UNIVERSITY OF NAPLES "FEDERICO II"

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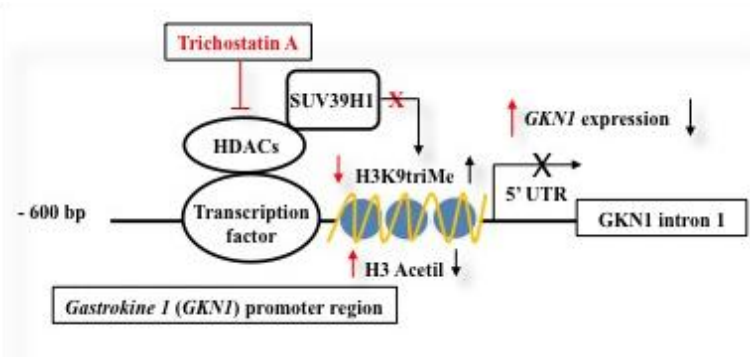
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY
XXIX CYCLE

Chiara Stella Di Stadio

Epigenetic mechanisms underlying *Gastrokine 1* gene silencing in gastric cancer progression



ACADEMIC YEAR
2015-2016

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Epigenetic mechanisms underlying *Gastrokine 1* gene silencing in gastric cancer progression

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ABSTRACT

Gastric cancer (GC) is still one of the leading causes of cancer-related deaths worldwide and high mortality rate is mainly due to late-stage diagnosis. New insights show that epigenetic alterations contribute significantly to the development and progression of GC and if nowadays the role of somatic mutations as drivers of carcinogenesis in the alimentary tract is well established, the importance of gene silencing by epigenetic mechanisms is increasingly recognized. Gastrokine1 (GKN1) is a highly expressed stomach protein important for maintaining the physiological function of the gastric mucosa. GKN1 is down-regulated in gastric tumor tissues and derived cell lines so it has recently emerged as a potential biomarker for gastric cancer. It has also been demonstrated that GKN1 expression induces apoptosis in gastric cancer cells thus suggesting a possible role of the protein as tumor suppressor. The mechanism by which *GKN1* gene is inactivated in GC remains still unknown, so here I have investigated on the possible causes of *GKN1* gene silencing in order to determine if epigenetic mechanisms could also contribute to its down-regulation. To these aim, chromatin immunoprecipitation (ChIP) assays for the repressive trimethylation of histone 3 at lysine 9 (H3K9triMe) and its specific histone-lysine N-methyltransferase (SUV39H1) were performed on biopsies of normal and tumor human gastric tissues. The results showed that *GKN1* downregulation in gastric cancer tissues is associated with high levels of H3K9triMe and with the recruitment of SUV39H1 on *GKN1* promoter, suggesting the presence of an epigenetic transcriptional complex that negatively regulates *GKN1* expression in gastric tumor.

It was also investigated whether underacetylation might contribute to *GKN1* transcriptional inhibition using TSA to increase general histone acetylation. The results showed that inhibition of HDACs leads to *GKN1* restoration at transcriptional level, but no at traslational level. These findings led to hypothesize that a second regulatory block occurs at translational level, perhaps by mechanisms mediated by microRNAs (miRNAs), resulting in translational repression and gene silencing. So, the possible involvment of miRNAs in this process was investigated. The results demostrated that *GKN1* 3'UTR was a direct target of hsa-miR-544a and miR-1245b-3p and showed an increase of miR-544a expression in the gastric cancer cell lines after TSA treatment.

The up-regulation of miR-544a could be the cause of the GKN1 translational repression, thus suggesting its potential role as biomarker and therapeutic target in GC patients.

These findings indicate that epigenetic mechanisms leading to the inactivation of GKN1 play a key role in the multi-step process of gastric

carcinogenesis and would provide an essential starting point for the development of new therapeutic strategies based on epigenetic targets for alternatives gene.

Abbreviations

AMP18, 18 kDa antrum mucosal protein; ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; DNMT1, DNA (cytosine-5)-methyltransferase 1; EZH2, enhancer of zeste homolog 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC, gastric cancer; GKN1, gastrokine 1; G6PD, glucose-6-phosphate dehydrogenase; EBNA1, Epstein-Barr nuclear antigen 1; EBV, Epstein- Barr virus; HAT, histone acetyltransferases; HDAC, histone deacetylase; H3K9triMe, trimethylation of Histone 3 at lysine 9; IM, intestinal metaplasia; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; RNU6, U6 Small nucleolar RNA; Tip60, Histone acetyltransferase; TSA, trichostatin A; SUV39H1, Histone-lysine N-methyltransferase; 3'UTR, 3'-untranslated regions.

1. INTRODUCTION

1.1 Gastric cancer

Gastric cancer (GC), despite its declining incidence rate, is still the fourth most common cancer and the second cause of cancer-related death worldwide. Most patients are typically asymptomatic and diagnosed at an advanced stage, thus GC continues to be a highly aggressive malignancy that is associated with poor prognosis and a low survival rate [1, 2].

According to the classification criteria developed by Lauren in 1965 [3], GC is frequently divided into the intestinal type and the diffuse type, each having different epidemiological and pathophysiological features. The intestinal-type generally evolves through a relatively well-defined sequence of histological lesions, namely nonatrophic gastritis, chronic atrophic gastritis, intestinal metaplasia, and dysplasia. The diffuse subtype has instead a poorer prognosis and develops from normal gastric epithelium through unknown genetic and morphological events. The intestinal type is more common in males and older age groups, while the diffuse type is more common in younger age groups; the incidence has no association with sex difference [4].

The etiology of GC is multifactorial including environmental, genetic and infectious factors [5-7]. Multiple evidence demonstrated the important role of *Helicobacter pylori* (*H. pylori*) infection in the development of GC [8-10]. In fact, exposure of gastric epithelial cells to the bacterium determines the release of cytokines and reactive oxygen species (ROS) leading to the inflammatory and immune response of the stomach epithelium, that in turn may cause genetic alterations and an increased risk of developing GC. Although the molecular pathways and histologic changes involved in progression to cancer are less characterized, GC, as other neoplasias, is thought to result from a combination of environmental factors and the accumulation of genetic and epigenetic alterations, which affect, tumor suppressor genes or oncogenes.

The understanding the molecular pathophysiology of GC and the identification of novel prognostic and diagnostic biomarkers are necessary to reduce the mortality rates through cancer screenings in high-risk populations, to increase early diagnosis, and to develop new target therapies.

1.2 Gastrokine 1

In order to search for possible informative biomarkers for GC, a new protein named gastrokine 1 (GKN1) was identified by proteomic approach. Gastrokine1 protein, previously known as 18 kDa antrum mucosal protein (AMP-18) was subsequently called by the "Human Gene Nomenclature Committee" GKN1 for its tissue-specific expression and its highly conserved

presence in the gastric mucosa of many mammalian species [11- 14]. The gene coding for GKN1 (CA11, accession number: BK0017373) is located in a 6 kb region of the chromosome 2p13 and contains 6 exons separated by relatively short introns that encode small (from 181- to 184-amino acid) proteins containing a conserved central structural BRICHOS domain of about 100 amino acids including two conservative cysteine residues most likely involved in disulfide bridges; a COOH-terminal segment, showing considerable divergence between the GKN1 paralogs and the hydrophobic NH2-terminal signal peptide, that acts as a transmembrane anchor and/or signal peptide; the processing of which is predicted to generate about 160 mature amino acid proteins [15].

Molecular studies on the BRICHOS domain function have suggest that it has a range of possible roles, including intracellular trafficking, pro-peptide processing, chaperone function and secretion. The hydrophobic region and BRICHOS domain in GKN1 seem to suppress GC cell growth, reduce cell viability, proliferation and colony formation in AGS cells. It might be the main functional domain for the tumor suppressor activity [16, 17].

GKN1 is a secretory protein that tends to localize within the granules just under the apical plasma membrane [18, 19], and its expression is confined to the gastric epithelium, except for trace levels in the placenta [19]. The protein is highly expressed in the gastric mucosa of healthy individuals, but markedly down-regulated in samples derived from *H. Pylori* infected patients [20]. Furthermore, GKN1 was also found to be down-regulated or completely absent in GC tissues [21, 22].

Although the biological function of this protein is currently unknown, a role in cell proliferation and differentiation has been hypothesized, as well as the potential involvement in the replenishment of the surface lumen epithelial cell layer and the maintaining of mucosal integrity [23, 24]. After injury of the gastric mucosa, restoration may occur very rapidly in the presence of GKN1 [25]. In contrast, if the protein is down regulated, the repair process may be hampered. In fact, application of GKN1 to gastrointestinal cells promoted epithelial restoration by increasing accumulation of specific tight and adherens junction proteins thus promoting stabilization of actin network [18]. All these data show that GKN1 has a very important role in maintenance of gastric mucosal homeostasis and its early down regulation may lead to a defect in the gastric mucosal barrier, rendering gastric mucosa subject to exposure to carcinogens and eventually evoke subsequent genetic alterations of tumor suppressor genes or oncogenes involved in gastric carcinogenesis. GKN1 can also induce apoptosis and senescence. In fact restoration of GKN1 expression resulted in cell cycle arrest at the G1/s or G2/M phases caused by a down regulation of positive cell cycle regulators, including CDK4, cyclin D1, E2F, cdc25 and cyclin B, and an up regulation

of negative cell cycle regulators, including p16 and p21 [26]. Furthermore our group investigated the effect of GKN1 on gastric cell line. The overexpression of the protein in AGS and MKN28 cells activated the expression of Fas receptor, while treatment with an anti-Fas antibody significantly increased apoptosis [27]. Moreover, treatment of tumor cells with recombinant human GKN1 reduced the proliferation of AGS cells compared with human embryonic kidney cells (HEK 293) and non-gastric cancer cells (H1355) [28]. Recent evidence has also demonstrated that GKN1 is involved in gastric mucosal inflammation by regulating production of inflammatory mediators, including NF-kB, COX-2 and cytokines and inhibits the carcinogenetic potential of *H. pylori* trough the direct binding to CagA at the extracellular level and increasing the expression of antioxidant enzymes at the intracellular level [29]. Subsequent study found that GKN1 is involved also in the epithelial mesenchymal transistion, a process observed in response to injury, organ fibrosis and cancer. In particular the recovery of GKN1 expression suppresses cell migration and invasiveness abrogating the expression of PI3K/akt pathway proteins, concomintant with the re-expression of E-cadherin [30]. These data suggest that GKN1 may be a gastric specific tumor suppressor. GKN1 could also be considered a biomarker for GC because individuals with a lower expression of the protein have an increased risk of developing gastric diseases.

1.3 Epigenetic and gastric cancer

GC is a very complex and heterogeneous disease, and although much has been learned about the different genetic changes that eventually lead to its development, the detailed mechanisms still remain unclear. Malignant transformation of gastric cells is the consequence of a multistep process involving different genetic and epigenetic changes in numerous genes in combination with host genetic background and environmental factors [31].

Increasing evidence suggests that epigenetic changes play a key role in cancer development, including gastric cancer [32].

Epigenetics refers to a number of modifications in the chromatin structure that affect gene expression without altering the primary DNA sequence, and these changes lead to transcriptional activation or silencing of the gene. Epigenetic regulation of chromatin is a normal and vital process of cell differentiation and development. It explains the ability of stem cells from a single origin to differentiate into all of the necessary cells of the human body; then remain differentiated without reverting back to a stem cell or other cell type. In tumorigenesis however, there is dysregulation of the physiologic epigenetic process, and this contributes to the development of malignancy.

At present, the most important epigenetic mechanisms involved in the carcinogenic process include DNA methylation, histone modification and

non-coding RNAs, mainly microRNAs. DNA methylation, that consists in the addition of a methyl group to cytosine residues (5mC) in CpG dinucleotides, contributes to cancer through DNA hypo-or hypermethylation. It has been observed that almost half of the tumor suppressor genes that causes familial cancers through mutations are also inactivated by promoter hypermethylation in sporadic cancers. The methylation changes that occur within CpG islands, which are present in about 70% of all mammalian promoters, plays an important role in transcriptional regulation, and it is commonly altered during malignant transformation [33]. Recent studies have describe a number of genes that are silenced by hypermethylation in gastric cancer associated with *H. pylori* or Epstein Barr virus infection (EBV) the most important of which include: cycle regulators p16(INK4a), p14(ARF) and APC; DNA-repair genes, hMLH1, BRCA1 and MGMT; the cell adherence gene E-cadherin [34, 35]. Recent research focused on exploring the role of hystone modification in the pathogenensis of gastric cancer. Histone modifications, which have been recently recognized to generate a 'histone code' that affects chromatin structure and gene expression, also play an important role in the establishment of gene silencing during tumorigenesis. Histones are evolutionarily highly conserved proteins characterized by an accessible aminoterminal tail and a histone fold domain that mediates interactions between histones to form the nucleosome scaffold. The N-terminal of histone polypeptides are modified by post-translational modifications, including methylation, acetylation, phosphorylation, ribosylation, ubiquitination, sumoylation, carbonylation and glycosylation.

Acetylation and methylation of histones H3 and H4 seem to be the most widespread and functionally important modifications, playing a major role in the regulation of gene expression and they are also the only modification that have been clinically associated with pathological epigenetic disruption in cancer cells. The acetylation and deacetylation of key lysine residues of histone H3 and H4 are controlled by histone acetyltransferases and histone deacetylases (HDACs).

Transcription becomes active when histones are acetylated by histone acetyltransferases (HAT), silenced when histones are deacetylated. The other modification includes methylation of arginine and lysine residues of histones. This methylation is catalyzed by histone methyltranferase (HMT) and, in general, lysine methylation at H3K9, H3K27 and H4K20 is associated with gene silencing, whereas methylation at H3K4, H3K36 and H3K79 is associated with gene activation. Recent studies identified candidate genes with significant differences in H3K27triMe in gastric cancer samples compared to adjacent non-neoplastic gastric tissues [36]. These genes included oncogenes, tumor suppressor genes, cell cycle regulators, and genes involved in cell adhesion. It has also been reported that methylation of

histone H3 plays an important role in carcinogenesis by silencing tumor suppressor genes [37]. Park et al. [38] observed global histone modification patterns using immunohistochemistry and reported that the trimethylation of H3K9 positively correlates with tumor stage and lymphovascular invasion in gastric cancer [38]. They also observed that the level of H3K9triMe was correlated with patient survival, because stronger methylation corresponded to a worse prognosis and intermediate methylation to an intermediate prognosis. On the other hand, the acetylation of histone, which occurs mostly from lysine residues of N-terminal domains, is known to be associated with transcriptional activation. The acetylation of histone H3 at K9 has been shown to be associated with a poorly differentiated or diffuse type of histology [38]. Histone H4 acetylation is reduced in gastric cancer compared to normal mucosa. The reduction of histone H4 acetylation correlates with a more advanced stage, deeper invasion and a greater extent of lymph node metastasis [39].

Deacetylation of histones may also be due to the activity of histone deacetylase (HDAC), responsible for removing the acetyl group to lysine residues. Aberrant histone deacetylation in gastric cancer lead to chromatin condensation and transcriptional inactivation of key genes important for normal gastric cells regulation. Deregulation of HADC activity has been strongly implicated in aberrant gene silencing and tumorigenesis. In fact HDAC has been reported to be up regulated in 17 of 25 gastric carcinomas and its expression is associated with aggressive behavior of primary gastric [40]. The class III HDACs play an important role in cell survival via deacetylation of key cell cycle and apoptosis regulatory molecules including p53 and Rb [40-42]. Histone H3 in the p21(WAF1/CIP1) promoter is hypoacetylated in gastric cancer; this hypoacetylation is associated with reduced p21(WAF1/CIP1) expression in gastric cancer specimens. Treatment of gastric carcinoma cell lines with HDAC inhibitor, trichostatin A, increases the acetylation level and restores p21(WAF1/CIP1) expression [43].

Aberrant DNA methylation and histone deacetylation are also linked to the silencing of the *SLC5A8* gene in gastric cancer [44]. SLC5A8 is a sodium co-transporter, solute carrier family 5 member 8 gene and a putative tumor suppressor. Aberrant methylation of *SLC5A8* gene is detected in both cell lines and in primary gastric cancers and acetylation of histone H3 correlates directly with SLC5A8 expression and inversely with DNA methylation.

Recently, microRNA have emerged as molecular regulators that can have key roles in pathogenesis and progression of different malignancies, including gastric cancer. miRNAs are a class of small non-coding RNAs (19–25 nucleotides) that act as important epigenetic players in many cellular processes, such as differentiation, proliferation and apoptosis, exerting a great influence in cancer pathogenesis [45, 46].

The mature miRNAs act as post-transcriptional regulators interacting with the 3'-untranslated regions (3'UTR) of the target transcripts and resulting in either mRNA degradation or inhibition of translation. Changes in miRNA expression profiles have been observed in a variety of human tumors, including gastric cancer [47].

Further studies demonstrated that miRNAs may function as tumor suppressors and oncogenes. These findings have shown great potential of miRNAs as a novel class of therapeutic targets [47]. In addition, it was found that some miRNAs were directly involved in patients with gastric cancer, including prognosis prediction, treatment selection, and in the search for unknown primary sites.

All these findings suggest that epigenetic pathways play a significant role in gastric cancer because alteration of both methylation and acetylation of histones or the involvment of miRNA may give rise to the expression of oncogene and silence of tumor suppressors in cancer cells. Whereby a greater understanding of epigenetics and the therapeutic potential of intervention into these processes is necessary to help gastric cancer treatment.

1.4 *GKN1* inactivation

Althougt it is well know that GKN1 expression is undetected in gastric tumors, the molecular mechanisms underlying its inactivation remain unclear. Recently, Yoon et al. 2011 [48] investigated this aspect in a sample group of 81 gastric carcinomas and 40 gastric adenomas [48]. No mutation was detected in gastric tumors and hyper-methylation of the *GKN1* promoter was only observed in two tumors, whereas DNA copy number and *GKN1* mRNA levels were significantly decreased in all GC samples. More recently, the Epstein–Barr nuclear antigen 1 (EBNA1) protein was reported to directly bind *GKN1* and *GKN2* promoters [49]. Treatment of AGS-Epstein–Barr virus (EBV) and AGS-EBNA1 cell lines with 5' azacytidine showed that *GKN1* and *GKN*2 were transcriptionally silenced by DNA methylation, and that latent EBV infection further reduced GKN1 and GKN2 expression in AGS cells. EBNA1 depletion by small interfering RNA partially alleviated this repression. However, the ectopic expression of EBNA1 slightly increased *GKN1* and *GKN2* basal mRNA levels, but reduced their responsiveness to demethylating agents. These findings indicated that EBNA1 contributes to the transcriptional complex and epigenetic deregulation of *GKN1* and *GKN2* tumor suppressor genes in EBV-positive GC. These studies indicate that epigenetic modifications play an important role in the initiation and progression of cancer and may in part explain the mechanism of *GKN1* gene silencing in the early stages of tumorigenesis.

2. AIM

Although these studies suggest that epigenetic modifications are involved in the deregulation of *GKN1* in GC, no investigation on histone modifications or the recruitment of histone-modifying enzymes and *GKN*1 co-repressors in GC have yet been done. Therefore, in the present study, we attempted to clarify whether epigenetic mechanisms are associated with *GKN1* silencing in GC and to determine whether this event might be involved in the development and progression of GC.

3. MATERIALS AND METHODS

3.1 Materials

Dulbecco's modified Eagle's medium (DMEM-F12) and fetal bovine serumz (FBS) were purchased from Cambrex (Rutherford, NJ, USA). Mouse GKN1 monoclonal antibody (M01), clone 2E5, was purchased from Abnova (Taipei, Taiwan). Rabbit monoclonal to Histone H3 (tri methyl K9) [ab8898] and to HDAC1 [ab109411] antibodies were from Abcam (Cambridge, MA, USA), mouse SUV39H1 (clone MG44) [05–615] and rabbit acetyl-Histone H3 [06–599] antibodies were from Millipore (Temecula, CA, USA). DMSO and Trichostatin A (TSA) were from Sigma (Milan, Italy). Rabbit GAPDH monoclonal (glyceraldehyde-3-phosphate dehydrogenase) and rabbit Lamin A/C polyclonal antibodies were from Santa Cruz Biotecnology (Dallas, TX, USA).

3.2 Cell cultures, transfection, human tissues and Western blotting

Human gastric adenocarcinoma cell lines (AGS, MKN28, KATO III, NCI N87) and human embryonic kidney 293 (HEK293) cells were grown in DMEM-F12 and DMEM, respectively, supplemented with 10% heat inactivated FBS, 1% penicillin/ streptomycin and 1% L-glutamine at 37°C in a 5% CO2 atmosphere.

AGS were transfected with 4 μg of vector pcDNA 3.1, pcDNA3.1-flGKN1(His)6 encoding the full length GKN1 (flGKN1, containing the first 20 amino acids leader peptide and His6-Tag sequence at the C-terminal) as already described [10]. The efficiency of transfection of gastric cancer cells with flGKN1 was always evaluated by a parallel transfection using EGFP vector as control. In general, after transfection, the average value of the ratio between number of green fluorescent cells/total number of cells was 0.5 ± 0.1.

Human gastric tissues were from patients with GC recruited at Hospital A. Cardarelli, Naples, Italy. All patients were interviewed regarding smoking habit, alcohol intake and chronic use of drugs. Hospital Pathologist performed the macro dissection of tumor and non-tumor tissues of GC patients during surgery. Gastric cancer was staged and graded according to the American Joint Committee on Cancer criteria [50]. The characterization of non-tumoral gastric mucosa was based on macroscopic aspects of normal compared with tumoral tissue as evaluated by the hospital pathologist [51], and from our previous work showing that GKN1 was highly expressed in gastric non-tumoral tissues but down-regulated or totally absent in GC tissues [3]. The study reported in the manuscript has been carried out in the frame of a research protocol entitled "Role of gastrokine 1 in gastric cancer" that has the approval from the Ethic Committee of the University of Naples Federico

II (Comitato Etico Università Federico II). The assigned protocol number of the study was 34/15 [51].

Proteins from cell extracts (about 20 µg) were analyzed by Western blotting using mouse anti-GKN1 at 1:500, rabbit anti-Histone H3 (tri methyl K9) at 1:1000, rabbit anti-HDAC1 at 1:1000, mouse anti-SUV39H1 at 1:500, anti-GAPDH at 1:1000 and rabbit anti-Lamin A/C at 1:1000 dilution. Detection was performed using the enhanced chemiluminescence detection kit (SuperSignal West Pico) following manufacturer's instructions. Western blot band intensity was measured with ImageJ 1.46r software.

3.3 RNA isolation and real-time qPCR

Total RNA was extracted from normal and cancer human tissue or from using TRIzol reagent (Invitrogen) and quantified by Nanodrop (Thermo Scientific, Wilmington, DE). cDNA was synthesized using the reverse transcription kit from Roche Molecular Systems (Roche, Penzberg, Germany) according to the manufacturer's protocol. *GKN1* transcript levels was quantified using SYBR Green PCR MasterMix (Applied Biosystems) IQ SYBR green Supermix (Bio-Rad) on a CFX96 Real-Time System instrument (Bio-Rad) using using the following primers pairs:
5'-CTTTCTAGCTCCTGCCCTAGC-3';
5'-TGGTTGCAGCAAAGCCATTT-3'.

Real-Time qPCRs were performed in triplicate under the following conditions: 10 minutes at 95°C, followed by 40 cycles (15 seconds at 95°C and 1 minute at 60°C). The relative fold changes were calculated using the 2-ΔΔCt method by the formula: 2-(sample ΔCt – control ΔCt), where ΔCt is the difference between the amplification fluorescent thresholds of the gene of interest and the internal reference gene (G6PD) used for normalization [52].

TaqMan MiRNA Assay kit (Applied Biosystems, Foster City, CA, USA) was used to detect the expression miRNAs (miR-544a, miR-1245b-3p) in DMSO or TSA treated cells lines. Briefly, 100 ng of total RNA was reversely transcribed (RT) at 16 ºC for 30 min, 42 ºC for 30 min and 85 ºC for 5 min in 15 µl reaction volume. Two µl of RT product were used for PCR reaction in a final volume of 20 µl. The PCR reaction started with an initial denaturation step at 90 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 sec and 60 ºC for 1 min. Small nucleolar RNA RNU6 (Applied Biosystems, Foster City, CA) was used for normalization. PCR reactions were performed in triplicate and fold changes were calculated using $2^{-\Delta\Delta CT}$ method, where ΔCt is the difference between the amplification fluorescent thresholds of the miRNA of interest and the RNA of RNU6.

3.4 Chromatin Immunoprecipitation assay

Samples from normal and cancer human tissues were processed for chromatin immunoprecipitation (ChIP) assay. Cellular sospension was collected by centrifugation at 2000 rpm at 4°C for 10 minutes and then resuspended in 6× volume of cell lysis buffer [5 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) pH 8.0, 85 mM KCl, 0.5% NP-40] plus phenylmethylsulfonyl fluoride (PMSF) (1 mM) and trypsin inhibitor (10 μg/ mL) as protease inhibitors. Cells were then incubated on ice for 15 minutes and lysed using a dounce several times. Nuclei were collected at 5000 rpm at 4°C for 10 minutes and the pellet was resuspend in 5× volume of nuclei lysis buffer (50 mM TrisHCl pH 8.1, 10 mM EDTA, 1% SDS) plus the same protease inhibitors as the cell lysis buffer. The solution was incubate on ice for 20 minutes and subsequently freezed and thawed in liquid nitrogen 2 times to aid in nuclear lysis. After centrifugation at 5000 rpm at 4°C for 10 minutes, the obtained chromatin was sonicated according to the procedure described by Federico et al. 2009 [53]. Samples were subjected to IP with the following specific antibodies against histone modification anti-tri methyl K9-Histone3, anti-acetyl H3 and the specific histone methyltransferase anti-SUV39H1. For qRT-PCR, 2 μl aliquot of IP DNA (150 μl) were amplified with a set of three primers pairs (all primers are listed in the 5' to 3' direction); region A: F1, ggggtaggtttgg tgggagttgc, R1, atcacagctgaaaagccacgtgta; region B: F2, cgcccacagctttgactgggt, R2, tgccatgagccagtgtaccagga; region C: F3, tcctggtacactggctcatggca, R3, agcagtggacag aggagtaggca. GAPDH promoter amplicon was used as a negative control in all experiments (data not shown). IgGs were used as nonspecific controls, and input DNA values were used to normalize the values from quantitative ChIP samples. ChIP assay from AGS cells treated with dimethyl sulfoxide (DMSO) or TSA were processed as above reported. For each assay, about 5x106 AGS cells were used for chromatin preparation and IP.

3.5 Treatment of gastric cancer cell lines with TSA

AGS, MKN28 and KATO III cells were plated in 10 cm culture dishes and grown for 24 hours before drug treatment. The next day, about 8.8 x 106 cells were incubated in fresh culture medium containing a TSA/ DMSO solution up to a final concentration of 300 nM. Control cells were treated with an equivalent volume of DMSO. After 24 or 48 hours, cells were harvested and used either to evaluate the *GKN1* mRNA and miR expression by qRT-PCR or for ChIP assays.

3.6 Bioinformatic analysis

For the identification of putative miRNA which can target *GKN1* 3'UTR, 5 different software algorithms were used, namely TargetScan7.0 (http://www.targetscan.org), miRanda (http://www.microrna.org), miRDB (http://mirdb.org), PITA (http://genie.weizmann.ac.il), and DIANA-microT-CDS (v5.0) (http://www.microrna.gr/microT-CDS). Putative miRNA predicted by at least three algorithms have been selected for further studies.

3.7 Plasmid construction and Luciferase Reporter Assay

To understand the role of microRNAs in complex biological processes, it is important to experimentally assess the interaction between the four putative miRNAs and their targeting sequences in the 3'UTR of *GKN1* mRNA. To these aim a luciferase reporter assay was performed. The full-length *GKN1* 3'UTR (157bp) were amplified by PCR using AGS DNA with primer pairs containing XbaI and BamH1 sites. Oligonucleotide sequences were as follows:

5'-ATCATTCTAGAGGATCCACAATTTTTTAAAGCCACTATGG-3';
5'-ATCATTCTAGATGAATTCAATGCTAAATGATTTTAT-3'.

The PCR products were cloned into the pGL3-control vector, downstream from the Firefly luciferase 3'UTR (Promega, Madison, WI, USA). The orientation of the inserted fragments was established by digestion and confirmed by sequencing. The pGL3 constructs with the reverse orientation were used as negative control.

For luciferase assays, human HEK-293 cells were plated at in 24 well plates (40,000 cells/ well) 12 h before transfection. The pGL3 constructs (200 ng) were co-transfected with 100 nM pre-miRs (Ambion) and with the Renilla luciferase reporter plasmid (10 ng) as an internal control, using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured at 48 h after transfection of IMR90 cells using a dual luciferase reporter assay (Promega) according to manufacturer's instructions and performed on a 20/20n Luminometer (Turner BioSystems, Sunnyvale, CA, USA). Relative luciferase activity was calculated by normalizing the firefly luminescence to the Renilla luminescence. All transfection experiments were done in triplicate and each experiment was repeated three times.

3.8 Statistical analysis

Statistical analysis was performed by two-tailed paired Student's t-test using KaleidaGraph 4.1.1 software. Western blot band intensity was evaluated with ImageJ 1.41o software. Data were reported as means ± standard deviation (SD). The significance was accepted at the level of $p < 0.05$.

4. RESULTS

4.1 Characterization of non-tumoral and tumoral tissues

For this study, six gastric tissue specimens of paired samples of non-tumoral (N1–N6) and tumoral (T1–T6) human gastric tissues excised from the same patients during surgery were used. Tissue T1 was charactized by a well-differentiated adenocarcinoma of intestinal type, T2 showed a severe dysplasia grade associated with a small area of intraglandular adenocarcinoma that was moderately differentiated, T3 and T6 exhibited a poorly differentiated adenocarcinoma of diffuse type, T4 showed a poorly differentiated adenocarcinoma of intestinal type, and T5 showed a moderately differentiated adenocarcinoma of intestinal type.

The peritumoral areas of intestinal type GC presented a variable degree of gastric atrophy with diffuse intestinal metaplasia, while the peritumoral areas of diffuse type GC showed a variable degree of non-dysplastic inflammation. The clinicopathologic characteristics of GC patients are summarized in Table 1.

Table 1. Characteristics of Gastric Cancer Patients

Variable	Gastric Cancer patients (n = 6)
Age at surgery (Y)	
Mean	67 ± 13
Range	45 – 78
Sex ratio (M/F)	3/3
Tumor type	Intestinal 3, Diffuse 2 Dysplasia 1
Grade of differentiation	Well 1, Moderate 2, Poor 3

4.2 Expression levels of GKN1 in non-tumoral and tumoral tissues

Subsequenly, the expression levels of GKN1 in non-tumoral (N1–N6) and tumoral (T1–T6) samples were analyzed. As reported in Figure 1 (panels A and C), compared with non-tumoral tissues, the expression profiles of GKN1 evaluated by western blotting showed that in all tumoral samples a down-regulation or an almost total absence of GKN1, as evaluated from the expression profile of GAPDH as a control (panels B and D) and based on the

densitometric analysis of GKN1 expression (Fig. 1E). Figure 1C also shows the expression profiles of GKN1 in positive control samples taken from healthy individuals undergoing sleeve gastrectomy (C1 and C2).

To asses if the down-regulation of GKN1 also occurred at the transcriptional level, quantitative reverse transcription (qRT)-PCR on total RNA isolated from paired gastric non-tumoral and tumoral tissues were performed. As reported in Figure 1F, compared with non-tumoral tissues, a decrease of *GKN1* mRNA levels in tumoral tissues was observed thus supporting the western blot findings.

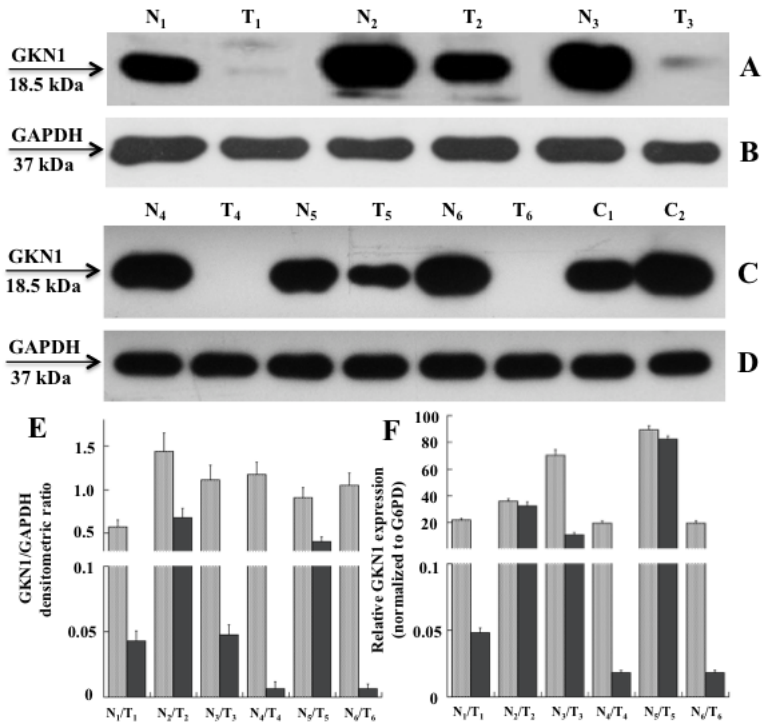


Figure 1. Expression levels of GKN1 in human gastric tissues. A and C. Western blot of tissue extracts analyzed in paired non-tumoral (N1-N6), tumoral (T1-T6) human gastric samples and samples taken from healthy individuals undergoing sleeve gastrectomy (C1 and C2), respectively, using mouse anti-GKN1 antibody. positive control E. Expression levels of GKN1 protein in non-tumoral (N1-N6) and tumoral (T1-T6) paired samples evaluated from the densitometry of GKN1 bands normalized towards the corresponding densitometry of GAPDH bands B and D. F. qRT-PCR analysis. Total RNA was prepared from gastric tissues and analyzed by qRT-PCR for *GKN1* mRNA level compared to *G6PD* mRNA as reference sample. Data from three experiments are reported as mean values ± SD.

4.3 The down-regulation of GKN1 in GC was associated with H3K9triMe on the *GKN1* gene promoter

To highlight if epigenetic alteration was associated to the strong down-regulation of GKN1 observed in the tumoral tissues analyzed, chromatin immunoprecipitation (ChIP) assays for the repressive trimethylation of histone 3 at lysine 9 (H3K9triMe) were performed. Using UCSC Genome Browser, a 600 bp promoter region of *GKN1* gene including the 5'-untranslated region (5'UTR) was identified and divided into three different segments (A, B, and C) of about 160 bp. The designed corresponding PCR primers (Fig. 2) used for ChIP assays revealed on the three DNA segments a significant increase in H3K9triMe modification in tumoral tissues compared with non-tumoral tissues. Figure 3 displays the results of an average of three independent experiments performed on six paired non-tumoral (N1–N6) and tumoral (T1–T6) specimens.

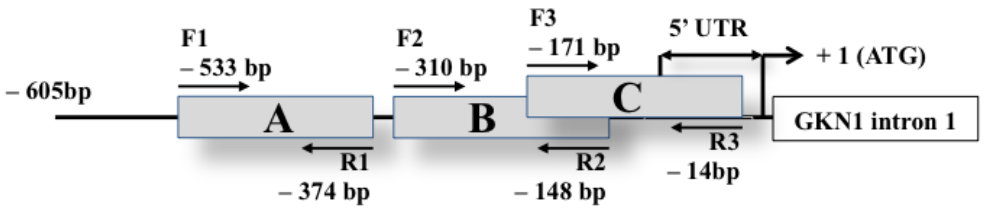


Figure 2. 5'-region of *GKN1* gene analyzed by ChIP assay and proposed gene expression regulation. The A, B and C regions of *GKN1* gene promoter delimited by the corresponding primer pairs (F1-R1, F2-R2, F3-R3) are boxed. The length of the 5'UTR of *GKN1* mRNA is indicated by double arrows. +1 indicates the position of the start codon.

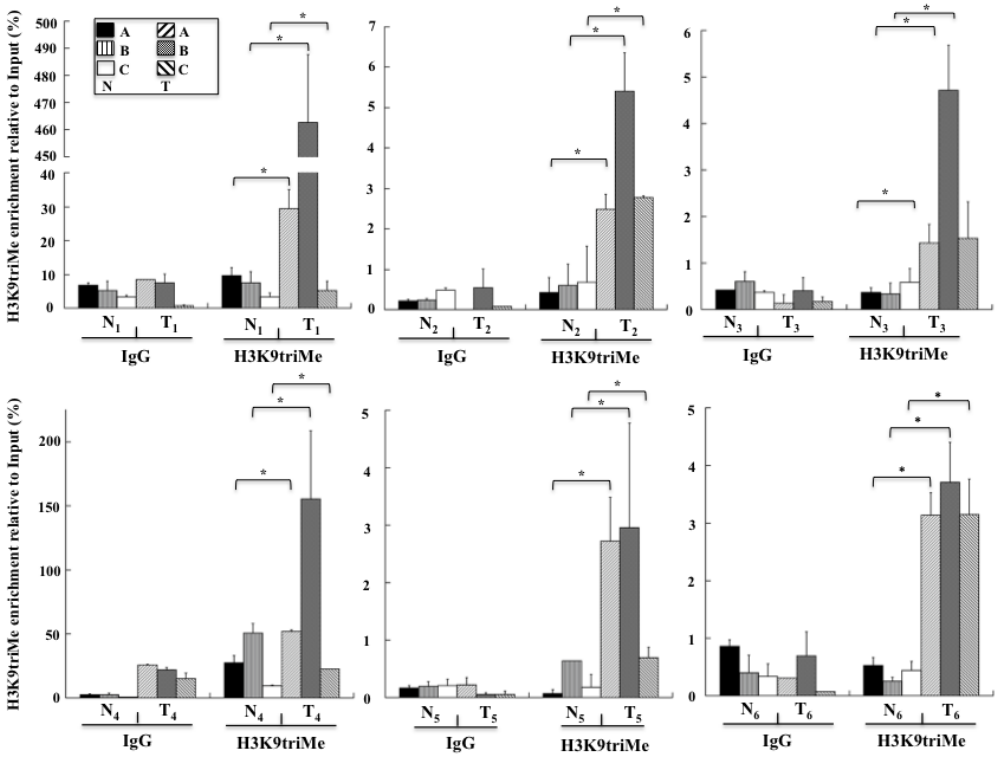


Figure 3: H3K9triMe levels on human *GKN1* gene promoter. Chromatin immunoprecipitation assays were performed on human non-tumoral (N1-N6) and tumoral (T1-T6) gastric samples, respectively. H3K9triMe enrichment relative to input is reported as $2\Delta Ct \times 100$, where ΔCt is the difference between CtInput and CtIP. All quantitative ChIP data were derived from three independent experiments, and for each experiment qPCR was performed in triplicate. * $p<0.05$, compared to corresponding control.

4.4 The increase of H3K9triMe was associated with the recruitment and/or with the activation of SUV39H1 on the *GKN1* gene promoter

Data from literature suggested that the H3K9triMe modification is generally associated to a specific histone methyltransferase, the histone-lysine N-methyltransferase SUV39H1, that trimethylates Lys-9 of histone H3 using mono-methylated H3 Lys-9 as a substrate [54]. Therefore, to asses the presence of this enzyme in the *GKN1* promoter region a ChIP assays was performed. The result reported in Figure 4 clearly shows that compared with non-tumoral tissues, a significant increase in the binding of SUV39H1 to the *GKN1* promoter region in tumoral samples was observed.

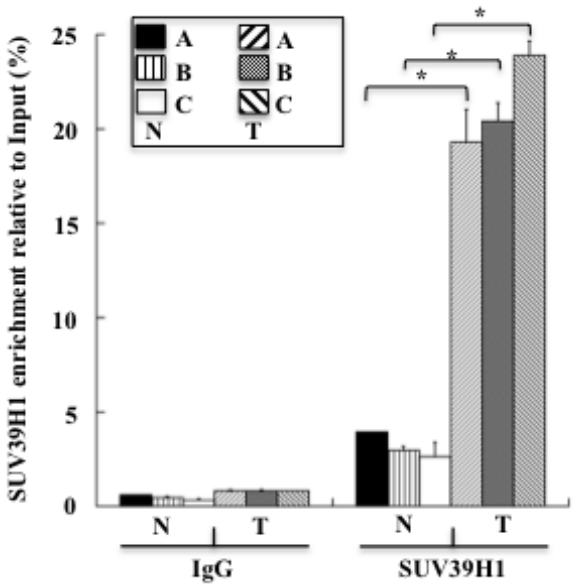


Figure 4. SUV39H1 levels on human *GKN1* gene promoter. ChIP assays was performed on human non-tumoral N1-N6 (N) and tumoral T1-T6 (T) human gastric samples, respectively. SUV39H1 enrichment relative to input is reported as $2\Delta Ct \times 100$, where ΔCt is the difference between CtInput and CtIP. All quantitative ChIP data were derived from three independent experiments, and for each experiment qPCR was performed in triplicate. * $p<0.05$, compared to corresponding control.

4.5 Expression levels of SUV39H1, HDAC1, and H3K9triMe in GC tissues

Then, it was analyzed if there was a relationship between the expression levels of GKN1 and that of SUV39H1, histone deacetylase 1 (HDAC1), and H3K9triMe. As reported in Figure 5 (panels A and D), compared with non-tumoral tissues (N1–N6), an increase in the expression of SUV39H1 in tumoral tissues (T1–T6), evaluated from the corresponding GAPDH western blot band intensity (Fig. 5, panels C and F), was observed. Similar behaviours for HDAC1 were seen in paired non-tumoral and tumoral gastric tissues (Fig. 5, panels B and E). Regarding H3K9triMe, it was possible to analyze its expression in the nuclear extracts of only one paired non-tumoral (N6) and tumoral (T6) sample. As shown in Figure 5G, compared with non-tumoral tissue, tumoral tissue showed higher level of H3K9triMe expression as

determined by the western blot band intensity ratio with respect to that of lamin A/C (Fig. 5H).

The expression levels of SUV39H1 and HDAC1 were next evaluated in healthy sleeve gastrectomy specimens (C1 and C2) (Fig. 5, panels D and E). In these cases, expression levels appeared similar to those of non-tumoral tissues (N4–N6).

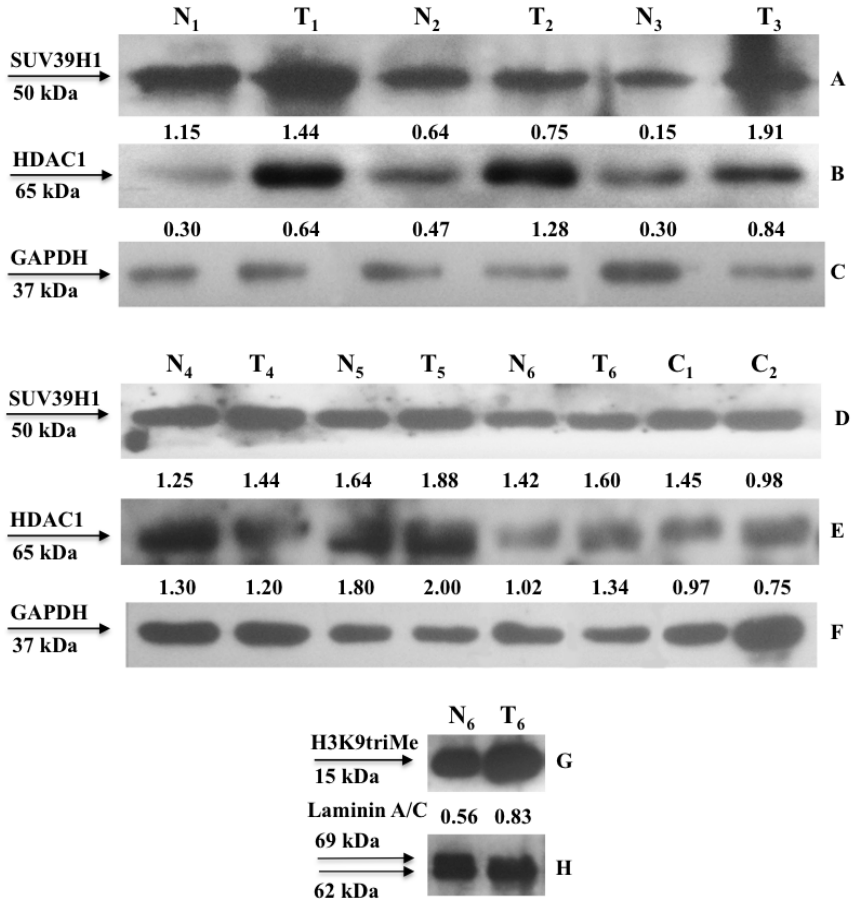


Figure 5. Expression levels of SUV39H1, HDAC1 and H3K9triMe in human gastric tissues. SUV39H1, HDAC1 expression in human non-tumoral (N) and tumoral (T) cell extracts (panels **A-F**) and H3K9triMe expression in nuclear extracts (panels **G-H**) was assessed by Western blot with the specific antibodies. Relative expression of SUV39H1, HDAC1 in sample tissues is reported as band intensity ratio with that of the corresponding GAPDH whereas that of H3K9triMe is shown as band intensity ratio with that of the corresponding lamin A/C.

4.6 Expression levels of SUV39H1, HDAC1, and H3K9triMe in cell lines

The expression levels of SUV39H1, HDAC1, and H3K9triMe proteins was also evaluated in GC cell lines. Because of the lack of a non-tumoral gastric cell line, we analyzed protein expression in GKN1-transfected and non-transfected GC cells (AGS) and in an additional non-transfected gastric cancer cell line (NCI-N87). As shown in Figure 6, compared with non-transfected cells, AGS cells transfected with GKN1 demonstrated lower expression levels of SUV39H1 (Fig. 6I), HDAC1 (Fig. 6J), and H3K9triMe (Fig. 6K) as determined by the western blot band intensity ratio with respect to that of lamin A/C (Fig. 6L).

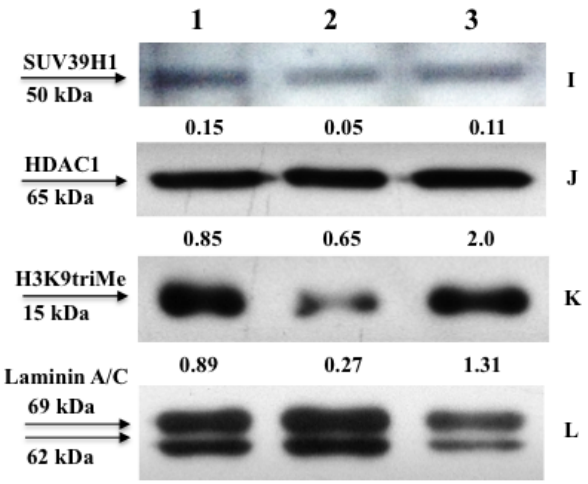


Figure 6. Expression levels of SUV39H1, HDAC1 and H3K9triMe in human gastric cell lines. Protein expression in gastric cancer cell lines (panels **I-L**) was assessed by Western blot with the specific antibodies on nuclear extracts of AGS cells (lane 1), AGS cells transfected with flGKN1 (lane 2) and NCI-N87 cells (lane 3). Relative expression of SUV39H1, HDAC1 and H3K9triMe is reported as band intensity ratio with that of the corresponding lamin A/C.

4.7 Trichostatin A induced the up-regulation of *GKN1* mRNA in GC cell lines

To further understand the effect of epigenetic modifications on GKN1 down-regulation, the possible role of histone acetylation was investigated. MKN28, AGS, and KATO III GC cell lines were treated with trichostatin A (TSA), an inhibitor of histone deacetylases (HDACs), and *GKN1* mRNA levels were

evaluated by qRT-PCR. As reported in Figure 7, treatment of AGS cells with TSA for 24 h led to an increase in *GKN1* mRNA expression of about 28-fold compared with untreated cells, whereas no effect was observed in MKN28 and KATO III cells. TSA treatment for 48 h increased *GKN1* mRNA expression by around 50-, 160- and 110-fold in MKN28, AGS, and KATO III cells, respectively. However, the increase in the *GKN1* mRNA levels were not associated with the re-expression of the protein as evaluated by western blotting (data not shown).

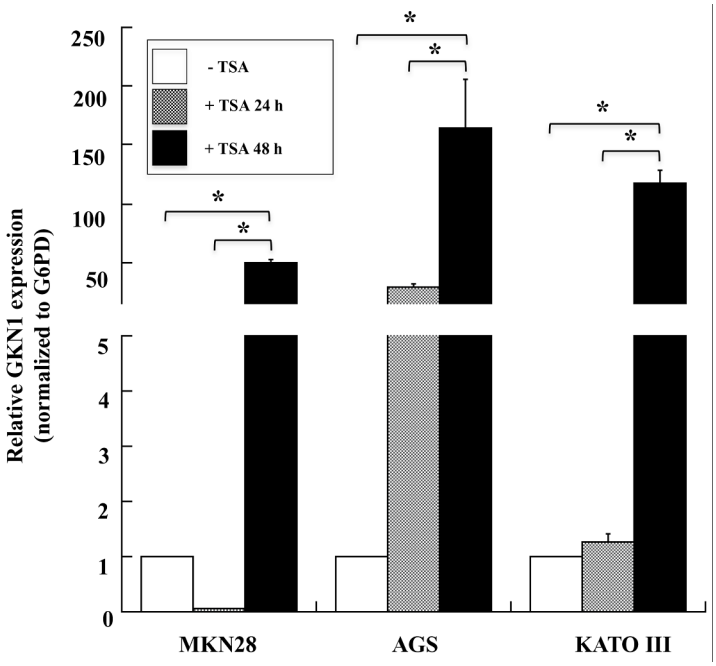


Figure 7. TSA induced the expression of *GKN1* mRNA in gastric cancer cell lines. qRT-PCR analysis of *GKN1* mRNA in MKN28, AGS and KATO III gastric cancer cell lines after TSA treatment of the cells for 24 and 48 hours. *G6PD* was used as internal standard for normalization The relative expression of *GKN1* was evaluated using as control cells treated with DMSO. Data from a representative experiment are reported as mean values ± SD. * $p<0.05$.

4.8 Trichostatin A induced in GC cell line the down-regulation of H3K9triMe and up-regulation of H3 acetylation

The up-regulation of *GKN1* mRNA observed in TSA-treated AGS cell line suggested the use of these cells as a positive control to confirm the results

obtained in human gastric tissues in a cellular gastric model. As reported in Figure 8, a ChIP assay showed that up-regulation of *GKN1* mRNA in AGS cells after TSA treatment was associated with a reduction of the H3K9triMe repressive modification (Fig. 8 A). This result confirmed what found in gastric tissues, and revealed an increase of H3-acetylation-activating modification in the same three regions of the *GKN1* promoter analyzed earlier (Fig. 8B).

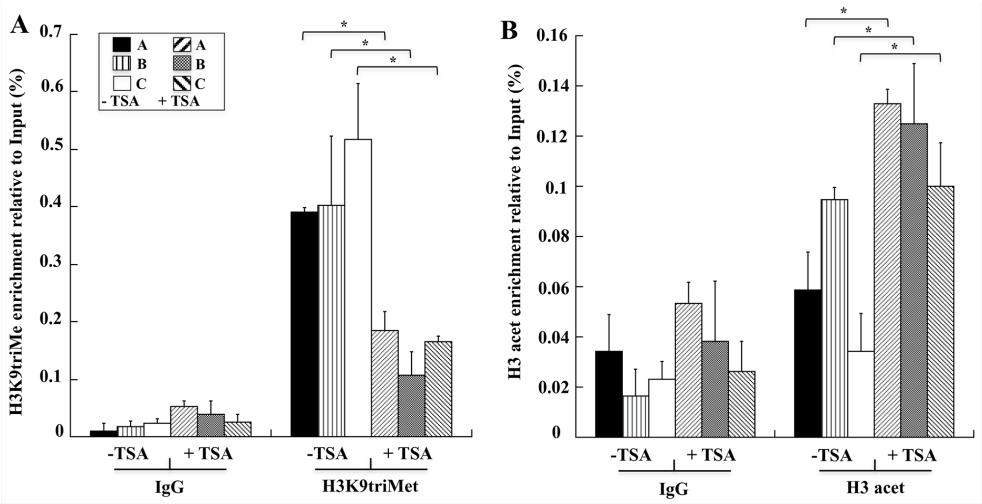


Figure 8. TSA induced in AGS cells decreased levels of H3K9triMe and increased levels of H3 acetylation. Levels of H3K9triMe (**A) and** Levels of H3 acetylation (**B)** determined by ChIP assays on AGS cells not treated (-TSA) and treated with TSA (+TSA). H3K9triMe and H3 acetylation enrichment relative to input are reported as $2\Delta Ct \times 100$, where ΔCt is the difference between CtInput and CtIP. All quantitative ChIP data were derived from three independent experiments, and for each experiment, qRT-PCR was performed in triplicate. * $p<0.05$, compared to corresponding control.

4.9 Bioinformatic analysis for miRNAs targeting *GKN1* 3'UTR

Although *GKN1* mRNA was strongly up-regulated in castric cancer cell lines after TSA treatment, the presence of the protein as evaluated by Wester blotting was still undetectable. Therefore, it was hypothesized the occurrence of a post-translation regulation of GKN1 expression mediated by miRNAs.

To search for putative miRNAs that could target the 3'UTR of *GKN1* mRNA, in silico analyses were performed using 5 different algorithms

(TargetScan 7.0, miRDB, miRanda, PITA, and DIANA-microT-CDS v5.0). Putative miRNAs were selected following a level of stringency that was based on the intersections of ≥3 different algorithms (Figure 9). This allowed evidentiate 4 putative miRNAs: hsa-miR-1245b-3p (3'-AUAUCCGGAAAUCUAGUAGACU-5', 22 nts), hsa-miR-186-5p (3'-UCGGGUUUUCCUCUUAAGAAAC-5', 22 nts), hsa-miR-325 (3'-UGUGAAUGACCUGUGGAUGAUCC-5', 23 nts), hsa-miR-544a (3'-CUUGAACGAUUUUUACGUCUUA-5', 22 nts); the underlined sequence represents the miRNA seed region. Figure 10 shows the nucleotide sequence of *GKN1* 3'UTR and the corresponding segments where the identified miRNAs form base pairing.

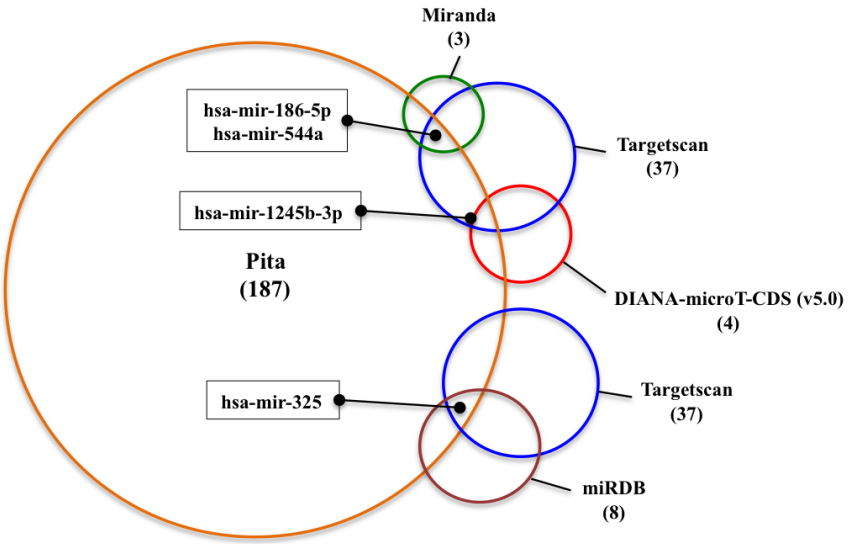


Figure 9. Venn diagram showing the algorithms intersection highlighting miRNAs targeting *GKN1* 3'UTR. miRNAs population of each algoritm is given in parenthesis.

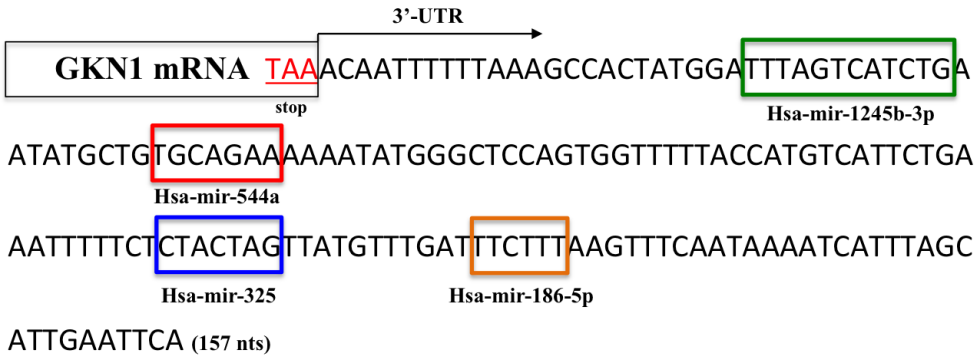


Figure 10. *GKN1* 3'UTR and miRNAs annealing regions. Nucleotide sequence of the 3'UTR of *GKN1* mRNA showing the corresponding miRNAs target sequences (boxed).

4.10 *GKN1* 3'UTR is direct target of hsa-miR-544a and miR-1245b-3p

The functional studies were performed using constructs containing the *GKN1* 3'UTR (WT) and *GKN1* reverse 3'UTR (MUT), as negative control, cloned downstream of the luciferase genes. These report plasmids were co-transfected in presence of premiR negative control (miR-SCR) or the 4 premiRs in HEK-293 cells and the luciferase activity was assessed. As shown in Figure 11, over-expression of miRNAs resulted in a significant inhibition of the activity of the reporter constructs bearing the wild-type (WT) 3'UTRs of *GKN1* gene when compared with miR-SCR-transfected cells only for miR-544a and miR-1245b-3p (Fig. 11C, D) whereas for miRNA-186-5p and miR-325, no effect was instead observed (Fig. 11A, B].

The transfection of the constructs containing the inverted 3'UTR regions (MUT) abolished the effect of miR-544a and miR-1245b-3p. These results indicated that *GKN1* 3'UTR is a direct target of miR-544a and miR-1245b-3p.

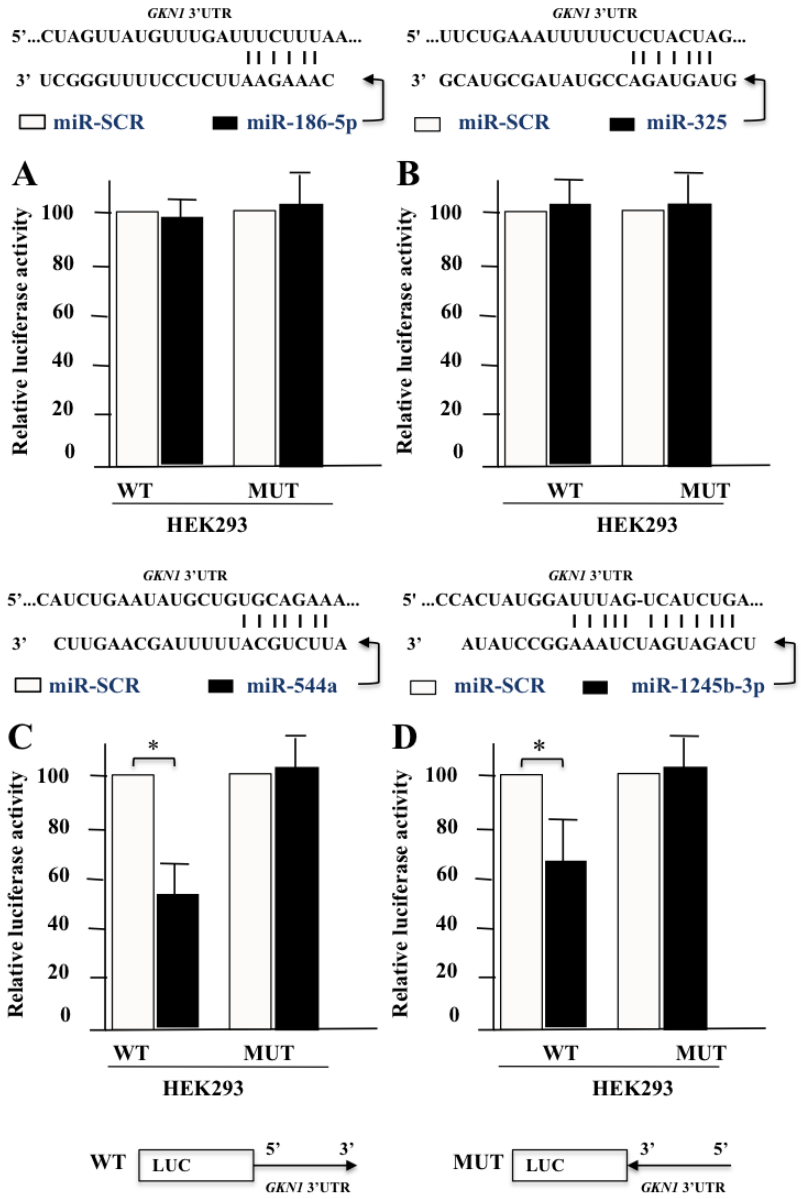


Figure 11. Validation of putative miRNAs by luciferase assay in HEK293 cells. The luciferase constructs bearing WT or inverted (MUT) *GKN1* 3'UTR were co-transfected in presence of premiR negative control (miR-SCR) or the 4 premiRs in HEK-293 cells. Renilla luciferase was used as an internal control. Dual luciferase assays were performed as described in Materials and Methods. Triplicate transfection ratio (±SD) firefly/Renilla luciferase activity were averaged and expressed as percentage of the corresponding transfections with miR-SCR control. *$p < 0.05$.

4.11 TSA-induced up-regulation of *GKN1* mRNA is accompained in GC cell lines by up-regulation of miR-544a

To assess the possible involvment of miRNAs in the post-translation regulation of GKN1 expression, it was evalueted the expression level of miR-544a and miR-1245b-3p in MKN28, AGS and KATO III cell lines after TSA treatment by TaqMan MiRNA Assay. The results showed an increase of miR-544a expression of about 20-, 140- and 60-fold in MKN28, AGS, and KATO III cells, respectively, compared with untreated cells (Fig. 12A); the expression of 1245b-3p was detectable only in AGS cells with an increase of about 7-fold (Fig. 12B), whereas no effect was observed in MKN28 and KATO III cells.

These results suggest that the up-regulation of miR-544a could represents one of the leading cause of the GKN1 translational repression.

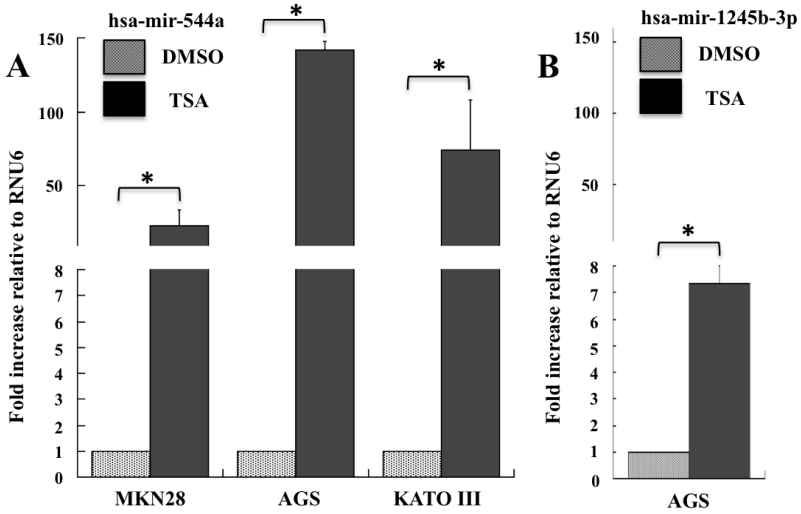


Figure 12. Expression level of miR-544a and miR-1245b-3p in TSA-treated cell lines. qRT-PCR analysis of miR-544a (A) and miR-1245b-3p (B) in MKN28, AGS and KATO III gastric cancer cell lines after TSA treatment of the cells for 48 hours. *RNU6* was used as internal standard for normalization The relative expression was evaluated using as control cells treated with DMSO. Data from a representative experiment are reported as mean values ± SD. * $p<0.05$.

5. DISCUSSION

One of key risk factor in the development of many types of cancers harbour from tissues inflammation and in particular, stomach chronic inflammation could lead to the histopathologic progression of chronic gastritis across gastric atrophy, intestinal metaplasia, dysplasia, and finally GC [55]. A major step in the initiation and development of GC is *Helicobacter pylori* infection of the gastric mucosa and the consequent chronic inflammation [56]. Hypermethylation of promoters of tumor-related genes is quite often detected in premalignant gastric lesions [57]. These findings suggest a relationship with the induction and or promotion of GC [58, 59]. In fact, aberrant DNA methylation is one of the leading inactivating mechanisms of tumor suppressor genes frequently associated with *H. pylori* infection [60, 61].

It is already well estabilished that the expression of gastrokine 1 (GKN1) decreases throughout the progressive stages of neoplastic transformation. Earlier investigations performed on GC tissues showed no CpG hyper-methylation of the *GKN1* promoter [48] and altered GKN1 expression associated with the severity of gastritis and DNA methylation in non-neoplastic gastric mucosa [62]. Other studies showed that GKN1 expression in AGS cells induced endogenous micro RNA (miR)-185 that directly targets the epigenetic effectors DNA (cytosine-5)-methyltransferase 1 (DNMT1) and histone-lysine N-methyltransferase enzyme (EZH2). In particular, the histone acetyl transferase Tip60 was found to be up-regulated and HDAC1 to be down-regulated in an miR-185-independent manner, thus inducing cell cycle arrest by regulating cell cycle proteins in GC cells [63]. However, the exact relationship between gastritis and GKN1 expression was not evaluated.

In this thesis, the possible causes of *GKN1* gene inactivation have been investigated by evaluating if other possible epigenetic mechanisms could be involved in this process. Because DNA methylation, histone deacetylation, and histone H3 methylation at lysine 9 are the three best-characterized covalent modifications associated with a repressed chromatin state, the attention was focused on these modifications [64, 65]. For the first time it has been shown that a mechanism comprising histone modifications appears to be involved in the dysregulation of *GKN1* transcription in GC. In fact, by using ChIP assays, comparing human non-tumoral tissues with corresponding tumoral ones, it was revealed an increase of the repressive histone modification H3K9triMe (Fig. 3) associated by recruitment of the specific histone methyltransferase SUV39H1 (Fig. 4; Fig. 5A, D) in tumoral tissues. SUV39H1 and H3K9triMe upregulation both at transcriptional and translational level has been demonstrated in several cancers therefore, SUV39H1 and H3K9triMe have important roles in cancer development and progression. Moreover, the pharmacological inhibition of SUV39H1

represents a promising therapeutic approach for cancer treatment [66, 67]. The present thesis documents in gastric carcinoma the overexpression of Suv39H1 and histone tri-methylated H3K9.

Interestingly, the results obtained showed that eventhough the H3K9triMe enrichment levels observed in the six human specimens appeared quantitatively different, the promoter region of *GKN1* gene where this modification was mostly enriched was the same in all samples (region B). No significant difference in H3K9triMe in non-tumoral gastric tissues was instead observed compared with tumoral ones. This observation suggests a correlation with the expression of GKN1 protein shown in Figure 1. In fact, the H3K9triMe enrichment observed by ChIP assay (Fig. 3) was associated with tumoral samples in which strong down-regulation of GKN1 expression was observed. The important role of H3K9triMe in GC was also supported by the finding that H3K9 trimethylation positively correlates in gastric carcinoma with tumor stage, lymphovascular invasion, and cancer recurrence whereas higher levels of H3K9 trimethylation correlate with poor survival [38].

One of the main determinants of chromatin structure is given by histone acetylation [68] that can be regulated dynamically through the involvement of transactivating factors with intrinsic histone acetylase activity or through the recruitment of deacetylase complexes that repress gene expression [69]. HDAC1 is in fact up-regulated in many cancer cell lines and tissues [70], including GC, at both the transcriptional and translational levels [71]. The findings here reported are in agreement with these aspects (Fig. 5) and imply that increased HDAC1 expression causes histone hypoacetylation and the silencing of several tumor suppressor genes in GC.

The model hereafter reported could describe the mechanisms by which H3K9triMe acts on the *GKN1* promotor (Fig. 13) mediated by SUV39H1. A transcription factor functions as a negative regulator by recruiting SUV39H1 and HDACs on the *GKN1* promoter to induce histone deacetylation and methylation, thus resulting in *GKN1* repression.

Figure 13. Proposed *GKN1* gene expression regulation. Scheme showing the proposed mechanism of histone/SUV39H1 actions on *GKN1* gene promoter.

This model is in agreement with recent findings showing that restoration of GKN1 protein suppressed GC cell growth through an miRNA-mediated mechanism for DNA epigenetic modification [63]. Therefore, the loss of GKN1 function contributes to malignant transformation and the proliferation of gastric epithelial cells in gastric carcinogenesis.

On the bases of the above findings, it was then investigated whether underacetylation could contribute to *GKN1* transcriptional inhibition using TSA to increase general histone acetylation in an attempt to bypass the inhibitory effects of DNA methylation. Because TSA in the absence of cytotoxicity can affect the cell cycle, regulates cell differentiation, induces apoptosis, and inhibits cell migration [72-74], to reduce the possible inhibition of cell proliferation [75] milder experimental conditions (TSA, 90 ng/ml; time of treatment, 24 and 48 h) were used.

Under these same experimental conditions, TSA was found to reduce AGS cell viability by less than 10% [76], and to inhibit in a dose-dependent manner MKN28 cell growth up to a concentration of 500 ng/ml [77]. Here, treatment of GC cell lines MKN28, AGS, and KATO III with TSA strongly increased *GKN1* mRNA expression (Fig. 7), thus suggesting that histone deacetylation represents an important mediator of *GKN1* repression associated with DNA methylation. In fact, treatment of AGS cells with TSA led to a reduction of H3K9triMe and an increase of histone acetylation (Fig. 8). Because histone acetylation is a fundamental regulatory mechanism for controlling gene accessibility, the results obtained indicate that histone methylation is a unique mechanism for establishing local histone deacetylation, and generating maintainable epigenetic chromosomal states.

However, it should be considered that in the experimental condition used, the up-regulation of *GKN1* mRNA level was still very low because the cycle numbers required for its amplification resulted of about 10-fold lower than those required for the amplification of housekeeping glucose-6-phosphate dehydrogenase (data not shown). Neverthless, no GKN1 protein re-expression was detected under these conditions by Western blotting. This finding could have been caused by proteosome-mediated degradation of the GKN1 protein. To ascertain this possibility, AGS cells were treated with proteosome inhibitor (MG132). No GKN1 expression was observed (not shown). This finding suggested the presence of further regulation at the translational level, perhaps by mechanisms mediated by miRNAs, resulting in translational repression and gene silencing. For example, in a recent work, it has been reported that miRNA-544 directly targets the 3'UTR of the newly-identified tumor suppressor gene IRX1, whose hypermethylation decreases expression of the protein in GC [78]. Therefore, miRNAs and promoter hypermethylation are important epigenetic mechanisms for transcriptional inactivation of tumor suppressors. In fact, miRNAs are post-

transcriptional regulators that by interacting with their targets in the 3'UTR of transcripts lead to either mRNA degradation or to translation inhibition.

For the identification of putative miRNA which can targets *GKN1* 3'UTR, a bioinformatic approach was undertaken by analyzing 5 different software algorithms. The intersection of ≥3 programs resulted in a list of 4 potential miRNAs: hsa-miR-1245b-3p, hsa-miR-186-5p, hsa-miR-325, hsa-miR-544a. To verify if the selected miRNAs were able to target the 3'UTR of *GKN1* mRNA, a luciferase reporter assay was performed. The results of these experiments showed that *GKN1* 3'UTR was mainly a direct target of hsa-miR-544a and to a lower extent to miR-1245b-3p (Fig. 11).

On the bases of these results, it was then tryied to highlight the possible involvment of these miRNAs in the post-translation regulation of GKN1 expression. Therefore, it was evaluated the expression level of miR-544a and miR-1245b-3p in MKN28, AGS and KATO III gastric cancer cell lines after TSA treatment in which we observed a strong increase of *GKN1* mRNA expression that was not accompained by the expression of the protein. Compared with untreated cells, the results showed an evident up-regulation of miR-544a expression of about 20-, 140- and 60-fold in MKN28, AGS, and KATO III cells, respectively. The expression of miR-1245b-3p was instead detectable only in AGS with an increase of about 7-fold only, whereas no effect was observed in MKN28 and KATO III cells. Therefore, one might postulate that the up-regulation of miR-544a could be the cause of *GKN1* translational repression, thus suggesting its potential role as biomarker and as therapeutic target in GC patients. The data here presented are hopeful since miR-544a has been shown to play an important role in gastric cancer by inducing epithelial-mesenchymal transition (EMT) [79].

These findings suggest that epigenetic mechanisms leading to the inactivation of *GKN1* play a key role in the multi-step process of gastric carcinogenesis and would provide an essential starting point for the development of new therapeutic strategies based on epigenetic targets for alternatives gene.

6. CRITICISMS AND FUTURE PERSPECTIVES

One first criticism of the present study regards the number of GC speciments used. Although the results obtained on six GC patients were similar, to be confident that a mechanisms underlyning *GKN1* gene silencing is based on the up-regulation of histone methylation H3K9triMe by SUV39H1 and deacetylation mediated by HDAC1, a larger number of samples should be analyzed. However, this aspect is not easy to afford because the difficulty in the recruitment of GC patients, in the quality and quantity of tissues specimens that can be obtained after surgery and in the intrinsic difficulty of the ChIP assay experimental procedure. In fact, the number of experiments carried out were much higher with respect to those here reported and not in all of them the same reproducibility was observed. Nevertheless, it has been already planned with Hospital Partner the recruitment of a new set of samples in order to enlarge the statistical significance of the data obtained.

It was also tried to clarify the controversial behaviour of H3K4triMe that with respect to the up-regulation of H3K9triMe it should be instead dow-regulated. However, there are several reports in which is described that H3K4triMe remains almost unchanged during inactivation of tumor suppressor genes. In agreement to what reported in these works, ChIP assay performed on two pairs non-tumoral and tumoral samples confirmed in these cases no variations in the H3K4triMe levels.

It was also searched for possible transcription factors that could have been involved in *GKN1* positive or negative transcription regulation. Literature data suggested to assay the expression profile of RUNX3 and CBX7 by WB. Using the collection of paired non-tumoral and tumoral GC tissues specimens, not substantial differences were observed. Also ChIP assay performed for RUNX3 did not shown the presence of this factor on the *GKN1* promoter region.

Another important criticism concerning the project is the lack of a normal gastric cell line expressing GKN1. Although there are in the world two research groups that claim to possess this cell line, no success was achieved when these cells were received since no GKN1 expression was detected. For this reason, in order to study the functions of miRNAs we need an *in vitro* model of the gastric mucosa.

Therefore, I am now developing a method to culture normal human gastric epithelial cells from endoscopic biopsies, using enzymatic digestion to isolate cells.

Human gastric epithelial cells have been difficult to maintain in culture for prolonged periods of time. In order to increase the life span of normal human gastric epithelial cells in culture, I plan to transfect these cells with the SV40 Large T-Ag in order to immortalize them. At the moment, several trials to amplify a primary culture are in progress. In fact, having available a gastric

cell line expressing GKN1, functional effects of the selected miRNAs by treating the gastric cells with the corresponding premiR could be evaluated and a reduction in the expression level of GKN1 should be observed.

However, some criticisms linked to the identification of miR-544a and miR1245b-3p as possible negative regulator of GKN1 expression should be taken in consideration.

First of all, the up-regulation of miR-455a observed following treatment of gastric cancer cell line might not be directly induced by the increase of *GKN1* mRNA but to TSA treatment of the gastric cells. In fact, although TSA was used at mild concentration, it might had a general effect on histone deacethylation of the promoter regions of other genes and thus somehow induced the up-regulation of miRNAs. However, it must be pointed out that the increase of *GKN1* mRNA and the up-regulation of miRNA-544a in the three cell lines show a similar behaviour. This observation might suggest that the trasfection of the gastric cancer cell lines after TSA treatment with the corresponding anti-miRNAs should possibly lead to the re-expression of the protein.

Comparison of the expression levels of miR-544a and miR-1245b-3p in paired non-tumoral and tumoral gastric tissues also represents an important experimental approach. In particular, the evaluation should be carried out in a large number of samples and in association with the histopathologic characteristic of the gastric cancer tissues. In fact, miRNA up- or down-regulation might be very sensitive to the state and progression of the disease. In these circumstance, it might also function as possible tumor marker especially when its established up-regulation is associated to a tumoral stage.

In preliminary experiments, assaying the expression level of miR-544a and miR-1245b-3p, it was found that compared to the normal gastric tissue, miR-544a was found up-regulated in a sample from a patient owing intestinal metaplasia. The increase observed was about 3-fold compared to the control thus suggesting that this miRNA might be up-regulated during a particular stage of gastric cancer progression. Of coarse, this encouraging observation should be confirmed by analyzing other well characterized tissues.

7. CONCLUSIONS

In conclusion, I found that GKN1 expression was significantly reduced in several specimens from patients with gastric carcinoma showing dysplasia and tumor gastric mucosa, and that GKN1 down-regulation was inversely correlated with the recruitment on the *GKN1* promoter of H3K9triMe and Suv39H1. GKN1 expression in tumoral tissues was also associated with an overall increase of the expression profiles of H3K9triMe, Suv39H1, and HDAC1 proteins. These findings strongly suggested that that epigenetic mechanisms leading to the inactivation of GKN1 play a key role in the multi-step process of gastric carcinogenesis. The results obtained can be considered relevant and reliable since they were obtained *in vivo* on human specimens. Nevertheless it will be necessary to confirm them in a larger number of samples.

TSA-mediated down-regulation of H3K9triMe and up-regulation of H3 acetylation observed in gastric cancer cells supported the obtained data whereas, TSA-induced increase of *GKN1* mRNA not accompained by the expression of the protein suggested a miRNAs mediated post-translation regulation. Of coarse, the identification of miR-544a and perhaps miR-1245b-3p as additional possible regulators of GKN1 expression in GC should be better characterized. This will enable obtain a greater understanding of these mechanisms and to determine whether they are involved in the development and progression of GC.

8. APPENDICES

8.1 Example of qRT-PCR calculation for H3K9triMe Chip assay performed on the A, B and C regions of the *GKN1* gene promoter

Patient n. 2

Region A		**Dct**	**2^{-Dct}**	**2^{-Dct} x100**
N Input	N IgG	N Igg-N Input		
27,42200216	32,90889809	5,486895927	0,02229871	2,229871017
27,24634822	32,57791753	5,331569307	0,024833488	2,483348753
27,35886712	33,01756375	5,658696633	0,019795322	1,97953223
N Input	N Met	N Met-N Input		
27,42200216	36,11472032	8,692718158	0,002416746	0,241674646
27,24634822	36,25444646	9,008098241	0,001942192	0,194219229
27,35886712	34,23138517	6,872518054	0,008534261	0,853426099
T Input	T IgG	T Igg-T Input		
27,57182797	44,97860592	17,40677795	5,7549E-06	0,00057549
28,09594252	44,97860592	16,8826634	8,27584E-06	0,000827584
27,35157109	44,97860592	17,62703483	4,94008E-06	0,000494008
T Input	T Met	T Met-T Input		
27,57182797	32,74959045	5,177762478	0,027627283	2,762728347
28,09594252	33,67432301	5,578380492	0,020928598	2,092859841
27,35157109	32,5944488	5,242877707	0,026408063	2,640806295

Region B		**Dct**	**2^{-Dct}**	**2^{-Dct} x100**
N Input	N IgG	N Igg-N Input		
27,9044421	33,02732687	5,122884767	0,028698422	2,869842222
27,76507613	33,25170743	5,486631302	0,022302801	2,230280067
28,05589741	33,72531676	5,669419356	0,01964874	1,964874045
N Input	N Met	N Met-N Input		
27,9044421	33,81718792	5,912745814	0,016599162	1,659916161
27,76507613	35,04311039	7,278034263	0,006443078	0,644307783
28,05589741	34,27208766	6,216190248	0,013450557	1,345055723
T Input	T IgG	T Igg-T Input		
28,65401001	34,58360595	5,929595936	0,016406418	1,640641757
28,36326492	34,03490014	5,671635228	0,019618585	1,961858462
28,40308657	35,02161751	6,618530936	0,010177091	1,017709108
T Input	T Met	T Met-T Input		
28,65401001	32,65128316	3,99727315	0,062618243	6,261824347
28,36326492	32,86650539	4,503240471	0,04409502	4,409501967
28,40308657	32,56866913	4,165582556	0,055723027	5,572302712

Region C		**Dct**	**2^{-Dct}**	**2^{-Dct} x100**
N Input	N IgG	N Igg-N Input		
28,29850371	34,08240063	5,783896927	0,018149871	1,81498706
30,17438951	33,25085767	3,076468154	0,118547065	11,85470647
27,55576253	34,37842784	6,822665304	0,008834319	0,883431932
N Input	N Met	N Met-N Input		
28,29850371	36,91310832	8,614604615	0,002551206	0,255120649
30,17438951	36,0490027	5,874613189	0,017043752	1,704375243
27,55576253	37,56824645	10,01248392	0,000968149	0,096814857
T Input	T IgG	T Igg-T Input		
28,34489232	38,52636523	10,18147291	0,000861135	0,086113503
28,09345669	38,52636523	10,43290854	0,000723405	0,072340514
28,2191745	38,52636523	10,30719072	0,000789271	0,07892715
T Input	T Met	T Met-T Input		
28,34489232	33,48637577	5,141483446	0,028330828	2,833082754
28,09345669	33,26998208	5,176525388	0,027650984	2,765098361
28,2191745	33,39080956	5,171635055	0,027744872	2,774487176

Statistical evaluation of the results with Student-t-test using KaleidaGraph 4.1.1 software.

Region A
Student t Test for paired data

Group 1: N IgG
Group 2: N Met

	Group 1	Group 2
Count	3	3
Mean	2,23092	0,000632361
Variance	0,06342586	3,02E-08
Std. Dev.	0,25191908	0,000173
Std. Err	0,14544406	0,000100406
Mean Difference	2,23029	
Degrees of Freedom	2	
t Value	15,345	
t Probability	0,00422	

Region B
Student t Test for paired data

Group 1: N IgG

Group 2: N Met

	Group 1	Group 2
Count	3	3
Mean	2,355	1,54007
Variance	0,2164 08	0,23044 1
Std. Dev.	0,4651 97	0,48004 2
Std. Err	0,2685 81	0,27715 3
Mean Difference	0,8149 29	
Degrees of Freedom	2	
t Value	2,8581	
t Probability	0,1037	

Region C

Student t Test for paired data

Group 1: N IgG

Group 2: N Met

	Group 1	Group 2
Count	3	3
Mean	4,8510 4	0,0791271
Variance	37,005 4	4,75E-05
Std. Dev.	6,0832 1	0,00688867
Std. Err	3,5121 4	0,00397718
Mean Difference	4,7719 1	
Degrees of Freedom	2	
t Value	1,3574	
t Probability	0,3075	

Region A

Student t Test for paired data

Group 1: T IgG

Group 2: T Met

	Group 1	Group 2
Count	3	3
Mean	0,42977	2,4988
Variance	3	0,1273
Std. Dev.	0,13517	06
Std. Err	4	0,3567
	0,36766	99
	1	0,2059
	0,21226	98
	9	
Mean Difference	-2,06902	
Degrees of Freedom	2	
t Value	-9,0633	
t Probability	0,01196	

Region B

Student t Test for paired data

Group 1: T IgG

Group 2: T Met

	Group 1	Group 2
Count	3	3
Mean	1,2164	
Variance	3	5,41454
Std. Dev.	0,2702	
Std. Err	74	0,87644
	0,5198	
	79	0,936184
	0,3001	
	52	0,540506
Mean Difference	-4,19812	
Degrees of Freedom	2	
t Value	-17,349	
t Probability	0,0033	
	06	

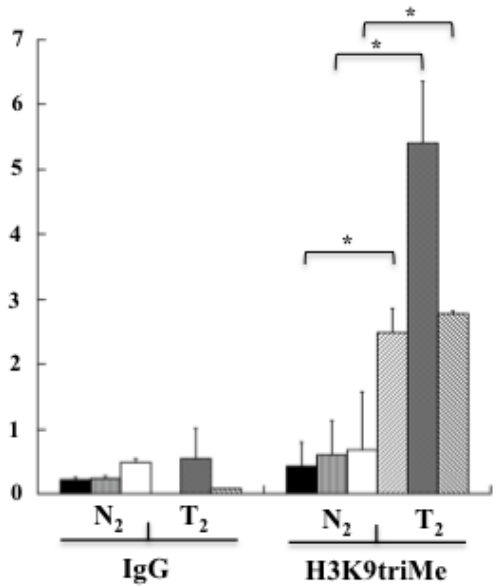
Region C

Student t Test for paired data

Group 1: T IgG

Group 2: T Met

	Group 1	Group 2
Count	3	3
Mean	0,6854	
Variance	37	2,79089
Std. Dev.	0,7849	0,0013572
Std. Err	42	4
	0,8859	
	69	0,0368408
	0,5115	
	15	0,02127
Mean Difference	-2,10545	
Degrees of Freedom	2	
t Value	-4,0245	
t Probability	0,0505	
	5	



Graph of the results included in the Fig. 2 of the thesis

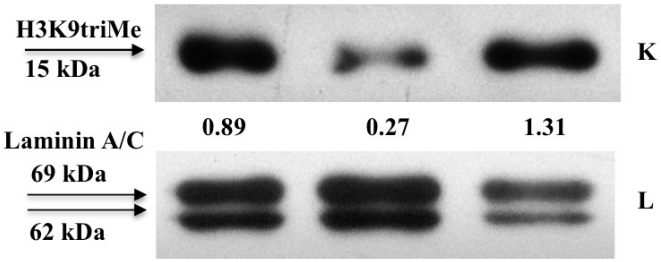
8.2 Example of qRT-PCR calculation for *GKN1* mRNA expression levels performed on AGS cells before and after treatment with TSA

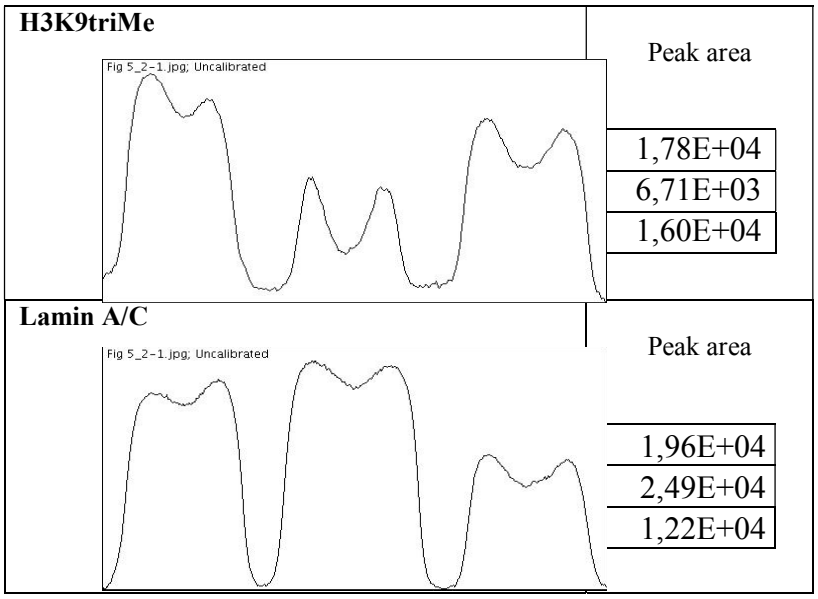
Sample	cDNA2	G6PD	CDNA2/G6PD	Dct	2^{-Dct}
AGS DMSO	35,45140035	23,58581968	11,86558068	0	1
	35,00294388	23,57034208	11,43260179	0	1
	35,07313792	23,68654877	11,38658916	0	1
AGS 24h	33,7267492	26,76284773	6,963901462	-4,901679216	29,89182789
	33,18307826	26,38631757	6,796760685	-4,635841109	24,86149434
	33,00561147	26,52359121	6,482020256	-4,904568901	29,95176054
AGS 48h	36,39986532	32,44236017	3,957505142	-7,908075536	240,1972021
	37,02686188	32,20084112	4,826020765	-6,606581029	97,44937511
	36,34090629	32,24629255	4,094613739	-7,291975418	156,712388

Statistical evaluation of the results with Student-t-test using KaleidaGraph 4.1.1 software.

AGS 24			AGS 48		
Student t Test for paired data			Student t Test for paired data		
Group 1: - TSA			**Group 1: - TSA**		
Group 2: + TSA 24h			**Group 2: + TSA 48h**		
	Group 1	Group 2		Group 1	Group 2
Count	3	3	Count	3	3
Mean	1	28,235	Mean	1	164,786
Variance	0	8,53645	Variance	0	5143,13
Std. Dev.	0	2,92172	Std. Dev.	0	71,7156
Std. Err	0	1,68686	Std. Err	0	41,405
Mean Difference	-27,235		Mean Difference	-163,786	
Degrees of Freedom	2		Degrees of Freedom	2	
t Value	-16,145		t Value	-3,9557	
t Probability	0,003814		t Probability	0,05037	

8.3 Example of WB band densitometry evaluation using ImageJ 1.14o software





WB were analyzed using ImageJ to calculate the ratio of sample band peak areas with that of the corresponding control

Graph of the results included in the Fig. 6 of the thesis

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11. LIST OF PUBLICATIONS

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Book Chapter

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