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 cytokinin-associated gene A (CagA) and gastrokine 1 (GKN1) on cell proliferation, apoptosis, reactive oxygen species (ROS) production, epithelial–mesenchymal transition (EMT) production, epithelial–mesenchymal transition (EMT) production, and modulation of 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.

Materials and methods

Generation of CagA gene deleted H. pylori strains

The isogenic mutant H. pylori 26695 (CagA::aphA), in which most of CagA gene was replaced by a aphA (kanamycin resistant gene from pl1433) cassette, was made using PCR products generated with primers ‘kanF’ (5'-GATAAAACCCGCGAACATC-3’) and ‘aphAR’ (5’-CTAAGATATTTCATACCGGTAATG-3’) and CagA F5’ (5’-ATGCTGGGTTTATCATTGATTGCTTCTTTGACA TCGTACACGCCGACCACAATAG-3’) (552bp; upstream of deleted cagA segment); ‘CagA F5’ (5’-TTCTGATGAAAT GTTTTATGACATAATGCAAAGGTGGTGGAGATAGCTACTAATGCAAAATACGTCCTGCAACAC-3’) (425bp; downstream of deleted cagA segment). Natural transformation was carried out by adding 7 µl of purified PCR product containing this ΔcagA::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% O2, 10% CO2, and 85% N2) 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 × 105 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.

Abbreviations: CagA, cytotoxic-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; TEMPO, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl.
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 pSP65Rαpha vector containing a hemagglutinin (HA) tag. Dr Hatakeyama (Tokyo University, Tokyo, Japan) kindly provided the CagA construct, AGS, MKN1 and 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. Real-time qPCR was performed as described previously 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-tetramethyl-piperidine-1-oxyl (TEMPOL, Sigma–Aldrich), or H2O2 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-Dimethylthiazol-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 NaVO4 and 1.0% protease inhibitor cocktail (Sigma, St Louis, MO) as described previously (19). Equal copy number of GKN1 were found in 12 or 19, were found in 13 or 12 κ-1412κ-enhanced was κ). A 96-well plate clonogenic assay was also performed for 2 weeks in order to examine cell viability, proliferation and colony formation.

Measurement of DNA copy number change in CagA and GKN1-transfected AGS cells
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. Real-time qPCR was performed as described previously 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-tetramethyl-piperidine-1-oxyl (TEMPOL, Sigma–Aldrich), or H2O2 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).

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′-dichlorodihydrofluorescein 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.

Statistical analysis
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 antipapoptotic 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-kB-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 mucosa (Figure 1B). Furthermore, we found that GKN1 inhibits the CagA–SHP-2 complex formation in AGS cells and human gastric mucosa (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 mucosa (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 mucosa 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 mucosa (Figure 3C). In addition, CagA enhanced the expression of NF-κB-related proteins in both cell lines and CagA-positive human gastric mucosa, 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 mucosa; 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 zona occcludens-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- 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 mucosa (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
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 mucosa (Figure 5B–E). However, presence of GKN1 induced increased expression of MnSOD and catalase in cell lines and human gastric mucosa (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.
GKN1 as an inhibitor of *Helicobacter pylori* CagA

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.
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 H2O2 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. 3. GKN1 suppresses the CagA-induced cell cycle progression and activation of NF-κB 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-IκKα/β, p-IκB, 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.
GKN1 as an inhibitor of *Helicobacter pylori* CagA

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.

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. 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 mucosa (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.

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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 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

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
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.
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 eradicateating 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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References

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