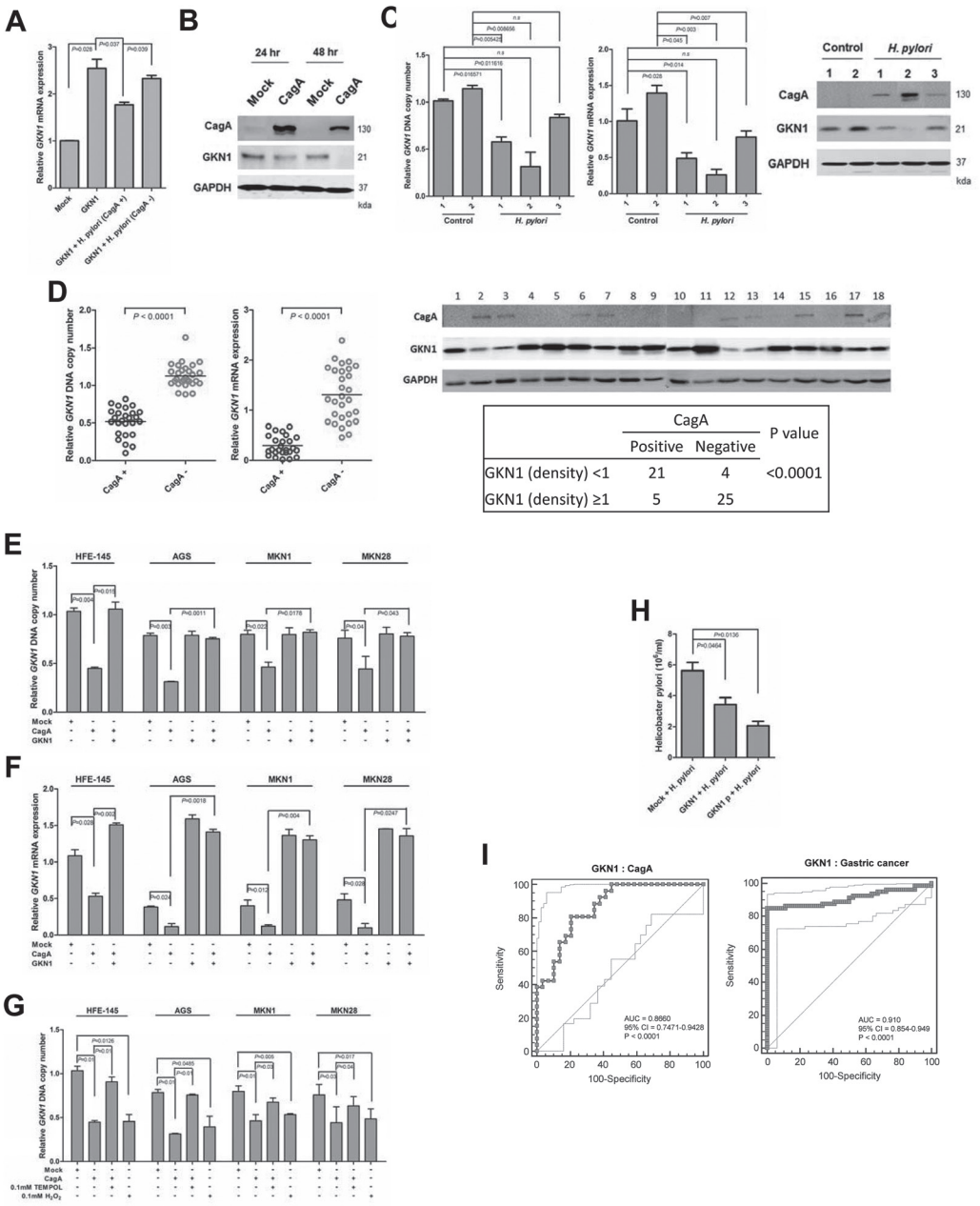


Fig. 5. GKN1 suppressed the *Helicobacter pylori* CagA induced ROS production. (**A**). *CagA* transfection induced ROS production, whereas GKN1 suppressed CagA-induced ROS production in AGS, MKN1, MKN28 and HFE-145 cells. (**B** and **C**) CagA decreased mRNAs (B) and protein (C) expression of *MnSOD* and *catalase* in both cells, but GKN1 increased the expression of these antioxidant enzymes. (**D** and **E**) Human gastric mucosae also showed decreased mRNA (D) and protein (E) expression of these antioxidant enzymes. The data are representative of three separate experiments. Data are presented as the mean ± SEM of triplicate experiments.

Interestingly, we found that GKN1 binds to the CagA protein, and inhibits the formation of CagA/SHP-2 complex as well as independent phosphorylation pathway of CagA (Figure 1), suggesting that GKN1 may suppress the CagA function by inhibiting the transport of CagA into gastric epithelial cells. In addition, CagA was involved in the interaction with activated Met, leading to the sustained activation of PI3K/Akt signaling. This, in turn, resulted in the activation of β-catenin and NF-κB signaling pathway (Supplementary Figure 2, available at *Carcinogenesis* Online), which promotes proliferation and inflammation (37). Moreover, activation of the NF- κB signaling induced synthesis of cytokines, such as TNF-α, IL-1β, -6, -8 and -10. The synthesized cytokines were associated with cell proliferation, inflammation, antiapoptosis in various cancer cells (38–41). We previously reported that GKN1 inhibited β-catenin, cyclin D1 nuclear translocation and NF-κB signaling (13,14). Here, CagA demonstrated an increase in cell viability, proliferation and colony formation by inhibiting apoptosis and the expression of negative cell cycle regulators, while activating NF-κB and PI3K/Akt signaling pathways in gastric cells and CagA-positive human gastric mucosae (Figures 2–4). However, ectopic expression of GKN1 completely rescued these tumorigenic activities of CagA (Figures 2–4). Cumulatively, these results suggest that GKN1 inhibits the carcinogenic potentials of CagA that promote cell proliferation and antiapoptosis in gastric epithelial cells and, ultimately, may facilitate the development of gastric cancer.

During tumor progression, subsequent invasiveness is thought to herald the onset of the last stage of this multi-step process that eventually leads to metastatic dissemination with life-threatening consequences (42). It has been proposed that turning epithelial cancer cells into mesenchymal cells, the so-called EMT, is a critical process in tumor progression, whereby epithelial cancer cells acquire invasive and metastatic phenotype. Recently, it has been reported that CagA induces actin-cytoskeletal rearrangements involved in the host cell scattering and elongation (43). Additionally, increased expression of the PI3K catalytic subunit genes and an increase in Akt activity were observed in a variety of metastatic human cancer cells (44,45). We therefore examined whether CagA could modulate EMT and cell migration and, if so, whether GKN1 is able to inhibit these effects of CagA. Ectopic expression of CagA induced cell elongation and accelerated cell migration and invasiveness, but GKN1 abrogated these activities of CagA (Figure 4). In gastric cells and mucosal tissues infected with *H.pylori*, CagA led to the increased expression of p-Akt, β-catenin, slug, snail and vimentin, but ectopically expressed GKN1 restored the expression of E-cadherin and downregulated expression of EMT-inducible proteins. We also found that CagA binds to E-cadherin, resulting in activation of β-catenin, whereas GKN1 inactivated β-catenin by inhibiting the CagA/E-cadherin complex formation (Figure 4), an important change related to the adhesive and migratory properties necessary for local tumor invasion. These data



	CagA		P value
	Positive	Negative	
GKN1 (density) <1	21	4	<0.0001
GKN1 (density) ≥1	5	25	

Fig. 6. *Helicobacter pylori* CagA downregulates GKN1 copy number and expression. (**A**) AGS cells were treated with mock, *GKN1* and *GKN1* plus CagA-positive or CagA-negative *H.pylori*. Infection with CagA-positive *H.pylori* reduced *GKN1* mRNA expression, whereas that with CagA-negative *H.pylori* did not. (**B**) In HFE-145 cells, CagA completely downregulated GKN1 expression at 24 and 48 h after transfection. (**C**) Gastric mucosae from the mice infected with *H.pylori* also showed a decrease in *GKN1* copy number, mRNA transcript and protein expression. (**D**) *GKN1* copy number, mRNA and protein expression level were significantly lower in CagA-positive gastric mucosae than CagA-negative cases out of 54 non-cancerous tissues, illustrated by scatter plot ($P < 0.01$). (**E** and **F**) At 24 h after transfection with *CagA*, AGS, MKN1, MKN28 and HFE-145 cells showed decreased *GKN1* copy number (E) and mRNA (F) transcripts. However, the ectopic expression of GKN1 reverted the effects of CagA in these cells. (**G**) CagA or H_2O_2 treatment significantly reduced *GKN1* copy number, but treatment with antioxidant TEMPOL restored *GKN1* copy number in AGS cells. (**H**) *GKN1* transfection and recombinant GKN1 (GKN1p) treatment suppressed the *H.pylori* colonization. The data are representative of three separate experiments. (**I**) Receiver operating characteristics curve analysis of GKN1 was used to distinguish patients with CagA infection and gastric cancer. GKN1 yielded an area under the curve value of 0.866 (95% confidence interval [CI], 0.7471–0.9428; $P < 0.0001$) and 0.910 (95% CI, 0.854–0.949; $P < 0.0001$) in distinguishing the presence of CagA and gastric cancers, respectively. Data are presented as the mean ± SEM of triplicate experiments.

suggest that GKN1 may contribute to the progression of gastric cancer by inhibiting the CagA-induced EMT and migration of cancer cells.

Helicobacter pylori is known to induce the production of ROS and DNA damage in gastric epithelial cells and frequently causes chromosomal aberrations (33,34). Moreover, *H.pylori* infection downregulates the activity and expression of base excision and mismatch repair (46). Clinical observations have supported the integral link between the *H.pylori* CagA infection and increased disease pathology and chromosomal abnormalities in premalignant and malignant gastric tissues (47). In three gastric cancer cell lines and mice, CagA-positive *H.pylori* infection and ectopic CagA expression markedly reduced the *GKN1* gene copy number and protein expression (Figure 6). Expectedly, CagA increased ROS production and reduced the expression of antioxidant proteins including MnSOD and catalase (Figure 5A), whereas inhibition of ROS production by antioxidant TEMPOL resulted in the restoration of *GKN1* copy number

(Figure 6G). In addition, *GKN1* copy number and protein expression were also significantly decreased in CagA-positive gastric mucosae than in CagA-negative mucosae (Figure 6), consistent with the previous report of Nardone *et al*. (10). Another study observed increased expression of *GKN1* transcript after *H.pylori* eradication (48). These results indicate that *H.pylori* CagA-induced ROS may reduce the expression of the GKN1 protein by decreasing *GKN1* DNA and mRNA copy numbers. However, GKN1 inhibited the CagA-induced ROS production by overexpression of antioxidant enzymes (Figure 5) and the effect of CagA on *GKN1* copy number and expression (Figure 6). Furthermore, ectopically expressed GKN1 considerably suppressed *H.pylori* colonization (Figure 6). Although the molecular mechanisms for the antibacterial effects of GKN1 remains to be elucidated, our data suggest that GKN1 may counteract CagA-induced genetic alterations by inhibiting CagA injection into cell through binding to CagA at the extracellular level and by regulating antioxidant enzymes at intracellular level. Further studies are necessary to identify the role of GKN1 in nucleic acid synthesis, cytoplasmic membrane function and energy metabolism of *H.pylori*.

In conclusion, the present study reveals a novel additional function of the tumor suppressor gene, GKN1, which inhibits the CagA-induced carcinogenic potentials, including cell proliferation, antiapoptosis, EMT and invasion in gastric carcinogenesis. Although CagA reduced *GKN1* copy number and expression, ectopic expression of GKN1 inactivated NF-κB and PI3K/Akt signaling pathways, as well as cellular ROS production. GKN1 further inhibited the CagA-induced antiapoptosis and expression of EMT-inducible proteins and restored the expression of E-cadherin, which may inhibit migration and invasiveness of gastric cancer cells. Finally, these results strongly suggest that GKN1 may suppress the CagA-induced malignant transformation of gastric epithelial cells and progression of gastric cancer. Additional functional and translational studies of GKN1 will broaden our understanding of the pathogenesis of gastric cancer and provide us with novel diagnostic and therapeutic modalities for eradicating *H. pylori* and treating gastric cancer.

Supplementary material

Supplementary Materials and methods, Tables S1–S3 and Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/

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