

# Regulation of GKN1 expression in gastric carcinogenesis: A problem to resolve (Review)

JUDIT ALARCÓN-MILLÁN<sup>1</sup>, DINORAH NASHELY MARTÍNEZ-CARRILLO<sup>1</sup>,  
OSCAR PERALTA-ZARAGOZA<sup>2</sup> and GLORIA FERNÁNDEZ-TILAPA<sup>1</sup>

<sup>1</sup>Clinical Research Laboratory, Faculty of Biological Chemical Sciences,  
Guerrero Autonomous University, Chilpancingo, Guerrero 39070;

<sup>2</sup>Direction of Chronic Infections and Cancer, Research Center in Infection Diseases,  
National Institute of Public Health, Cuernavaca, Morelos 62100, México

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**Abstract.** Gastrokine 1 (GKN1) is a protein expressed on the surface mucosa cells of the gastric antrum and fundus, which contributes to maintaining gastric homeostasis, inhibits inflammation and is a tumor suppressor. The expression of GKN1 decreases in mucosa that are either inflamed or infected by *Helicobacter pylori*, and is absent in gastric cancer. The measurement of circulating GKN1 concentration, the protein itself, or the mRNA in gastric tissue may be of use for the early diagnosis of cancer. The mechanisms that modulate the deregulation or silencing of GKN1 expression have not been completely described. The modification of histones, methylation of the *GKN1* promoter, or proteasomal degradation of the protein have been detected in some patients; however, these mechanisms do not completely explain the absence of GKN1 or the reduction in GKN1 levels. Only NKX6.3 transcription factor has been shown to be a positive modulator of *GKN1* transcription, although others also have an affinity with sequences in the promoter of this gene. While microRNAs (miRNAs) are able to directly or indirectly regulate the expression of genes at the post-transcriptional level, the involvement of miRNAs in the regulation of GKN1 has not been reported. The present review analyzes the information reported on the determination of GKN1 expression and the regulation of its expression at the transcriptional, post-transcriptional and post-translational levels; it proposes an integrated model that incorporates the regulation of GKN1 expression via transcription factors and miRNAs in *H. pylori* infection.

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## 1. Introduction

The gastric epithelium is continually renewed over a lifetime, and is maintained through the proliferation and differentiation of pluripotent stem cells from the isthmus of the gastric gland (1). Stem cells generate precursors that migrate to the gastric lumen and, in turn, generate parietal, gastric zymogenic and foveolar cells. Parietal cells produce hydrochloric acid, while gastric zymogenic cells have a half-life of ~6 months and synthesize trefoil factor 2 and mucin 6 (1-3). Foveolar cells or surface mucous cells (SMCs), whose half-life is 2-3 days, produce mucous granules, mucin 5AC, gastrokine 1 (GKN1) and trefoil factor 1 (1-3) and play an important role in the restitution of the gastric mucosa in the event of *Helicobacter pylori* infection (4). The integrity and continuity of the gastric epithelium are rapidly restored after damage, prior to cell proliferation (5). Epithelial restitution is achieved through the migration of epithelial cells from the adjacent area or the cell stratum below the surface cells in the injured area. Epithelial cell restitution in the stomach of mammals takes place in minutes (5).

GKN1 is a protein secreted by SMCs of the gastric antrum and fundus (6), which contributes to maintaining gastric homeostasis, inhibits inflammation and acts as a tumor suppressor (7-16). The expression of GKN1 decreases due to *H. pylori* infection, inflammation or atrophy, and is absent in gastric cancer (17-25).

Although methylation of the GKN1 promoter (20), modification of histones (26) and proteasomal degradation of GKN1 in some cases (27) have been reported, the mechanisms that

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**Correspondence to:** Dr Gloria Fernández-Tilapa, Clinical Research Laboratory, Faculty of Biological Chemical Sciences, Guerrero Autonomous University, Avenida Lázaro Cárdenas S/N, Col. Haciendita, Chilpancingo, Guerrero 39070, México  
E-mail: gferti@hotmail.com

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cause a decrease in GKN1 levels or its absence entirely have not been completely described. A total of ~10% of gastric tumors contain the Epstein-Barr virus (EBV), while the Epstein-Barr nuclear antigen 1 (EBNA1) binds to the promoter region of the *GKN1* gene and induces the reduction of its transcription (28,29).

Epigenetic modifications are as important to the regulation of gene expression and the initial stages of disease as genetic modifications. Differential changes have been documented in the expression profile of microRNAs (miRNAs) in gastritis or cancer patients infected with *H. pylori* (17,30-33), gastric cancer cell lines (34-36), CD4 T lymphocytes, macrophages, monocytes and dendritic cells (37-39). It has been proposed that some components of *H. pylori* induce the activation of signals that modify the expression of miRNAs in the host cells, and that changes in the global expression profile of miRNAs are related to the genotype of the bacteria (31).

The reduction of GKN1 cannot be explained by mutations in its gene, the methylation of its promoter or the proteasomal degradation of the protein (20,26,27). It is probable that some miRNAs modulate the decreased translation of GKN1 mRNA and, consequently, the reduction of the level of protein in the gastric mucosa. The role of miRNAs in the regulation of GKN1 expression in the normal gastric mucosa, or mucosa infected with *H. pylori*, affected by preneoplastic lesions or with gastric cancer, has not been explored. The present review comprises an analysis of the information published on the regulation of GKN1 expression, proposing a model that integrates the probable regulatory mechanisms at the transcriptional, post-transcriptional and translational levels.

## 2. GKN1

GKN1 (also known as CA11, AMP-18, foveolin or TFIZ2) is a small protein of 181-184 amino acids, specifically expressed in the stomach. The *GKN1* gene is located on chromosome 2p13.3 and is composed of six exons separated by relatively short introns (6). GKN1 is composed of: i) A hydrophobic signal peptide in the extreme NH<sub>2</sub>-terminal, whose processing generates a protein of 160 amino acids with a molecular mass of 18 kDa; ii) a BRICHOS domain with three conserved amino acid residues, one aspartic acid residue and two cysteine acid residues; and iii) a COOH-terminal domain (6,19,40-43). GKN1 is a member of the BRICHOS superfamily of proteins, which includes proteins associated with the development of cancer. It is a protein with both an autocrine and paracrine function, which promotes the healing of the mucosa and facilitates cellular restitution and proliferation (43). GKN1 modulates the progression of the cell cycle, cellular proliferation and viability, and apoptosis.

Additionally, GKN1 regulates the production of reactive oxygen species (ROS) and the PI3K/Akt signaling pathway, thus influencing epithelial mesenchymal transition (EMT) and the migration of cancerous cells. GKN1 significantly inhibits the expression of the mRNA of DNA (cytosine-5)-methyltransferase 1 (DNMT1) and histone-lysine N-methyltransferase EZH2 (EZH2) and the activity of DNMT1, functions that link this protein to the inhibition and progression of cancer (43,44).

In the normal gastric mucosa, GKN1 is expressed by epithelial cells on the surface, but not at the depth of the glands of the gastric mucosa (21,45,46). GKN1 reduces the expression of the gastrin receptor, gastrin/cholecystokinin type B receptor, thus inhibiting the cell proliferation induced by this hormone (13). GKN1 activates the p16/Rb and p21 signaling pathways, inhibits cell growth and drives cells to senescence (46). GKN1 modulates the expression of cytokines and other inflammatory mediators associated with gastric carcinogenesis, inducing the increased expression of interleukin (IL)-8 and IL-17 and the decreased expression of nuclear factor (NF)-κB, IL-6 and IL-10. Thus, it regulates the immune response and inhibits the progression of epithelial gastric cells to cancerous cells. GKN1 suppresses the activation of NF-κB, and thus inhibits the oncogenic signaling regulated by this transcription factor (9).

## 3. GKN1, *H. pylori* infection and gastric cancer

*GKN1* and *H. pylori* infection. Infection with *H. pylori cagA*<sup>+</sup> strains increases the risk of gastric cancer and is related to the reduced expression of GKN1 in the mucosa (47). In mice infected with *H. pylori-cagA*<sup>+</sup>, the increased expression of the antiapoptotic proteins Bcl-2, Bcl-XL and induced myeloid leukemia cell differentiation protein Mcl-1, as well as NF-κB and proteins related to EMT, is found, while the expression of p53, p21, p16 and stress response genes decreases (48). The ectopic expression of GKN1 suppresses the effects of *H. pylori-cagA*<sup>+</sup> in the human gastric cancer cell lines AGS, MKN1 and MKN28. Based on these findings, it has been suggested that GKN1 suppresses the malignant transformation of gastric epithelial cells and the progression to gastric cancer (48).

The expression of GKN1 decreases at the mRNA and protein levels in dyspeptic patients and is not detected in the mucosa of subjects with intestinal-type gastric cancer, both with and without *H. pylori* infection (19,20,21,22,30,49-52), or with a diffuse-type cancer (19,23,25).

*GKN1* and gastric cancer. GKN1 is absent in human gastric tumors and acts as a tumor suppressor, regulating cell proliferation, apoptosis, migration and invasion in gastric cancer cell lines (10). Stimulating the expression of Fas receptor and the activation of caspase-3, this protein modulates apoptotic signals, playing an important role in the repair of tissues during the early stages of neoplastic transformation (7).

In AGS, MKN-1 and MKN-28 gastric cancer cell lines transfected with GKN1, the re-expression of p16 and a reduction in CDK4, cyclin D1 and E2F levels was observed (8). In gastric cancer SGC7901 cells, GKN1 reduces the expression of MMP2, through the deactivation of NF-κB (15), and induces the expression of miRNA (miR)-185. The extreme 3' untranslated region (UTR) of RhoA mRNA has sequences with affinity to miR-185 and, when this hybrid miRNA with RhoA mRNA reduces its translation, the silencing of RhoA is indirectly mediated by GKN1. c-Myc is a transcription factor that activates RhoA expression and is a target of miR-34a, a miRNA whose expression is promoted by GKN1. Thus, GKN1 also deactivates RhoA via miR-34a. These data suggest that GKN1 inhibits cell motility and invasion by means of the deactivation of RhoA (16).

#### 4. GKN1 as a potential biomarker of gastric carcinogenesis

In ~80% of gastric cancer cases, symptoms are scarce and non-specific at the early stages of the disease, with the majority of patients diagnosed at an advanced stage with metastasis already occurring (44,53). Thus, the treatment of this malignancy is ineffective and the prognosis for patients is unfavorable. Due to the late diagnosis and consequent limited therapy options for most patients, the 5-year survival rate is <20% (54). The lack of criteria and useful markers for early diagnosis has led to studies being conducted on the expression of genes associated with gastric carcinogenesis, with the objective of identifying biomarkers characteristic of premature stages of the disease. GKN1 is one of the proteins considered to be potential biomarker of carcinogenesis.

There are few reports in the available literature on the identification of GKN1 in samples taken from patients. Nardone *et al* (17) identified the presence of GKN1 in human gastric tissue, finding that its expression decreases in the event of *H. pylori* infection, deteriorates progressively from chronic gastritis to atrophic gastritis, and is not detected in areas in which intestinal metaplasia or *H. pylori*-positive tumors are found (17). GKN1 is absent in cases of gastric cancer without *H. pylori* infection (17-25). Villano *et al* (55) analyzed the level of *GKN1* mRNA in serum taken from patients with gastric cancer and apparently healthy volunteers, finding no statistically significant differences between patients with cancer and healthy volunteers. The aforementioned results indicate that *GKN1* mRNA is not a useful biomarker for the diagnosis of gastric cancer (55). Yoon *et al* (56) found that the serum levels of GKN1 are significantly lower in gastric cancer patients than in either apparently healthy subjects or patients with hepatocellular and colorectal carcinoma ( $P<0.0001$ ). These data suggest that the serum levels of GKN1 may be used for the differentiation of patients with gastric cancer from those with other malignancies of the digestive system and clinically healthy subjects. The authors concluded that the serum concentration of GKN1 may be an informative diagnostic biomarker for gastric cancer (56). Dokhaee *et al* (44) reported that *GKN1* mRNA is significantly reduced in the gastric tissue of patients with gastric cancer, compared to normal tissue. The results led to the hypothesis that GKN1 may be a reliable biomarker for the detection of gastric cancer in its early stages.

The aforementioned data indicate that the measurement of circulating GKN1 concentration, the protein or the mRNA in gastric tissue may be of utility for the early diagnosis of cancer. However, it is necessary to strengthen these findings with more research in patients with preneoplastic lesions (atrophic gastritis, intestinal metaplasia and dysplasia) and cancer in distinct stages of evolution.

#### 5. Regulation of GKN1 expression in mucosa infected with *H. pylori* or with gastric cancer

At the chromosomal level, cytogenetic aberrations, such as duplications, translocations, deletions or the loss of heterozygosity in the 2p13 chromosome (in which the *GKN1* gene is found) have not been detected (57,58).

The sequence of the *GKN1* gene was analyzed in 81 gastric tumors and 40 adenomas, confirming a lack of mutations (20)

These data suggested that the reduction of GKN1 cannot be attributed to cytogenetic aberrations or mutations, and that other mechanisms are involved in the deregulation of this protein.

Gene expression is regulated at different levels, from transcription to translation (59). At the transcriptional level, regulation occurs via epigenetic modifications, such as the modification of histones and the methylation of DNA (60,61). At the post-transcriptional level, the role of small RNAs (miRNAs) in the modulation of translation should be taken into account (62), while at post-translational level, ubiquitination, followed by the proteasomal degradation of the marked protein, is the best-known mechanism involved in the reduction in cytoplasmic levels of proteins (61).

**Transcription factors.** Transcription factors are able to activate or repress the expression of a gene (63,64). Little is known about the transcriptional regulation of *GKN1*. Yoon *et al* (65), using luciferase and chromatin immunoprecipitation assays, confirmed that NKX6.3 is a transcription factor for *GKN1*, and located the recognition sequence corresponding to NKX6.3 in the promoter region of the *GKN1* gene (Fig. 1A). NKX6.3 positively modulates the transcription of *GKN1*, which is reflected in the increased level of both mRNA and protein (65).

By means of *in silico* analysis, conducted using the MatInspector (66) (<http://www.genomatix.de/matinspector.html>), AliBaba2.1 (67) (<http://gene-regulation.com/pub/programs/alibaba2/index.html>) and TfsiteScan (68) (<http://www.ifti.org>) programs, transcription factors were identified with affinity to recognition sequences in the *GKN1* promoter region (Fig. 2A and Table I). It is likely that one or more of these transcription factors, predicted bioinformatically, are involved in the transcriptional regulation of *GKN1*. Experimental confirmation of the effect exerted by the proposed transcription factors on the modulation of *GKN1* expression will improve understanding of the mechanisms involved in the regulation of the expression of this protein.

In patients with gastric pathology, in murine models or in gastric epithelial cell lines, the expression of trans-acting T-cell-specific transcription factor GATA-3 (GATA-3), STAT-1, STAT-3, transcription factor Sp1 (Sp1), cyclic AMP-responsive element-binding protein 3-like protein 4 (CREB), AP-1 transcription factor (AP-1) and Oct-1 increases, while the levels of CCAAT enhancer binding protein- $\alpha$  (CEBP $\alpha$ ) and NKX6.3 decrease (65,69-82). The expression of GATA-3 was found to have increased at different stages of the carcinogenesis associated with *H. pylori* in patient biopsies, murine models and human gastric epithelial cells (73,74).

CagA and OipA of *H. pylori* induce the activation of transcription factors such as AP-1, NF- $\kappa$ B, STAT-3, CREB and nuclear factor of activated T cells (NFAT), which favor the expression of IL-6, and cytokines, which promote inflammation (83-89). IL-6 stimulates the activation of the signaling pathway gp130/STAT3 in gastric cancer cell lines (90), while CagA stimulates the expression of the NFAT transcription factor in AGS cells (88).

The protein Tipa, produced by *H. pylori*, activates the IL-6/STAT3 pathway (89). *H. pylori* cagA<sup>+</sup> strains induce signaling through the MAPK pathway, thus increasing proliferation and activating transcription factors such as AP-1 (70).

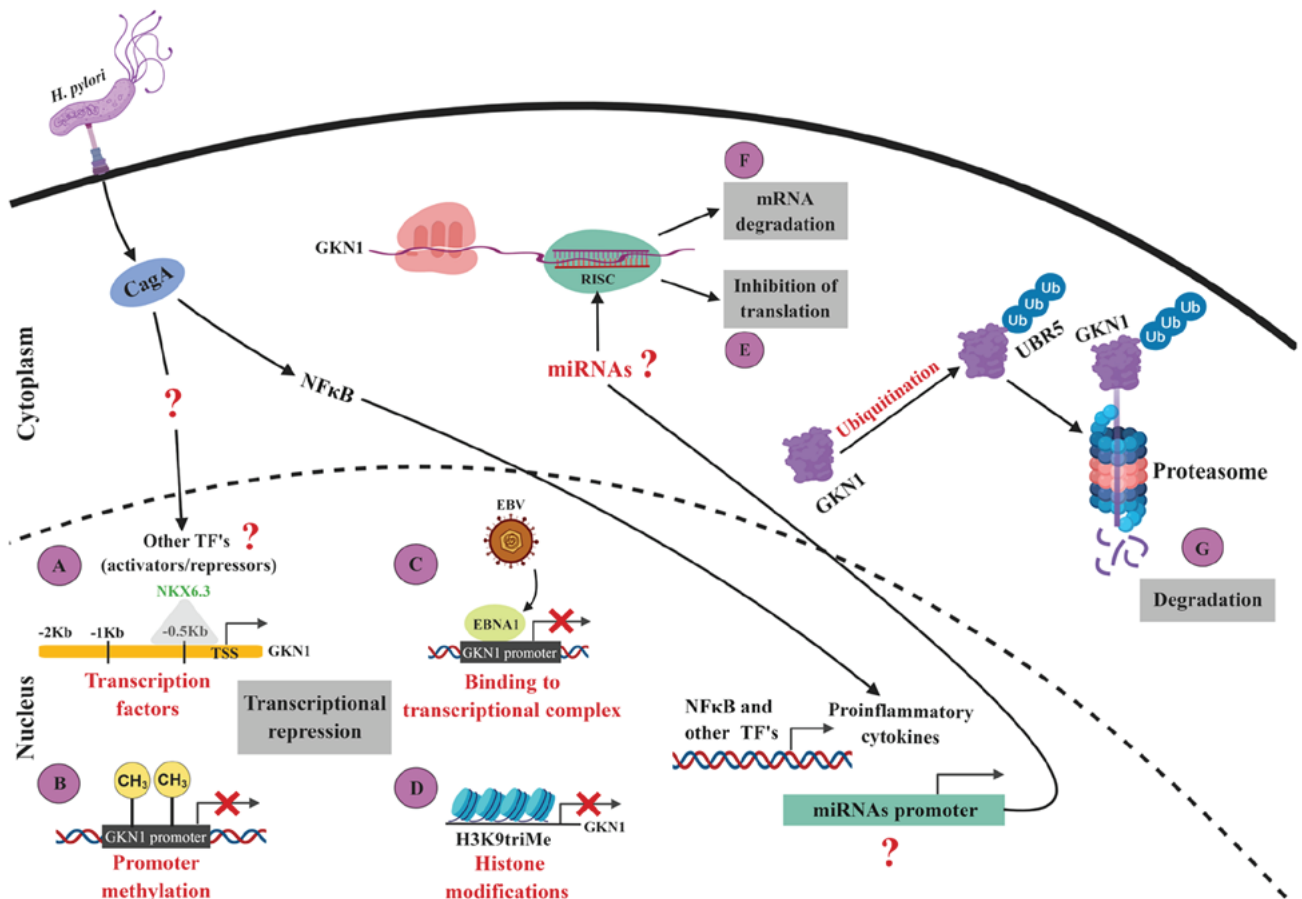


Figure 1. Regulation of GKN1 expression. (A) NKX6.3 is the only transcription factor validated as a positive regulator of GKN1 transcription. It is likely that another transcription factor or factors act as activators or repressors of GKN1 transcription during the infection of the gastric epithelium by *H. pylori*. Evidence indicates that *H. pylori* activates different signaling pathways that induce the expression of various transcription factors. The decrease in or loss of GKN1 expression in gastric cancer may be a consequence of: (B) GKN1 promoter methylation; (C) EBNA1 binding to the transcriptional complex; or (D) histone modification, such as trimethylation of lysine 9 in histone 3. Additionally, it is possible that the GKN1 mRNA is targeted by some miRNAs. By *in silico* analysis, miRNAs were found with sequences complementary to sites located in the 3' untranslated region of GKN1 mRNA, and are likely to contribute to its negative post-transcriptional regulation through: (E) Inhibition of translation; or (F) mRNA degradation. The expression of the proposed miRNAs may or not be induced by *H. pylori*. GKN1 is degraded in the cytoplasm of epithelial cells when (G) the ubiquitin ligase UBR5 marks GKN1 for its degradation in the proteasome. These and other mechanisms can act synergistically to promote the diminution or silencing of GKN1 expression, manifesting as a decreased or absent mRNA and protein expression in gastric tissue or in the circulation. Figure created using BioRender.com. TFs, transcription factors; GKN1, gastroskine 1; UBR5, E3 ubiquitin-protein ligase UBR5; EBNA1, Epstein Barr nuclear antigen 1; EBV, Epstein Barr virus; RISC, RNA-induced silencing complex; *H. pylori*, *Helicobacter pylori*; miRNA, microRNA; Ub, ubiquitin; NF-κB, nuclear factor-κB; TSS, transcriptional start site.

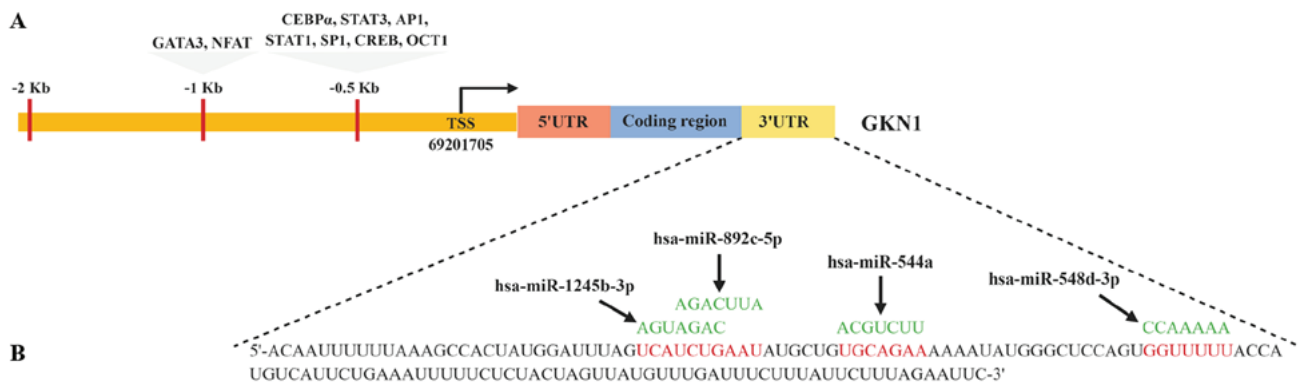


Figure 2. Transcription factors and miRNAs predicted *in silico* as regulators of GKN1 expression. By *in silico* analysis (A) transcription factors were identified with affinity to recognition sequences in the GKN1 promoter region and (B) miRNAs were found with sequences complementary to sites located in the 3'UTR of GKN1 mRNA. It is likely that the transcription factors act as activators or repressors of the transcriptional regulation of GKN1, and the miRNAs contribute to post-transcriptional regulation, inhibiting translation or inducing mRNA degradation. Figure created using BioRender.com. TSS, transcriptional start site; UTR, untranslated region; miRNA/miR, microRNA; GKN1, gastroskine 1; GATA3, T-cell-specific transcription factor GATA-3; NFAT, nuclear factor of activated T cells; CEBPα, CCAAT enhancer binding protein-α; AP-1, AP-1 transcription factor; Sp1, transcription factor Sp1; CREB, cyclic AMP-responsive element-binding protein 3-like protein 4.



Table I. Transcription factors with affinity to binding sequences in the gastrokeine 1 gene promoter region.

Transcription factor	Number of binding sites	Binding sequence	Genomic position of binding sequence <sup>a</sup>
GATA-3	7	CAGAGATAAAATG	68974044-68974056
CEBP $\alpha$	7	GAAATTGAGGAAGGT	68974539-68974553
Oct-1	6	GTCATGCAATTGATC	68973972-68973986
AP-1	4	TGATGAGTCAGGT	68974444-68974456
STAT-3	3	AGGTTTCCTGGTACACTGG	68974502-68974520
Sp1	2	GCTGTGGGCGTGAGTAT	68974361-68974377
STAT-1	1	AGTGTACCAGGAAACCTTT	68974500-68974518
CREB	1	AGGGTCCTATGTAATAAGATT	68973801-68973821
NFAT	1	CTTTGGAAATCTTATTACA	68973794-68973812

<sup>a</sup>Data obtained from MatInspector. GATA-3, T-cell-specific transcription factor GATA-3; CEBP $\alpha$ , CCAAT enhancer binding protein- $\alpha$ ; AP1-1, AP-1 transcription factor; Sp1, transcription factor Sp1; CREB, cyclic AMP-responsive element-binding protein 3-like protein 4 NFAT, nuclear factor of activated T cells.

Through toll like receptor (TLR)2 and TLR9, *H. pylori* activates the MAPK pathway and, downstream, the factors AP-1 and CREB, which positively regulate the transcription of cyclooxygenase 2 (COX-2) (91). CREB and STAT-3 are activated by *H. pylori* and positively regulate the transcription of COX-2 in gastric epithelial cells (72,83). Increased STAT-3 expression has been found in biopsies of the gastric mucosa infected with *H. pylori cagA*<sup>+</sup> (80), as well as in cell lines and murine models (86). CagA promotes the phosphorylation of STAT-3 in gastric epithelial cells (92).

*In vitro* and *in vivo* experiments have shown that the protein OipA of *H. pylori* stimulates the phosphorylation of STAT-1 (93) and that *H. pylori* alters the STAT-1 signaling induced by IFN- $\gamma$  in gastric epithelial cells. This event may represent an adaptation of the bacteria in order to modulate the immune response of the host mucosa, allowing the bacteria to survive in the stomach (94).

The expression level of the Sp1 transcription factor increases in gastric adenocarcinoma and is related to the cancer stage, the depth of infiltration and an unfavorable prognosis for patients (82). The expression of Sp1 differs between intestinal-type and diffuse-type cancer, while low-level expression of Sp1 is related to the progression and metastasis of intestinal-type cancer, in contrast to diffuse-type cancer (95). Sp1 is essential in the regulation of genes that determine the characteristics of cancer (96). In AGS cells, the ERK1/2 signaling pathway, activated in response to *H. pylori* infection, in turn activates Sp1, which modulates the transcription of vascular endothelial growth factor-A (69). It is probable that the factors AP-1, Oct-1, STAT-1, STAT-3, GATA-3, Sp1, CREB and NFAT, with recognition sequences in the *GKN1* promoter and being activated by *H. pylori*, repress the transcription of *GKN1* in infected mucosa or mucosa with gastric cancer.

In normal gastric mucosa, CEBP $\alpha$  is expressed in the foveolar epithelium and is reduced in the tumor tissue of patients with gastric cancer (79), and in the cell lines MKN45 and MKN74 (97). The ectopic expression of CEBP $\alpha$  in gastric cancer cell lines reduces cell viability (97). The level of CEBP $\alpha$  expression gradually decreases in line with the advancing carcinogenesis associated with *H. pylori* infection (73). Given

the function of CEBP $\alpha$  in the regulation of the viability of cancerous cells and the fact that GKN1 and CEBP $\alpha$  levels gradually decrease in line with the progress of the lesion, it is probable that this factor is an activator of *GKN1* transcription. The reduced expression of CEBP $\alpha$  and NKX6.3 in gastric cancer may be due to the negative regulation mediated by microRNAs.

**DNA methylation.** Changes in the methylation of DNA lead to changes in gene expression. The hypermethylation of CpG islands located in the promoter region of a gene results in the decrease or silencing of the expression of the gene.

The methylation of the *GKN1* promoter was previously studied, finding hypermethylation in the CpG islands of the promoter region in only two of 25 gastric tumors. This evidence indicates that the low or null GKN1 expression in the inflamed tissue, either tumoral or infected by *H. pylori*, is not due to the methylation of its promoter in all cases (Fig. 1B) (20).

The protein EBNA1 of EBV is able to bind to the promoter region of various genes of the host (28). It has been found to have an affinity with the sequences contained in the *GKN1* promoter and, binding at these sites, contributes to the deregulation of GKN1 in gastric cancer associated with Epstein-Barr infection (Fig. 1C) (29). Only a small proportion of gastric tumors contain EBV.

**Histone modification.** The modification of histones is an epigenetic mechanism influencing gene expression. Altieri *et al* (26) analyzed six gastric tumors in order to determine whether histone modification contributes to *GKN1* regulation. Chromatin immunoprecipitation assays were conducted on a fragment of 600 pb of the *GKN1* gene promoter, including the 5'UTR, finding trimethylation in lysine 9 of histone 3 (H3K9triMe), among bases -148 and -310 of the *GKN1* gene promoter in the six gastric tumors. H3K9triMe is a gene repression marker that generates binding sites for histone deacetylase I (HDAC1) (98) (Fig. 1D). The inhibition of HDAC1 activity with trichostatin A, a hypomethylating agent, is related to increased *GKN1* mRNA levels but not to the protein itself. These findings suggest that the regulation

of GKN1 may occur at the post-transcriptional level via miRNAs (26).

*miRNAs*. In the gastric mucosa, miRNAs can be expressed by epithelial cells, infiltrating inflammatory cells, transformed cells or cancerous cells (31). In the regulation of gene expression, miRNAs inhibit the translation or induce the degradation of target transcripts. It is likely that some miRNAs impede the translation of *GKN1* mRNA and, consequently, are responsible for the reduction in the protein level, although there are no reports indicating whether a miRNA is involved in the regulation of *GKN1* expression to the best of our knowledge.

In order to explore whether *GKN1* mRNA has binding sites for one or more miRNAs, an *in silico* analysis was conducted using programs for predicting miRNA targets: TargetScan (99) (2015, <http://www.targetscan.org>), miRanda (100) (<http://www.microrna.org>), miRDB (101) (<http://mirdb.org>), miRSystem (102) (<http://mirsystem.cgm.ntu.edu.tw/index.php>) and DianaTools (103) (<http://www.microrna.gr/microT-CDS>), based on thermodynamic and base complementarity analysis. miRNAs were found with sequences complementary to sites located in the 3'UTR region of *GKN1* mRNA (Fig. 2B), four of which are able to hybridize with canonical sites of *GKN1* mRNA and possess two or three guanine or cytosine residues in the seed region of the miRNA, conferring them greater binding stability. An adenine in position 1 of the 3'UTR region of *GKN1* mRNA ensures the recognition of the transcript by the RNA-induced silencing complex. These characteristics increase the probability that a miRNA will interact with the 3'UTR of *GKN1* mRNA (Table II).

Multiple prediction programs may be used to locate binding sites for miRNAs in gene transcripts. The results of the analysis facilitated the selection of miRNAs with a higher probability of binding to their target, miR-544a was predicted by five programs, according to the aforementioned criteria. It is highly probable that miR-544a is a regulator of *GKN1* (104,105). This proposal is strengthened by experimental data on the expression of miR-544a, which is found at increased levels in gastric cancer cell lines (106). To the best of our knowledge, research has not been conducted on the expression of hsa-miR-1245b-3p, hsa-miR-892c-5p and hsa-miR-548d-3p in the gastric mucosa, be that infected, inflamed, atrophic or with gastric cancer. However, the results of the *in silico* analysis suggested a high probability that these miRNAs regulate the expression of *GKN1*, either inhibiting the translation of the transcript or promoting its degradation (Fig. 1E and F).

Currently, >5,000 miRNAs are registered on miRBase, while *in silico* predictions estimate that more than one-third of the human transcriptome can be regulated by miRNAs. In gastric cancer, inflammatory processes and *H. pylori* infection, miRNAs fulfil an important function in the deregulation of gene expression (31,33). GKN1 is absent in cancer, both with and without *H. pylori*, and is reduced in patients with gastritis, in gastric mucosa infected by *H. pylori* and in atrophic gastritis (30). The reduction in or absence of GKN1 transcripts or protein is not completely explained by the studied mechanisms of transcriptional and post-translational regulation. It is likely that some miRNAs regulate *GKN1* expression directly or indirectly at the post-transcriptional level.

Table II. miRNAs proposed as a candidate to regulate GKN1 expression.

miRNA <sup>a</sup>	3'UTR GKN1 <sup>b</sup>	miRNA recognition site																Site type
hsa miR-544a	46-53	GKN1 5'	u	c	u	u	g	a	g	a	a	c	g	u	a	u	g	8mer
		miRNA 3'	u	u	g	g	a	a	g	c	g	c	u	a	u	u	a	8mer
hsa miR-548d-3p	70-77	GKN1 5'	u	a	u	g	g	u	u	c	g	a	u	g	u	u	a	8mer
		miRNA 3'	g	u	u	u	u	u	a	u	c	a	a	c	a	a	c	8mer
hsa miR-1245b-3p	30-37	GKN1 5'	a	c	u	u	a	g	g	a	g	g	g	g	u	u	a	8mer
		miRNA 3'	u	a	u	u	c	c	g	a	a	u	u	a	a	c	u	8mer
hsa miR-892c-5p	33-40	GKN1 5'	a	u	g	g	g	a	u	u	u	u	u	g	a	u	a	8mer
		miRNA 3'	a	c	u	g	a	a	c	g	a	g	a	c	u	a	u	8mer

<sup>a</sup>Programs used for predicting miRNA targets: TargetScan7.1, miRanda, miRDB, miRSystem and DianaTools. Capital letters indicate the seed region of each miRNA. The complementary sequences to the canonical sites in GKN1 mRNA are in bold. <sup>b</sup>Position in which the complementary sequences to the miRNA are found. miRNA/miR, microRNA; GKN1, gastrophilin 1.

Table III. miRNA expression in patients and *in vitro* models with *H. pylori* infection.

Author, year	Model	<i>H. pylori</i> strain	Method used to determine miRNA expression	Upregulated miRNAs	Downregulated miRNAs	Refs.
Cho <i>et al</i> , 2016	Murine	C57BL/6 mice	Mouse whole genome miRNA array (Agilent Technologies, Inc.) release 15	-	mmu-miR-1 mmu-miR-133a mmu-miR-133b mmu-miR-203 mmu-miR-205 mmu-miR-490-3p	(116)
Belair <i>et al</i> , 2011	Intestinal type gastric cancer cell lines	AGS	Reverse transcription-quantitative PCR	hsa-miR-21	hsa-miR-371-3p hsa-miR-372 hsa-miR-373 hsa-miR-19b hsa-miR-160b hsa-miR-320	(117)
Santos <i>et al</i> , 2017	AGS	26695 y P12	Cancer Pathway Finder miRNA PCR Array (MIHS-102Z; Qiagen, Inc.)	hsa-miR-150-5p hsa-miR-155-5p hsa-miR-3163	-	(35)
Chang <i>et al</i> , 2015	Patients diagnosed with gastric cancer with <i>H. pylori</i> infection	No data	Human miRNA microarray (Agilent Technologies, Inc.), release 16.0	hsa-miR-32-3p hsa-miR-514a-3p hsa-miR-181c-3p hsa-miR-146a-5p hsa-miR-4319 hsa-miR-99b-3p hsa-miR-543 hsa-miR-564 hsa-miR-645 hsa-miR-431-3p hsa-miR-650 hsa-miR-638 hsa-miR-139-3p hsa-miR-508-3p hsa-miR-2355-3p hsa-miR-934 hsa-miR-548f hsa-miR-1304-5p	-	(33)

*H. pylori*, *Helicobacter pylori*; miR/miRNA, microRNA.

Table IV. miRNAs expression in cell lines and patients with gastric cancer, grouped based on Lauren's classification (118).

Author, year	Model	Method used to determine the miRNAs expression	Upregulated miRNAs	Downregulated miRNAs	Refs.
Yu <i>et al</i> , 2012	Intestinal type gastric cancer cell lines	Human microRNA Microarray v.2 (Agilent Technologies), containing probes for 723 human microRNAs	hsa-miR-196a	hsa-miR-376a	(119)
			hsa-miR-615	hsa-miR-145	
			hsa-miR-196b	hsa-miR-143	
			hsa-miR-92b	hsa-miR-451	
			hsa-miR-149	hsa-miR-142-5p	
			hsa-miR-550	hsa-miR-1	
			hsa-miR-183	hsa-miR-377	
			hsa-miR-301	hsa-miR-495	
			hsa-miR-18a	hsa-miR-409-3p	
			hsa-miR-106a	hsa-miR-368	
	Diffuse type gastric cancer cell lines		hsa-miR-17-5p	hsa-miR-142-3p	
			hsa-miR-18b	hsa-miR-150	
			hsa-miR-19a	hsa-miR-497	
			hsa-miR-221	hsa-miR-214	
			hsa-miR-93	hsa-miR-199a	
			hsa-miR-20a	hsa-miR-146b	
				hsa-miR-133b	
				hsa-miR-127	
				hsa-miR-381	
				hsa-miR-195	
Guo <i>et al</i> , 2009	Patients diagnosed with gastric cancer	$\mu$ Paraflo™ microfluidic chip (LC Sciences)	hsa-miR-31	hsa-miR-17	(120)
			hsa-miR-133b	hsa-miR-18a	
			hsa-miR-139-5p	hsa-miR-18b	
			hsa-miR-195	hsa-miR-19a	
			hsa-miR-378	hsa-miR-20a	
			hsa-miR-497	hsa-miR-20b	
			hsa-miR-768-3p	hsa-miR-21	



Table IV. Continued.

Author, year	Model	Method used to determine the miRNAs expression	Upregulated miRNAs	Downregulated miRNAs	Refs.
Ueda <i>et al</i> , 2010		ArrayExpress, v3.0 (European Bioinformatics Institute), contains 1,100 microRNA probes, 326 human and 249 mouse	hsa-miR-373	hsa-miR-106a	(121)
			hsa-miR-498	hsa-miR-106b	
			hsa-miR-202	hsa-miR-340	
			hsa-miR-494	hsa-miR-421	
				hsa-miR-658	
Tsukamoto <i>et al</i> , 2010	Diffuse type	G4470A Human MiRNA Microarray (Agilent Technologies, Inc.), of 470 human and 64 virus mature miRNAs based on Sanger miRBase release 9.1	hsa-miR-18a	hsa-miR-375	(122)
			hsa-miR-106a	hsa-miR-29c	
			hsa-miR-17-5p	hsa-miR-148a	
			hsa-miR-146a	hsa-miR-30a-5p	
			hsa-miR-93	hsa-miR-30e-5p	
			hsa-miR-19a	hsa-miR-638	
			hsa-miR-20a		
			hsa-miR-20b		
			hsa-miR-25		
			hsa-miR-15b		
			hsa-miR-425-5p		
			hsa-miR-92		
			hsa-miR-194		
			hsa-miR-10a		
			hsa-miR-222		
			hsa-miR-7		
			hsa-miR-106b		
			hsa-miR-320		
			hsa-miR-21		
			hsa-miR-34a		
			hsa-miR-19b		
			hsa-miR-103		
			hsa-miR-215		
			hsa-miR-192		

Table IV. Continued.

Author, year	Model	Method used to determine the miRNAs expression	Upregulated miRNAs	Downregulated miRNAs	Refs.
Su <i>et al</i> , 2012		AFFX miRNA expression chips (Affymetrix; Thermo Fisher Scientific, Inc.)	hsa-miR-429		(123)
			hsa-miR-279		
			hsa-miR-223		
			hsa-miR-23a		
			hsa-miR-107		
			hsa-miR-200b		
			hsa-miR-24		
			hsa-miR-15a		
			hsa-miR-16		
			hsa-miR-455-3p	hsa-let-7g	
			hsa-miR-34a	hsa-miR-200b	
				hsa-miR-768-3p	
				hsa-let-7d	
				hsa-miR-104-3p	
Juzenas <i>et al</i> , 2015		TaqMan Array Human MiRNA Card A v2.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.), include 377 human miRNAs of miRBase v20		hsa-miR-27b	(124)
				hsa-miR-1207-5p	
				hsa-miR-663	
				hsa-miR-486-5p	
				hsa-miR-222	
				hsa-miR-574-3p	
				hsa-miR-768-5p	
				hsa-miR-378	
				hsa-miR-31	
			hsa-miR-146b-5p	hsa-let-7a-5p	
			hsa-miR-155-5p	hsa-let-7g-5p	
			hsa-miR-214-3p	hsa-miR-135a-5p	
			hsa-miR-223-3p	hsa-miR-148a-3p	
			hsa-miR-224-5p	hsa-miR-204-5p	
Katada <i>et al</i> , 2009		TaqMan miRNA assays	hsa-miR-331-3p	hsa-miR-26b-5p	(125)
			hsa-miR-484	hsa-miR-30b-5p	
				hsa-miR-375	
			hsa-miR-34b	hsa-miR128b	
			hsa-miR-34c	hsa-miR-129	
			hsa-miR128a	hsa-miR-148a	

Table IV. Continued.

Author, year	Model	Method used to determine the miRNAs expression	Upregulated miRNAs	Downregulated miRNAs	Refs.
Ueda <i>et al</i> , 2010		ArrayExpress, version 3.0 (European Bioinformatics Institute), A-MEXP-620, contains 1,100 microRNA probes, 326 human and 249 mouse	hsa-miR-105 hsa-miR-100 hsa-miR-125b hsa-miR-199a hsa-miR-99a hsa-miR-143 hsa-miR-145 hsa-miR133a	-	(121)
miRNA/miR, microRNA.					

It has been reported that ROS deregulate the expression of miRNAs in tissue infected with *H. pylori* or gastric cancer tissue (107,108). It is also known that ROS induce a decrease in the number of copies of *GKNI* mRNA in tissue infected by *H. pylori* (48). These data support the hypothesis that, in *H. pylori* infection, ROS alter the expression of miRNAs, among which are those with the *GKNI* transcript as a target. The cytotoxins VacA and CagA, lipopolysaccharide and peptidoglycan, among other components of *H. pylori*, are able to induce the increased expression of miRNAs that inhibit translation or induce the degradation of the *GKNI* transcript, thus modulating the decrease in the levels of this protein.

From the first stages of *H. pylori* infection, the inflammation associated with it causes changes in the expression of proteins and miRNAs, alterations in cell signaling, and unbalanced cell proliferation and apoptosis in gastric epithelial cells, promoting the progression of gastritis to pre-neoplastic and neoplastic lesions (109). The abnormal expression of miRNAs is common in different types of cancer (110), with the evidence indicating changes in the expression profiles of miRNAs in gastric cancer and in mucosa infected by *H. pylori*.

Alterations in the expression of miRNAs can manifest either as increases or decreases (111). In the gastric mucosa, both with and without *H. pylori* infection, it has been found that miRNAs with changes in their expression levels in response to *H. pylori* can be similar to or different from those observed in gastric cancer with a negative result for bacteria (31) (Tables III and IV). Chang *et al* (33) found that hsa-miR-99b-3p, hsa-miR-564 and hsa-miR-658 expression increased in cancerous tissue infected with *H. pylori*, while hsa-miR-204-5p, hsa-miR-338-5p, hsa-miR-375 and hsa-miR-548c-3p were found to be overexpressed in cancer tissue without *H. pylori* (33).

In infected mucosa, hsa-miR-223 expression was found to be increased, while in mucosa without *H. pylori*, hsa-miR-203, hsa-miR-204, hsa-miR-455, hsa-miR-141 and hsa-let-7f were found to be overexpressed (31). The levels of let-7, miR-125a and miR-500 were found to be significantly reduced in cells infected with *cagA*<sup>+</sup> strains, although not in those infected with *cagA*<sup>-</sup> strains. These results indicate that miRNAs participate in gastric pathogenesis, whether associated and not associated with *H. pylori*, and suggest that the CagA oncoprotein of *H. pylori* regulates the differential expression of miRNAs in epithelial gastric cells (31). Increased hsa-miR-127-5p, hsa-miR195, hsa-miR-196a, hsa-miR-206, hsa-miR-216 and miR-488 expression has been found, while decreased hsa-miR-103, hsa-miR-141, hsa-miR-17-3p, hsa-miR34a and let-7i expression has been found in gastric epithelial cells infected with different *H. pylori-cagA*<sup>+</sup> strains (36).

*H. pylori* is able to modify the expression of miRNAs by means of inflammatory effectors (112). In gastric epithelial cells, the pro-inflammatory cytokines IL-8, tumor necrosis factor- $\alpha$  and IL-1 $\beta$  induce the expression of miR-146a (113), while the oncoprotein CagA positively regulates *c-myc*, which is related to the decreased expression of miR-26a and miR-101. The decrease in the expression of these miRNAs contributes to increased levels of the histone methyltransferase EZH2 and methyltransferase DNMT3B, which promote the methylation of the let-7 promoter (114).

**Ubiquitination.** E3 ubiquitin-protein ligase UBR5 (UBR5) is an E3 ubiquitin ligase that participates in the ubiquitin-proteasome system, regulating protein concentration via ubiquitination and degradation, and is deregulated in different types of cancer (115). UBR5 increases in the cancerous tissues of gastric cancer patients, while an interaction between UBR5 and GKN1 has been observed through immunoprecipitation assays. These results suggest that UBR5 participates in the ubiquitination of GKN1, and that at least part of this protein is sent to be degraded by the proteasome (27) (Fig. 1G). Thus, UBR5 contributes to the regulation of gastric carcinogenesis, inducing the degradation of tumor suppressing proteins, such as GKN1 (27).

Therefore, promoter methylation, trimethylation of histones and ubiquitination are mechanisms that contribute to the regulation of the GKN1 expression in gastric cancer; however, they do not explain the absence of the protein in cell lines and cancerous human tissue.

## 6. Conclusion

GKN1 plays an important role in the maintenance of gastric homeostasis. In inflamed mucosa, both with and without *H. pylori* infection, GKN1 levels decrease, while this protein is absent in gastric cancer. The measurement of circulating GKN1 concentration, the protein itself or its mRNA in gastric tissue could be useful for the early diagnosis of cancer. However, little is known about the mechanisms that explain the reduction or silencing of GKN1 expression in gastric carcinogenesis. No mutations or polymorphisms have been found in the *GKN1* promoter region, which explains the reduction in the levels of this protein. While the modification of histones seems to be involved in the transcriptional regulation of *GKN1*, further research is required to confirm its level of participation in the regulation of GKN1 in the population. The information available suggests that the methylation of the *GKN1* promoter is an epigenetic mechanism that reduces the transcription rate of the gene. However, this mechanism only occurs in some cases of gastric cancer and, moreover, it is probable that it is determined by the genetic characteristics of the individual or the presence of EBV in the tumor. While only factor NKX6.3 has been confirmed as a positive regulator of *GKN1* transcription, *in silico* analysis suggests the existence of other transcription factors with affinity for sequences in the *GKN1* promoter region, among which are GATA-3, CEBP- $\alpha$ , Oct-1, AP-1, STAT-3, SP1, STAT-1, CREB and NFAT. It is unknown whether miRNAs regulate GKN1 expression at the post-transcriptional level. *In silico* analysis revealed that hsa-miR-544a, hsa-miR1245b-3p, hsa-miR-892c-5p and hsa-miR-548d-3p have sequences complementary to sites located in the 3'UTR of *GKN1* mRNA. It is likely that, together, they regulate the expression of GKN1 *in vivo*, in mucosa infected by *H. pylori* or in gastric cancer (Fig. 1). Functional studies are required to show whether miRNAs play a role in the regulation of GKN1 expression. At the post-translational level, UBR5 mediates the ubiquitination of GKN1, marking it for degradation in the proteasome; however, this mechanism does not explain the absence or minimal level of GKN1 expression in gastric cancer. Clarifying the mechanisms that regulate GKN1 expression will contribute useful information for evaluating

the possible clinical applications for the detection of this protein in mucosa, or in the circulation of patients with gastric diseases both associated and not associated with *H. pylori*.

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## Authors' contributions

JAM, DNMC, OPZ and GFT contributed the idea, wrote the text, and generated the tables and the figure.

## Ethics approval and consent to participate

Not applicable.

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## Competing interests

The authors declares that they have no competing interests.

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