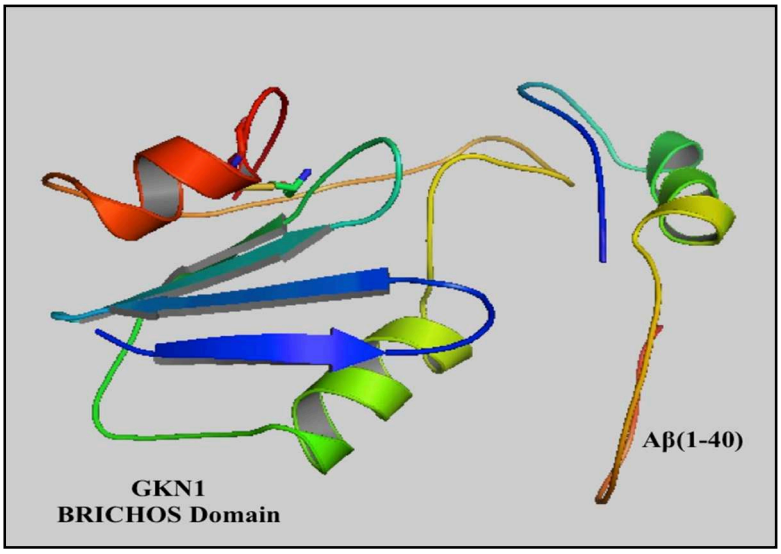
UNIVERSITA' DI NAPOLI FEDERICO II

DOTTORATO DI RICERCA
BIOCHIMICA E BIOLOGIA CELLULARE E MOLECOLARE
XXVI CICLO

Filomena Altieri

Does gastrokine 1 play a major role in gastric tissue?



Academic Year 2012/2013

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RINGRAZIAMENTI

Queste righe racchiudono tutta l'emozione che accompagna la fine di questo mio percorso di dottorato…un percorso a volte in salita, a volte meno…un percorso di sfida con me stessa che mi ha portato a crescere a livello umano e professionale…un percorso in cui ci siete anche voi ed è per voi questo GRAZIE!

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SUMMARY

Gastrokine1 (GKN1) is a stomach-specific 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. The protein is characterized by the presence of a BRICHOS domain consisting of about 100 amino acids found in several unrelated proteins associated with major human diseases like BRI2, related to familial British and Danish dementia; Chondromodulin-I (ChM-I), linked to chondrosarcoma; Lung Surfactant Protein C (SP-C), associated with respiratory distress syndrome. Literature data show that recombinant BRICHOS domains from BRI2 and SP-C precursor (proSP-C) prevent fibrils formation of amyloid-beta peptide (Aβ) that is the major component of extracellular amyloid deposits in Alzheimer's disease. Aβ derives from the partial hydrolysis of the amyloid precursor protein (APP) catalyzed by β-and γ-secretase. The hydrolysis produces amyloid peptides of 40 (about 90%) or 42 (about 10%) amino acid residues.

Main target of this thesis is to investigate the interaction between recombinant human GKN1 (rhGKN1) and Aβ(1-40) to explore whether also GKN1 was endowed by a chaperone activity toward amyloidogenic peptides. To this aim, Aβ was incubated in presence or absence of rhGKN1 and chicken cystatin, as negative control, at 10:1 molar ratios. Samples were then analyzed by SDS-PAGE. The aggregation of Aβ was also evaluated using Thioflavine T binding assay. Mass spectrometry and size-exclusion chromatography analysis were performed to characterize the interaction. The data obtained showed that rhGKN1 prevented amyloid aggregation and fibrils formation by inhibiting Aβ(1-40) polymerization. Surface plasmon resonance analysis of rhGKN1/Aβ interaction led to calculate a dissociation constant (K_D) of 34 μM. These preliminary data strongly indicated that rhGKN1 possess anti-amyloid activity thus, it might play a role of molecular chaperone directed against unfolded segments with an ability to recognize amyloidogenic polypeptides and prevent their aggregation.

Regarding the mechanism by which *GKN1* gene is inactivated in gastric cancer, this remains still unknown; so the second part of my work is focused to clarify the causes of *GKN1* gene silencing and to determine if epigenetic mechanisms, such as histonic modifications, could also contribute to its down-regulation. To this aim, chromatin immunoprecipitation assays (ChIP) for H3K9triMe and its specific HMT SUV39H1 were performed on different samples from biopsies of normal and tumor human gastric tissue. The results showed that GKN1 down-regulation 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.

RIASSUNTO

La Gastrochina1 (GKN1) è una proteina stomaco-specifica importante per il mantenimento della funzione fisiologica della mucosa gastrica. La GKN1 è down-regolata nei tessuti tumorali gastrici e linee cellulari derivate, per cui è recentemente emersa come potenziale marcatore per il cancro gastrico. La proteina è caratterizzata dalla presenza di un dominio BRICHOS costituito da circa 100 amminoacidi e trovato in diverse proteine, non correlate tra loro, associate alle principali patologie umane come BRI2, coinvolta nella demenza familiare britannica e danese; la Condromodulina-I (ChM-I), collegata al condrosarcoma; la Proteina C del Surfattante Polmonare (SP-C) associata alla sindrome da distress respiratorio. Dati di letteratura mostrano che il dominio BRICHOS ricombinante di BRI2 e del precursore della SP-C (proSP-C) previene la formazione delle fibrille del peptide beta-amiloide (Aβ) che è il maggiore componente dei depositi amiloidi extracellulari nel morbo di Alzheimer. Aβ deriva dall'idrolisi parziale della proteina precursore dell'amiloide (APP) catalizzata dalle β-e γ-secretasi. L'idrolisi produce peptidi amiloidi di 40 (circa il 90%) o 42 (circa il 10%) residui amminoacidici.

Obiettivo principale di questa tesi è quello di indagare l'interazione tra la GKN1 umana ricombinante (rhGKN1) ed Aβ(1-40) per valutare se anche la GKN1 sia dotata di un'attività da chaperone mirata contro peptidi amiloidogenici. A questo scopo, Aβ è stato incubato in presenza ed assenza di rhGKN1 e cistatina di pollo, usata come controllo negativo, in un rapporto molare di 10:1. I campioni sono stati poi analizzati su SDS-PAGE. L'aggregazione di Aβ è stata valutata anche attraverso il saggio di legame alla Tioflavina T. Per caratterizzare l'interazione sono state effettuate analisi di spettrometria di massa e di cromatografia ad esclusione di massa. I dati ottenuti hanno mostrato che rhGKN1 previene l'aggregazione dell'amiloide e la formazione delle fibrille inibendo la polimerizzazione di Aβ(1-40). Analisi dell'interazione rhGKN1/Aβ svolte mediante risonanza plasmonica di superficie hanno permesso di calcolare una costante di dissociazione (K_D) DI 34 μM. Questi risultati preliminary indicano fortemente che rhGKN1 possiede un'attività anti-amiloide per cui, potrebbe svolgere un ruolo di chaperone molecolare

diretto contro segmenti non correttamente ripiegati, con l'abilità di riconoscere polipeptidi amiloidogenici e prevenirne l'aggregazione.

Resta ancora da chiarire il meccanismo attraverso il quale il gene della *GKN1* viene inattivato nel cancro gastrico. La seconda parte del mio lavoro è stata volta a chiarire le cause del silenziamento del gene della *GKN1* e a determinare se ci siano anche meccanismi epigenetici, come le modifiche istoniche, a contribuire alla sua down-regulation. A tale scopo sono stati effettuati saggi di immunoprecipitazione della cromatina (ChIP) su differenti campioni derivanti da biopsie di tessuto umano gastrico normale e tumorale, immunoprecipitando per la modifica istonica H3K9triMe e per la sua HMT specifica SUV39H1. I risultati hanno mostrato che il decremento della GKN1 in tessuti tumorali gastrici è associato ad alti livelli di H3K9triMe ed al reclutamento di SUV39H1 sul promotore della GKN1, suggerendo l'intervento di un complesso trascrizionale epigenetico a regolare negativamente l'espressione della GKN1 nel cancro gastrico.

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INTRODUCTION

1.1 Gastrokine 1

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 mammals species (1, 2). The gene coding for GKN1 (*CA11*) (accession number: BK0017373), is located in a 6 kb region of the chromosome 2p13 and contains 6 exons. Sequence analysis of the gene showed that the human transcript contains two potential translation start sites (ATG). The first start codon would generate a protein of 199 amino acids whereas the second ATG, located 42 bp downstream, would make a protein of 185 amino acids. Among the two starting sites, the second appears more possible since it contains a Kozak sequence (GCAGCCAACATG). Comparison of the translated amino acid sequence of human GKN1 with that of other species showed homology only after the second ATG, and its product is predicted to be of 18 kDa. In addition, amino acid sequencing of native GKN1 from pig and N-terminal Edman's degradation of native human GKN1 confirmed that the protein was made of 185 amino acids containing a 20 amino acid extracellular signal peptide localized in the N-terminal region (Fig. 1) (1). The human protein is expressed in the stomach of healthy individuals but is absent in gastric adenocarcinoma tissues (1-3). Moreover, our research team observed that the protein is down-regulated in samples from *H. pylori* infected gastric mucosa that is considered as one of the leading cause for gastric cancer development (4, 5). The biological function of GKN1 is currently unknown, some findings indicate that the protein is involved in the replenishment of the surface lumen epithelial cell layer, in maintaining mucosal integrity and could play a role in cell proliferation and differentiation (6, 7). After injury of the gastric mucosa, restoration may occur very rapidly in the presence of GKN1 (6). 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, probably the protein may exert its protective effect by

increasing accumulation of specific tight and adherens junction proteins and also protecting their loss after injury (8, 9). We also has been studying the effect of GKN1 on gastric cancer cell lines (AGS and MKN28). We found that overexpression of GKN1 in these cell lines stimulated the expression of Fas receptor. Moreover, compared to control cells, a significant increase of apoptosis, evaluated by TUNEL, was observed when GKN1 transfected cells were treated with a monoclonal antibody (IgM) anti-Fas. The activation of Fas expression was also observed by the overexpression of GKN1 in other cancer cell lines (10).

In addition, in order to characterize the structural and functional properties of this protein, we produced a recombinant human GKN1 (rhGKN1) and we tested it on normal and tumor cells trought a MTT assay which showed that the protein reduced cell proliferation of gastric cancer cells (AGS) compared to human embryonic kidney cells (HEK 293) and non-gastric cancer cells (H1355) (11). These data suggest that GKN1 was able to exert its function as modulator of apoptotic signals specifically in gastric cancer suggesting a possible role of the protein as tumor suppressor, moreover GKN1 may play also an important role as biomarker in carcinogenic process. In fact, it was seen that individuals with a lower expression of the protein have an increased risk to develop gastric diseases.

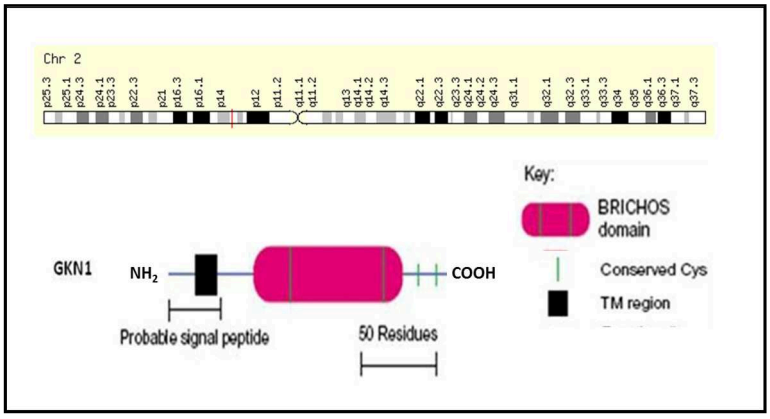


Figure 1. GKN1 genomic location and GKN1 human transcript. (**Upper panel**) Chromosome bands according to Ensembl and genomic location according to GeneLoc. (**Lower panel**) GKN1 protein structural prediction (12).

1.2 GKN1 and amyloidogenic property of BRICHOS domain

GKN1 contains a conserved central structural BRICHOS domain of about 100 amino acids containing two conservative cysteine residues most likely involved in disulfide bridges (Fig. 2). The BRICHOS domain has been found in proteins with a wide range of functions and disease associations and, in particular, the acronym "*BRICHOS*" refers to three proteins in which the domain was observed originally: BRI2, which is expressed in neurons and related to familial British and Danish dementia (FBD and FDD); Chondromodulin-I (ChM-I), a cartilage-specific glycoprotein related to chondrosarcoma and Lung Surfactant Protein C (SP-C), related to respiratory distress syndrome (RDS) (12). The putative association of GKN1 with such domain structure, and with at least three different possible functions, has been proposed, but not yet been conclusively demonstrated. Recent insights indicate that the tumor suppressor role of GKN1 might be ascribed to the presence of the BRICHOS domain. In fact, it has been found that the NH_2-terminal hydrophobic region (signal peptide) and BRICHOS domain of GKN1 were shown to suppress gastric cancer cell growth and recapitulate protein functions. The paper showed that the hydrophobic region and BRICHOS domain had a synergistic anti-cancer effect with 5-FU on tumor cell growth in contrast to what observed for mutants as control, implying that the NH_2-terminal region and BRICHOS domain of GKN1 are sufficient for tumor suppression (13).

Although GKN1 seems to play an important role in gastric mucosa and in carcinogenic process, a full characterization of its structural and biological activities is still lacking. To accomplish this deficiency, we have recently reported the characterization and secondary structural properties of recombinant human GKN1 (rhGKN1) (11). Using bio-informatics tools, we found that GKN1 BRICHOS domain showed structural features similar to those endowed by BRICHOS domain containing protein family. In particular, GKN1 3D model showed that its BRICHOS domain displayed a structural organization resembling that of the corresponding BRICHOS domain of Surfactant Protein C precursor (proSP-C), a transmembrane (TM) protein expressed in epithelial type II cells that contains a discordant helix forming the TM region. The proSP-

C α-helix can misfold and form amyloid fibrils associated with pulmonary disease. It has also been shown that the C-terminal domain (CTC) of proSP-C and its BRICHOS domain protects the TM part of the protein from aggregation into amyloid.

Recent literature data show that recombinant BRICHOS domains from BRI2 and proSP-C prevent fibrils formation of amyloid-beta peptide (Aβ) that is the major component of extracellular amyloid deposits in Alzheimer's disease (14-16). The Aβ peptide originates from the amyloidogenic processing of the amyloid precursor protein (APP), a type I ubiquitary membrane protein that undergoes a first cleavage by β-secretase to liberate the soluble APP domain (sAPP) in the extracellular space, and the membrane bound APP-C99 fragment (CTF). Then, APP-C99 is processed by the intramembrane aspartyl-protease gamma-secretase (γ-secretase) composed of four subunits (presenilin, nicastrin, anterior pharynx-defective protein 1 and presenilin enhancer 2) to release Aβ peptides (40 or 42 residues) in the lumen, and the APP intracellular domain (AICD) in the cytosol. Alternatively, APP can undergo the non-amyloidogenic processing, in which it is first cleaved by α-secretase to generate the sAPPα domain and the APP-C83 fragment (CTF). The latter is further cleaved by γ-secretase into the nontoxic p3 peptide and the AICD.

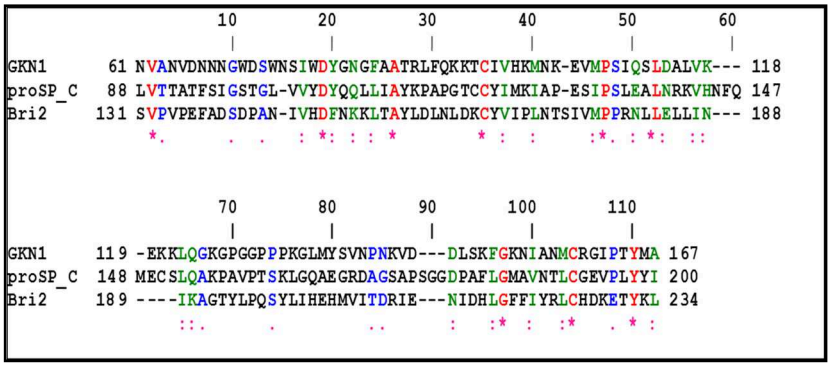


Figure 2. Amino acid sequences alignment of GKN1, proSP-C and BRI2 BRICHOS domain. The alignment was made with ClustaW. Asterisks and double dots mark identical residues and conservative replacements, respectively.

1.3 Gene-environment interaction in gastric cancer: epigenetics and GKN1

Gastric cancer 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.

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. Recent evidences indicate that epigenetic modifications play a central role in gastric carcinogenesis and novel therapeutic approaches that target DNA methylation and histone modifications have emerged. A greater understanding of epigenetics and the therapeutic potential of intervention into these processes is necessary to help gastric cancer treatment (17). At present, the major components of epigenetic regulatory network include DNA methylation, histone modifications and non-coding RNAs, mainly microRNAs. DNA methylation is introduced by addition of a methyl group to the fifth carbon of a cytosine pyrimidine ring of DNA, which typically occurs in a CpG dinucleotide. In normal cells, approximately 80% of all CpGs are methylated. DNA of cancer cells is generally hypomethylated, while promoters of certain genes are hypermethylated, both of which are implicated in carcinogenesis. Promoter-specific increased methylation leads to silencing of the affected genes that may function as tumor suppressors and result in heritable transcriptional silence. Aging and chronic inflammation can induce methylation in CpG islands. Recent studies have described a number of genes that are silenced by hypermethylation in gastric cancer association with H. pylori or EBV infection including: cell growth-related genes p16(INK4a), p14(ARF) and APC; DNA-repair genes, hMLH1, BRCA1 and MGMT; the cell adherence gene E-cadherin; as well as LOX, FLNC, HRASLS, HAND1, THBD and p41ARC.

Recent advances underscore the importance of histone

modifications in the pathogenesis of gastric cancer. Histones are the basic unit of the nucleosome, consisting of two copies of each of the core histones, H2A, H2B, H3 and H4. H3 and H4 histones have long tails protruding from nucleosome that can be covalently modified. This allows regulatory proteins to access DNA and regulate transcription. Modifications of histone tails include methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, citrullination and ADP-ribosylation. 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 and silenced or activated when methylated by histone methyltransferases (HMT). Both histone H3 lysine K9 and H3K27 trimethylation are associated with gene silencing, while histone H3 lysine 4 is associated with active genes (18). Recent studies identified candidate genes with significant differences in H3K27triMe in gastric cancer samples compared to adjacent non-neoplastic gastric tissues. These genes included oncogenes, tumor suppressor genes, cell cycle regulators, and genes involved in cell adhesion. Moreover, higher levels of H3K27triMe produce gene expression changes in MMP15, UNC5B, and SHH. LAMB3 and LAMC2 gene are overexpressed in gastric cancer samples in comparison with non-neoplastic adjacent tissue samples. Overexpression of these genes is a result of the enrichment of H3K4TtriMe in the gene promoter. Park *et al.* showed that higher levels of global H3K9triMe, which is a repressive mark, was associated with higher T stage, lymphovascular invasion, and recurrence in gastric tumors. 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. These results have suggested that histone methylation results in a worse prognosis by inactivating certain tumor suppressor genes. Moreover, Li *et al.* used gastric cancer cell lines to demonstrate that the PRC1 member CBX7 initiated trimethylation of H3K9 at the p16 locus through recruitment and/or activation of the HMT SUV39H1 to the target locus. This finding links two repressive epigenetic landmarks, H3K9triMe

formation and PRC1 binding within the silenced domains in euchromatin, and builds up a full pathway for epigenetic inactivation of p16 by histone modifications. Histone hypoacetylation of the HLTF gene are linked to its reduced expression in gastric cancer. HLTF is a homolog to the SWI/SNF genes, which encode chromatin-remodeling enzymes and serves as a tumor suppressor. 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. Aberrant histone deacetylation are also linked to the silencing of the SLC5A8 gene in gastric cancer. SLC5A8 (solute carrier family 5, member 8) is a sodium co-transporter and a putative tumor suppressor (19).

The mechanism by which *GKN1* gene is silenced in gastric cancer and if epigenetic changes could also contribute to silencing *GKN1* gene remains still unknown. Recently, Hwan Yoon *et al.* investigated this aspect in a sample group of 81 gastric carcinomas and 40 gastric adenomas: no mutation was detected in gastric tumors, hyper-methylation of GKN1 gene promoter was found only in two tumors, while DNA copy number and mRNA levels of GKN1 were significantly decreased in all gastric cancer samples (20). Another paper investigated Epstein-Barr Nuclear Antigen 1 (EBNA1) binding and epigenetic regulation of GKN1 in gastric carcinoma cells: Epstein-Barr Virus (EBV) latently infects ~10% of gastric carcinomas (GC). Epstein-Barr Nuclear Antigen 1 (EBNA1) is expressed in EBV-associated GC, and can bind host DNA, where it may impact cellular gene regulation. The authors have been shown that EBNA1 binds directly to DNA upstream of GKN1 gene. They generated AGS-EBV, and AGS-EBNA1 cell lines to study the effects of EBNA1 on GKN1 mRNA expression with or without 5' azacytidine treatment. Results shown that GKN1 is transcriptionally silenced by DNA methylation and latent EBV infection further reduces GKN1 expression in AGS gastric carcinoma cells, and that siRNA depletion of EBNA1 partially alleviates this repression. However, ectopic expression of EBNA1 slightly increased GKN1 and GKN2 basal mRNA levels, but reduced their responsiveness to demethylating agent. These findings demonstrate that EBNA1 binds to the divergent promoter of the GKN1

gene in gastric cancer cells, and suggest that EBNA1 contributes to the transcriptional complex and epigenetic deregulation of the GKN1 tumor suppressor gene in EBV positive gastric cancer (21). In conclusion, these studies indicate that epigenetic modifications could be involved in the deregulation of *GKN1* gene in gastric cancer. However, in literature there are not yet investigations for histone modifications and for the recruitment of histone-modifier enzymes and co-repressors on *GKN1* gene in gastric cancer. Under this aspect, it would be important to clarify the epigenetic causes of *GKN1* gene silencing associated to gastric cancer and to determine whether this event might be involved in the development and progression of gastric cancer. Additional functional and translational studies of GKN1 will broaden our understanding of the pathogenesis of gastric cancer, and provide us with novel diagnostic and therapeutic modalities for the disease.

1.4 Scientific hypothesis and aim of the work

On the basis of this data and in order to better understand the functional role of GKN1, the main target of this thesis was to explore whether also GKN1 was endowed by a chaperone-like activity toward amyloidogenic peptides. To this aim it was analyzed using biochemical, spectroscopic and mass spectrometry investigations how rhGKN1 interacts with and prevents fibril formation of Aβ(1-40), derived from the TM part of its precursor protein APP.

The second part of the work aimed to clarify the causes of *GKN1* gene inactivation and to determine if the epigenetic mechanism of histone modifications could also contribute to regulate the gene expression.

MATERIALS AND METHODS

2.1 Materials

Amyloid β-peptide (1-40) (DAEFRHDSGYEVHHQKLVFFAEDVGS NKGAIIGLMVGGVV) was purchased from Abnova and stored as lyophilized powder at -80 °C until its use. To obtain monomeric starting solutions, the peptide was dissolved in dimethyl sulfoxide (DMSO) (Merck) at a concentration of 138.5 μM before being diluted in experimental buffer. Chicken cystatin was purchased from Sigma-Aldrich and dissolved at a concentration of 76.8 μM before being diluted in experimental buffer.

2.2 GKN1 expression and Purification

Recombinant human GKN1 (rhGKN1) was expressed and purified as described previously (11). Stock solutions with concentration of 313.3 μM were used for the experiments.

2.3 Aβ(1-40) aggregation and fibrils formation

Aβ(1-40) was dissolved in DMSO at a concentration of 138.5 μM. Experiments were performed by co-incubating Aβ(1-40) (17.3 μM) with rhGKN1 (1.7 μM) at 37 °C in 10 mM sodium phosphate buffer (NaP) pH 7.0 and 150 mM sodium chloride (NaCl) containing 10% (v/v) DMSO under agitation. At various time points, samples were removed to determine the level of aggregation. The samples were centrifuged for 6 minutes at 14000 rpm and the supernatants were removed and centrifuged for an additional 2 minutes at the same speed. The supernatant from the last centrifugation was then analyzed by SDS-PAGE on 16% Tris-Tricine gels under nonreducing conditions and stained with Coomassie. As control, Aβ(1-40) was incubated with 1.7 μM chicken cystatin in the same manner as described above (22).

2.4 Thioflavin T assay for Aβ(1-40) aggregation

Thioflavin T binding was assessed on Aβ(1-40) incubated in the presence or absence of rhGKN1 at 5:1 molar ratio in 10 mM NaP pH 7.0 and 150 mM NaCL 10% (v/v) DMSO at 37 °C under agitation. At various time

points, 100 µL aliquots were removed and 160 µL of Thioflavin T 50 µM were added in 10 mM NaP pH 7.0 and 150 mM NaCl. A sample with rhGKN1 alone was also included. The samples were incubated for 5 minutes in the dark before fluorescence was measured. The wavelengths for excitation and emission were 450 and 482 nm, respectively. Each sample was measured in duplicate.

2.5 MALDI-TOF mass spectrometry

MALDI-MS experiments were performed in positive linear reflectron mode on a MALDI-TOF micro MX (Waters Co.), equipped with a pulsed nitrogen laser (λ=337 nm). The instrument was calibrated using a three-point external calibration using a mixture (10 pmol/mL) of trypsinogen (24 kDa), cytochrome C (12 kDa) and insulin (5.8 kDa) as standard proteins (Sigma-Aldrich) using a polynomial equation, as suggested by the manufacturer. All spectra were processed and analyzed using the MassLynx 4.0 software. The instrument source voltage was set to 12 kV. The pulse and detector voltages were optimized at 1999 V and 5200 V, respectively. Measurements were performed in the mass range m/z 3000-30000 with a suppression mass gate set to m/z 1000 and an extraction delay of 600 ns. Data were recorded by accumulating and averaging at least 10 spectra randomly acquired over the well surface. After averaging, spectra were processed for peak smoothing. To observe at best the rhGKN1•Aβ(1-40) complex, we performed scouting experiments by changing the amount and type of matrices and the protein/peptide ratio. The complex was observed at best using sinapinic acid (solution 10 mg/mL in ethanol/NH_4HCO_3 at 1:1 molar ratio, v/v) as the ionizing matrix and at 1:1 molar ratio of rhGKN1:Aβ. Optimized spectra were therefore acquired on a solution containing 10 pmol/µL rhGKN1 in 5 mM NaP and 10 pmol/µL Aβ in 1% DMSO. All spectra were acquired by spotting 1 µL of matrix solution on the target plate dried at room temperature. Then, protein/peptide samples (1 µL) were applied on top of the matrix crystal layer and dried again. A second matrix layer (1 µL), was formed on top of the sample layer preparation resulting in a matrix-sample-matrix sandwich. Spectra were collected after complete solvent evaporation. MALDI-MS experiments were also performed separately on

10 pmol/μL rhGKN1 and Aβ peptide solutions, under the same conditions.

2.6 Size-Exclusion Chromatography (SEC)

SEC was performed on an AKTA Purifier System using a Superdex 75 prepacked gel filtration column for high-resolution (GE Healthcare). The column was equilibrated and eluted at a flow rate 0.5 mL/min with 100 mM NaCl and 50 mM NaP pH 7.0 and the absorbance at 280 nm was recorded. The elution volumes of the following standard proteins were used for column calibration: bovine serum albumin (BSA) (66 kDa), carbonic anhydrase (30 kDa) and cytochrome C (12 kDa). rhGKN1 (30 μg in 10 mM NaP pH 7.0 and 150 mM NaCl 10% (v/v) DMSO) was first loaded into the column alone and then the various fractions (tetramer, dimer and monomer) were incubated in the presence of Aβ(1-40) in a 1:3 molar ratio. Before loading, all the samples were incubated at 37 °C for 1 hour.

2.7 Surface Plasmon Resonance analysis

Surface plasmon resonance (SPR) measurements were performed using a Biacore 3000 instrument (GE Healthcare). Aβ peptide (1-40) was covalently immobilized to the dextran matrix of a CM5 sensor chip via the primary amine groups (amine coupling kit; GE Healthcare). The carboxymethylated dextran surface was activated by the injection of a mixture of 0.2 M N-ethyl-N'-(diethylamino-propyl)carbodiimide and 0.05 M N-hydroxysuccinimide (EDC/NHS chemistry) according to the manufacturer's instructions (23). The immobilization of ligand was efficiently performed at 15.0 μg/mL in 10 mM sodium acetate buffer (NaOAc) pH 4.0 containing 2.5% DMSO deriving from the peptide stock solution. The remaining N-hydroxysuccinimide esters were blocked by injection of 1 M ethanolamine hydrochloride (O-AEA) pH 8.5. Reference channel was prepared and used as control blank. All immobilization steps were performed at a flow rate of 5 μl/min using 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% P20 (HBS-EP, GE Healthcare) (pH 7.4) as running buffer. rhGKN1 was injected at different concentrations ranging from 7.5 μM to 130 μM. After each injection the surface was regenerated

with pulses of a solution of 10 mM sodium hydroxide (NaOH). The activity of the immobilized peptide was not affected by the regeneration conditions employed and the chip were reusable to achieve two independent sets of binding experiments. Analyses were performed at 25 °C at a flow rate of 20 µl/min in HBS-EP buffer (GE Healthcare). In all binding experiments, association phases ran for 180 seconds and dissociation phases for 300 seconds. Non-specific binding from the reference channel was subtracted from the working channels using the BIAevaluation analysis package (version 4.1, GE Healthcare). Data were fitted using the software GraphPad Prism 5, versus 5.0 (GraphPad Software).

2.8 Cell culture and transfection

Human gastric adenocarcinoma cell line (AGS) was grown in DMEM-F12 (Dulbecco's modified Eagle medium-Cambrex) supplemented with heat inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine at 37 °C in a 5% CO_2 atmosphere. Cells (250000/well) were seeded into six well plates 24 hours prior to transfection in DMEM-F12 antibiotics free (2 ml) and then transfected with 4 µg of vector (pcDNA 3.1 or pcDNA-GKN1) by using lipofectamine 2000 (LF2000) according to the manufacturer's instructions (Invitrogen). The efficiency of transfection of gastric cancer cells with GKN1 was always evaluated by a parallel transfection using EGFP vector as control. In general, after transfection, the average value of the ratio green fluorescent cells/total number of cells was of 0.5±0.1. As control, untransfected cells were treated with 2 µM DAPT or only DMSO for 24 hours.

2.9 Cytosolic extracts and Western blot analysis

Cells were trypsinized, washed twice in cold phosphate buffered saline (PBS) and resuspended in 20-40 µl of lysis buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 0.25% NaDC, 150 mM NaCl, 1 mg/ml aprotinin, leupeptin, pepstatin, 1 mM Na_3VO_4, 1mM NaF) for 30 minutes on ice and centrifuged at 14000 rpm for 20 minutes at 4°C. Protein concentration was determined by a modified Bradford method (24), using the Bio-Rad (Milan, Italy) protein assay and compared with BSA standard curve.

Equal amounts of cytosolic proteins (70 µg) were separated by SDS–PAGE, electrotransferred to PVDF membrane and reacted with the specific antibody anti-Amyloid Precursor Protein (Sigma-Aldrich). Immunoblots were visualized using HRP-conjugated secondary antibodies and ECL Western blot detection kit (GE Healthcare). All films were analyzed by using Image J software.

2.10 Pull-Down

Pull-down of GKN1 was performed using Ni-NTA agarose (Qiagen). Cell extracts (500 µg) were incubated with equal amounts of pre-equilibrated resin (200 µl slurry/1 mg protein extract) over night at 4 °C. After the incubation, the resin was washed two times (centrifugation for 2 minutes at 2000 rpm and 4 °C) in 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole to reduce nonspecific bound proteins, resuspended in 10 µl of 2× Laemmli loading buffer, heated to 95 °C for 5 minutes and subjected to Western blot analysis as described above.

2.11 mRNA isolation and qRT-PCR

Total RNA was extracted from normal and cancer human tissue samples using TRIzol reagent solution (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using the reverse transcription kit from Roche Molecular Systems (Roche) according to the manufacturer's protocol. GKN1 cDNA was amplified by qRT-PCR using forward and reverse primers 5′-CTTTCTAGCTCCTGCCCTAGC-3′ and 5′-TGGTTGCAGCAAAGCCATTT-3′, respectively, according to standard procedures (Applied Biosystems). qRT-PCR was performed with the SYBR Green PCR MasterMix (Applied Biosystems) under the following conditions: 10 minutes at 95 °C, followed by 40 cycles (15 seconds at 95 °C and 1 minute at 60 °C). Each reaction was performed in duplicate. We used the $2^{-\Delta\Delta CT}$ method to calculate the relative expression levels (25).

2.12 Chromatin Immunoprecipitation assay

Samples from normal and cancer human tissues were processed for chromatin immunoprecipitation 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 PIPES pH 8.0, 85 mM KCl, 0.5% NP-40) plus 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 supernatant obtained was the chromatin. Samples were subjected to IP with the following specific antibodies against istonic modification anti-tri methyl K9-Histone3 (Abcam) and against the specific histone methyltransferase anti-SUV39H1 (Millipore). For qPCR, 3 µl of 150 µl IP DNA were amplified with a set of three pairs of primers covering a promoter region from -427bp to +91bp around the transcriptional start site on *GKN1* gene.

Promoter Region:

A *Forward*: 5′-GGGGTAGGTTTGGTGGGAGTTGC-3′
Reverse: 5′-ATCACAGCTGAAAAGCCACGTGTA-3′

B *Forward*: 5′-CGCCCACAGCTTTGACTGGGT-3′
Reverse: 5′-TGCCATGAGCCAGTGTACCAGGA-3′

C *Forward*: 5′-TCCTGGTACACTGGCTCATGGCA-3′
Reverse: 5′-AGCAGTGGACAGAGGAGTAGGCA-3′

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.

RESULTS

Part A. Interaction between recombinant human GKN1 and the amyloid peptide Aβ(1-40)

3.1 rhGKN1 prevents the aggregation and fibril formation of Aβ(1-40)

SDS-PAGE was first utilized to study the effects of rhGKN1 on Aβ aggregation. Aβ(1-40) peptide was incubated with rhGKN1 and with the control protein chicken cystatin at 10:1 molar ratios. As reported in Fig. 3A, at zero time incubation, the amounts peptide was quite similar in all samples whereas, compared to the peptide alone (left panel) or the peptide incubated in presence of chicken cystatin (middle panel), no reduction in the amount of Aβ was observed up to 25 hours when the peptide was incubated in the presence of rhGKN1 (right panel). Similar results were also observed after 7 days incubation (Fig. 3B). In fact, a drastic reduction of the amount of Aβ was observed for the samples corresponding to Aβ alone (left panel) or the sample co-incubated with chicken cystatin (middle panel). The sample containing rhGKN1 (right panel) showed, instead, almost similar amounts of soluble Aβ as observed at time zero incubation. These results suggest that rhGKN1 was able to prevent Aβ aggregation.

The ability of rhGKN1 to prevent Aβ peptide aggregation was also analyzed by Thioflavin T binding experiments. As reported in Fig. 4, compared to Aβ peptide alone, rhGKN1 was able to prevent the polymerization of Aβ(1-40).

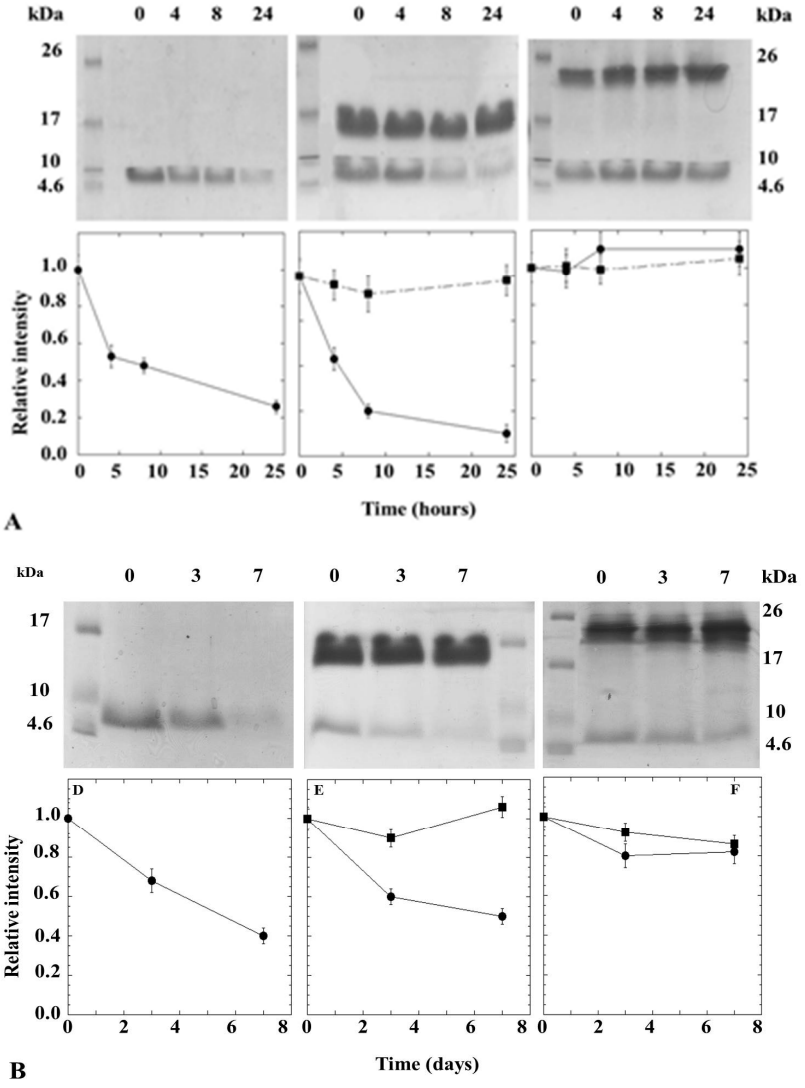


Figure 3. rhGKN1 prevents aggregation of Aβ(1-40). (**A**) 17.3 μM Aβ(1-40) incubated from 0 up to 24 hours alone in the absence or in the presence of 1.7 μM chicken cystatin (middle panel) or 1.7 μM rhGKN1 (right panel) and (**B**) 17.3 μM Aβ(1-40) incubated from 0 up to 7 days in the absence or in the presence of 1.7 μM chicken cystatin (middle panel) or 1.7 μM rhGKN1 (right panel). All incubations were performed in 10 mM sodium phosphate buffer (pH 7.0) and 150 mM sodium chloride with 10% (v/v) DMSO.

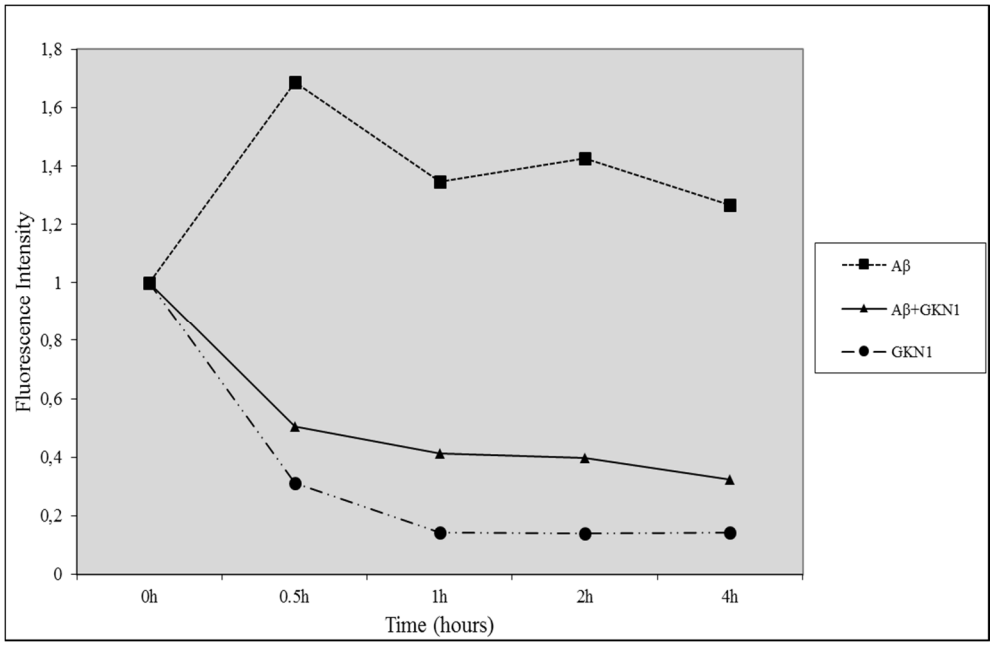


Figure 4. rhGKN1 inhibits Aβ(1-40) polymerization. Thioflavin T fluorescence binding was assessed at different times of incubation (0-4 hours) of 8.5 μM Aβ(1-40) alone or in the presence of 1.7 μM rhGKN1. A sample of 1.7 μM rhGKN1 alone was also included. Experiments were performed in duplicate with similar results.

3.2 rhGKN1 forms a complex with Aβ(1-40)

The putative interaction of rhGKN1 with β-amyloid peptide was investigated by MALDI-MS under non-denaturing conditions (Farmer TB, 1998). To observe at best the rhGKN1•Aβ(1-40) complex, MS analyses were performed under different experimental conditions by changing the amount and type of matrices and the protein/peptide ratio. The optimized conditions for detecting the complex included the use of sinapinic acid (solution 10 mg/mL in ethanol/NH4HCO3 at 1:1 molar ratio, v/v) as ionizing matrix and a 1:1 molar ratio of rhGKN1:Aβ, as described in the Methods section. The MS spectrum of Aβ(1-40) is shown in Fig. 5A. Only a signal corresponding to the peptide (m/z 4331.0, theoretical average [M+H]+= 4330.8 Da) was detected. Similarly, when rhGKN1 was analysed in isolation (Fig. 5B), a peak corresponding to the monomeric protein was expectedly observed at m/z 19406.0 (theoretical average [M+H]+= 19408.6 Da). By analysing the mixture of rhGKN1 and Aβ (Fig. 5C), an ion peak at m/z 23734.7 was clearly revealed, attesting the occurrence of a 1:1 rhGKN1•Aβ(1-40) complex (theoretical average [M+H]+= 23738.45 Da).

Size-Exclusion Chromatography (SEC) was then employed to better characterize the interaction between rhGKN1 and β-amyloid peptide (Fig. 6). The 280 nm absorbance elution profile for rhGKN1 fractions (tetramer, dimer and monomer), individually incubated in the absence (black line) and in the presence (red line) of Aβ(1-40), showed the tendence of the latter to dissociate the aggregated forms of the protein and to promote the monomeric form. It must be observed that the chromatographic profile did not show the presence of a peak corresponding to the rhGKN1•Aβ complex probably because, due to the low affinity, it dissociated during the chromatographic separation.

The possible interaction between rhGKN1 and Aβ(1-40) was also analysed using a bioinformatic approach such as that of generating a docking model. To this purpose, the pdb files corresponding to GKN1 BRICHOS domain and Aβ(1-40) were submitted at GRAMM-X Protein-Protein Docking Web Server v.1.2.0 (26, 27). The pdb GKN1 BRICHOS domain file was generated at Swiss-Model Server (28) using as template the recent crystal structure of pro-SP-C BRICHOS domain, Protein Data

Bank code 2yad (16) whereas the 2LFM.pdb file corresponded to the NMR structure of Aβ(1-40) (29). As illustrated in Figure 7, the docking model obtained supports the formation of a possible complex between GKN1 BRICHOS domain and Aβ(1-40).

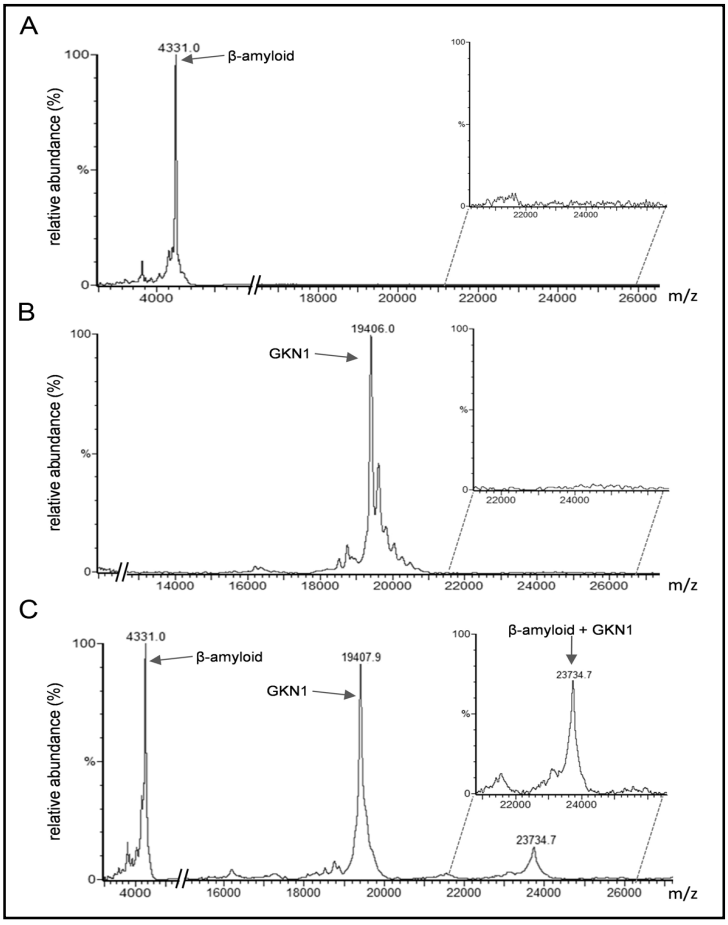


Figure 5. rhGKN1 forms a complex with Aβ(1-40). (**A**) MALDI-TOF spectra of Aβ; (**B**) rhGKN1 and (**C**) rhGKN1 in the presence of Aβ. A magnification of the ion peak at m/z 23734.7 corresponding to the 1:1 rhGKN1•Aβ(1-40) complex (theoretical average [M+H]+= 23738.45 Da) is reported in C.

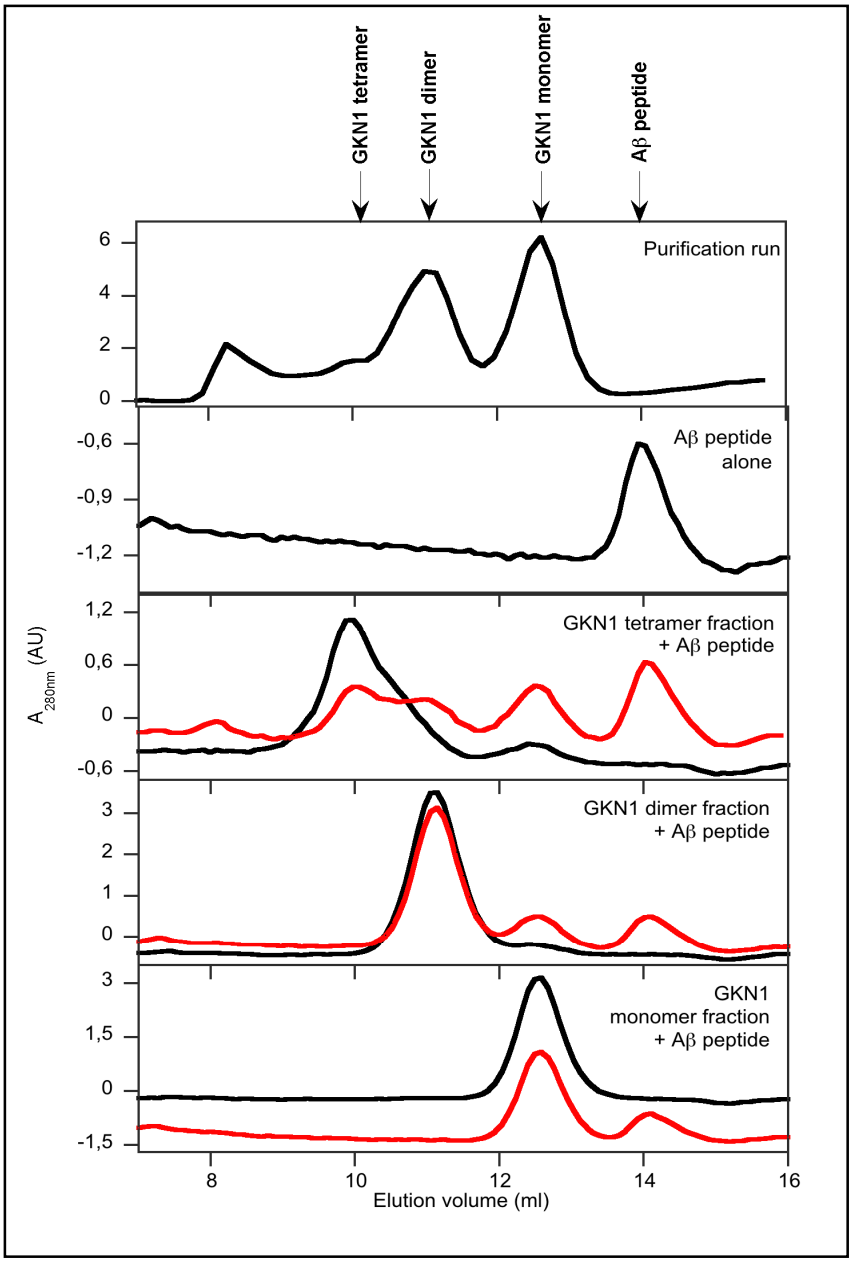


Figure 6. Size-Exclusion Chromatography analysis. Elution profiles at 280nm from the size-exclusion chromatography on Superdex 75 column obtained for rhGKN1 fractions in the absence (black line) and in the presence (red line) of Aβ(1-40).

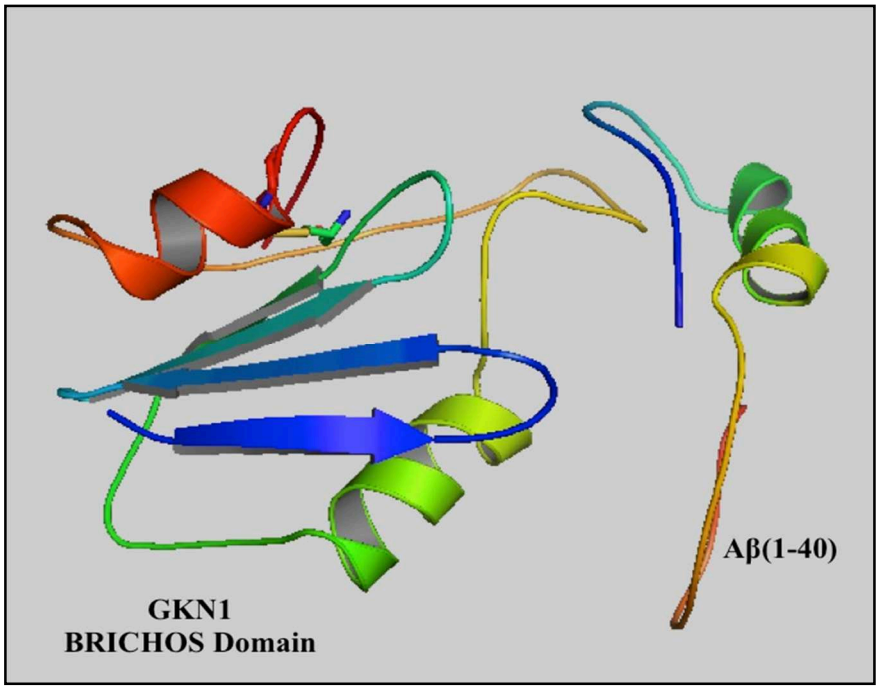


Figure 7. Imitation of a 3D model of the GKN1 (BRICHOS domain)•Aβ(1-40) complex. The representation was obtained from the molecular docking pdb file (Model_1-1.pdb) using PyMol software (DeLano Scientific LLC).

3.3 rhGKN1/Aβ interaction is specific but characterized by a low affinity

To probe the specificity of the interaction between rhGKN1 and Aβ and to determine their affinity constant in a quantitative manner, surface plasmon resonance analyses were performed. The β-amyloid peptide was successfully immobilized on the destran matrix of a CM5 sensor chip at about 1400 RU density. Fig. 8A depicts the binding sensorgrams of freshly prepared rhGKN1 in HBS buffer injected at different concentrations ranging from 7.5 to 130 μM on the immobilized β-amyloid surface. The specificity of binding was verified achieving saturation at the highest tested concentration of 130 μM. A dissociation constant (K_D) of $3.4 \pm 0.7 * 10^{-5}$ was extrapolated by data fitting of a plot of RUmax values from each binding determination against protein concentration, using a non-linear regression analysis (Fig. 8B). Chiken cystatin, used as negative control, did not show any reliable and measurable binding response to Aβ when tested at 60 μM (Fig. 8C), further confirming the specificity of binding.

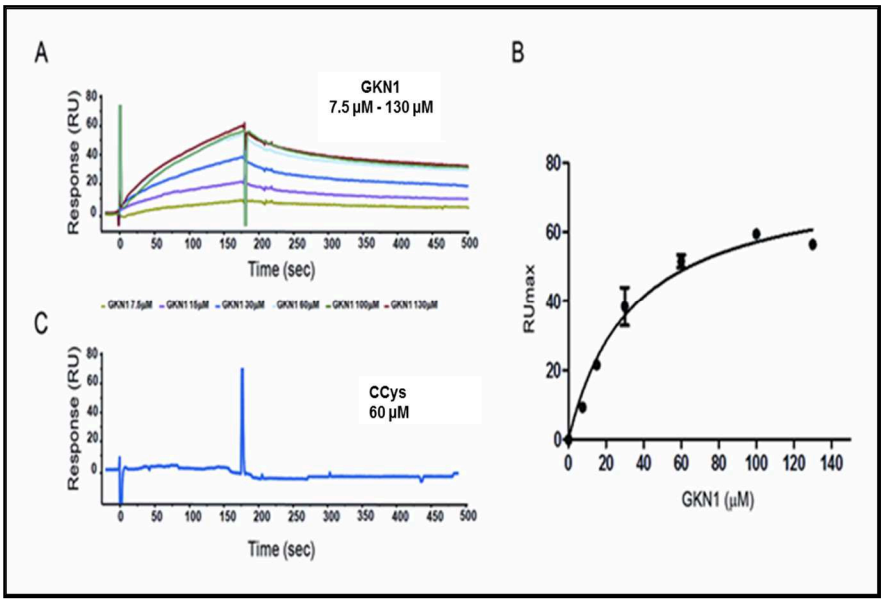


Figure 8. Surface plasmon resonance analysis. (**A**) Sensorgrams of the binding of rhGKN1 to Aβ(1-40) immobilized on CM5 sensor chip, tested at the concentrations between 7.5 to 130 mM. Experiments were carried out at a 25 °C, at a constant flow rate of 20 µL/min using HBS as running buffer. (**B**) Plot of RUmax from each single binding experiment *versus* concentration (µM). By data fitting using a nonlinear regression algorithm (GraphPad Prism 4) a $K_D = 3.4 \pm 0.7 * 10^{-5}$ M was calculated. (**C**) Sensorgram of the no-binding of chicken cystatin tested at the concentration of 60 mM to Aβ(1-40) immobilized on the surface of a CM5 sensor chip.

3.4 GKN1 binds amyloid precursor protein and inhibits γ-secretase activity

Since rhGKN1 was able to interact with Aβ peptide, it was tested if the protein was able to interact with amyloid precursor protein (APP) in a gastric cancer cell line. To this purpose, AGS cells were transfected with GKN1 cDNA His-tagged (pcDNA-GKN1) and after pull-down assay with Ni-NTA agarose were analysed by Western blot using anti-APP antibody. As reported in Fig. 9 (upper panel) transfected AGS cell specifically pulled-down for His-tagged GKN1 showed the presence of APP, thus indicating its interaction with GKN1. This result led to the hipothesis that GKN1 might participate in the processing of the Aβ precursor. Therefore, transfected AGS cells were analysed with specific antibody anti-APP able to recognize the APP processing products. As control, it was used a γ-secretase inhibitor (DAPT) able to highlight the internmediate processing products (CTFs). As shown in Fig. 9 (lower panel) there was an increase of the CTFs accumulation that was proportional to the time of transfection. This results strongly indicated that GKN1 exerts an inhibitory activity toward γ-secretase.

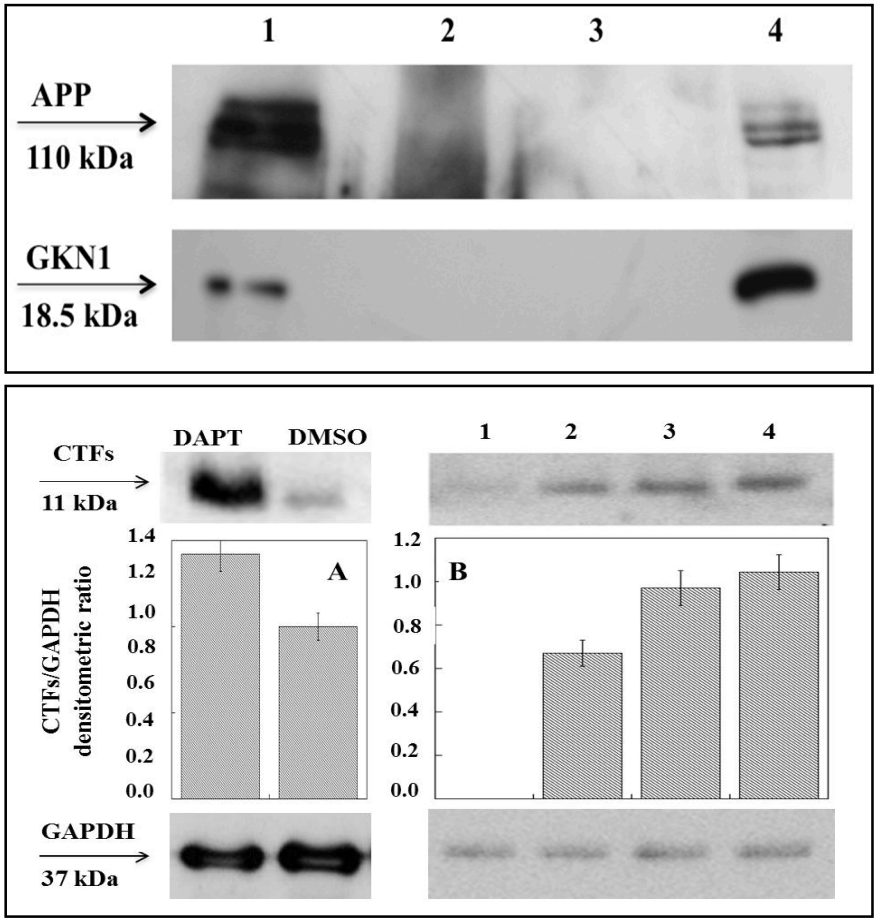


Figure 9. GKN1 and amyloid precursor protein. (**Upper panel**) Transfected AGS cells extracts were pull-down with Ni-NTA agarose and washed with 10 mM imidazole. The Ni-NTA resin was directly loaded on SDS-PAGE and analyzed by Western blot using anti-APP antibody. Lane 1, transfected AGS cells; lane 2, untransfected AGS cells; lane 3, Ni-NTA alone. (**Lower panel**) Inhibition of γ-secretase activity in APP processing in AGS cells. (**A**) CTFs levels in control samples in the presence or absence of DAPT. (**B**) CTFs levels in AGS cells transfected with pcDNA3.1 (lane 1) and AGS cells after transfection with pcDNA-GKN1 at 12, 24 and 48 hours (lane 2, 3 and 4, respectively). Histogram represents the percentage of band intensity with respect to GAPDH levels. Each measurement and Western blot was carried out in triplicate. Error bars indicate the maximum deviation from the mean value of two independent experiments.

Part B. Histone modification associated to *GKN1* gene silencing

3.5 GKN1 down-regulation in gastric cancer is associated with H3K9triMe on GKN1 promoter

To investigate the possible causes of *GKN1* gene inactivation, it was examined a group of three different samples derived from biopsies of normal and tumor human gastric tissue. Tissue extracts were first analysed to determine the GKN1 mRNA level by qRT-PCR and Western blot (Fig. 10 A and B). Subsequently, chromatin immunoprecipitation assays (ChIP) were performed for the repressive histone modification H3K9triMe. For this preliminary study, it was considered a promoter region of the human *GKN1* gene covering from -427bp to +91bp around the transcriptional start site. The results obtained showed that in the tumor tissue of the three subjects examined there is a very significant presence of this histone modification on *GKN1* gene promoter, contrary to what observed in the normal tissue of the same (Fig. 11 sample 1, 2 and 3).

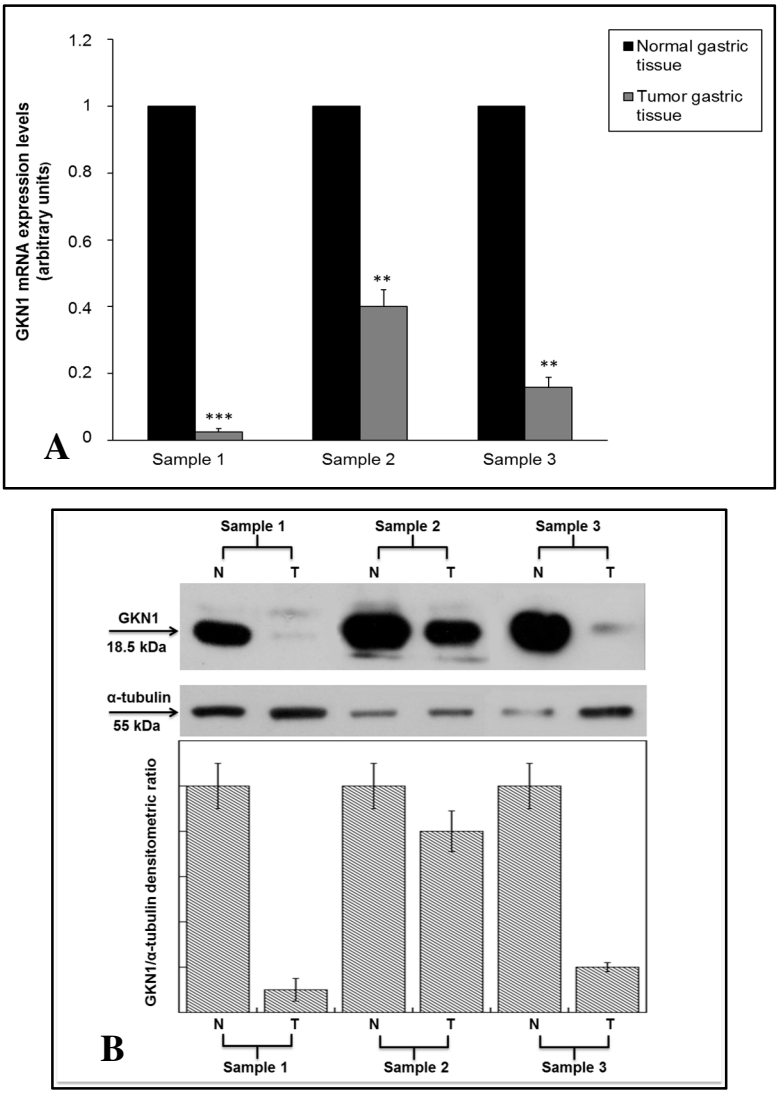
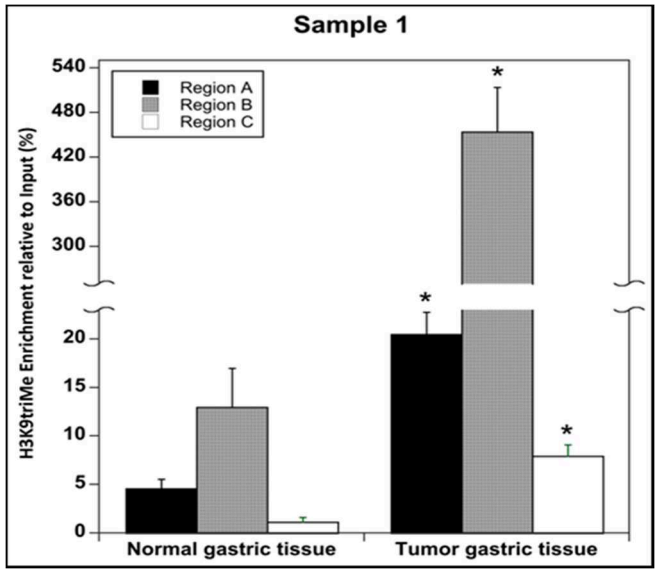
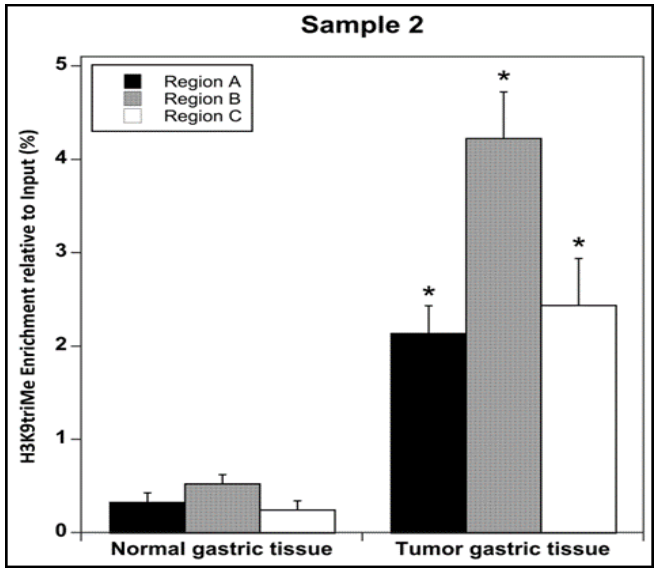


Figure 10. GKN1 expression in human gastric tissue. (**A**) GKN1 cDNA levels by qRT-PCR. Error bars indicate the maximum deviation from the mean value of two independent experiments. Asterisks indicate statistically significant differences at a $p < 0.001$ level. (**B**) GKN1 protein levels by Western blot and relative band intensity evaluation with respect to α-tubulin levels. Each measurement and Western blot was carried out in triplicate. Error bars indicate the maximum deviation from the mean value of two independent experiments.


Sample 1
Region A
Region B
Region C
H3K9triMe Enrichment relative to Input (%)
540
480
420
360
300
20
15
10
5
0
*
*
*
Normal gastric tissue
Tumor gastric tissue


Sample 2
Region A
Region B
Region C
H3K9triMe Enrichment relative to Input (%)
5
4
3
2
1
0
*
*
*
Normal gastric tissue
Tumor gastric tissue

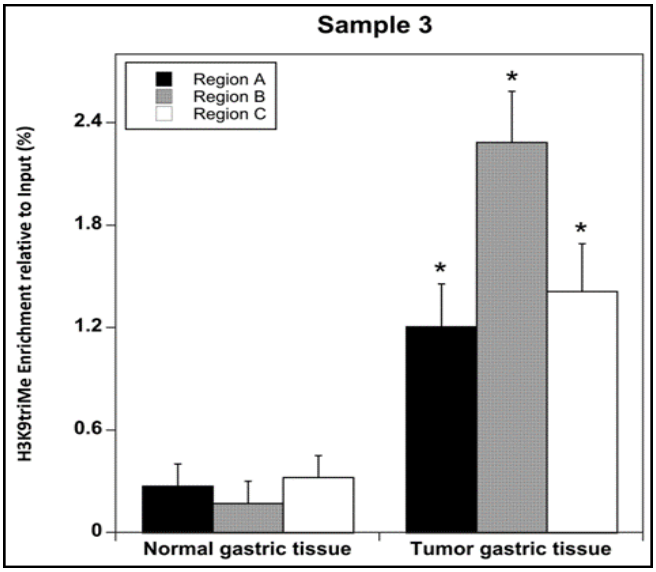


Figure 11. H3K9triMe levels on human *GKN1* gene promoter. Results of ChIP assays on human gastric tissue samples. Percent input was calculated as $2^{\Delta Ct} \times 100$, where ΔCt is the difference between Ct_{Input} and Ct_{IP}. NoIP (IgGs control) have been subtracted from IP. All quantitative ChIP data were derived from at least three independent experiments, and for each experiment, qPCR was performed in triplicate. Error bars indicate the maximum deviation from the mean value of two independent experiments. Asterisks indicate statistically significant differences at a $p < 0.001$ level.

3.6 High levels of H3K9triMe were associated with recruitment and/or activation of the specific HMT SUV39H1 on *GKN1* promoter

At this stage it was important to clarify who operates this modification on *GKN1* promoter. Literature data indicate that this histone modification is made by a specific histone methyltransferase, called SUV39H1 that trimethylates 'Lys-9' of histone H3 using monomethylated H3 'Lys-9' as substrate. So the next step was to verify by ChIP assays the presence of this enzyme on gene promoter in human gastric tissues. These preliminary findings show an increase in the binding of SUV39H1 on the same regions analyzed for histone modification (Fig. 12).

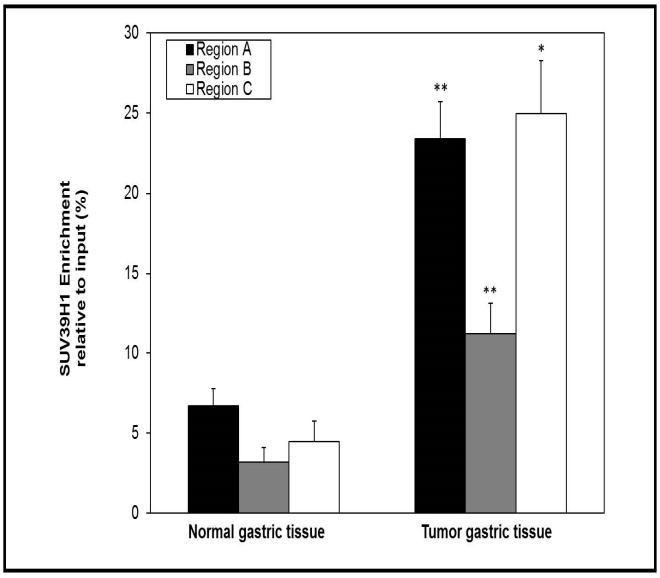


Figure 12. SUV39H1 levels on human *GKN1* gene promoter. Results of ChIP assays on human gastric tissue samples. Percent input was calculated as $2^{\Delta Ct} \times 100$, where ΔCt is the difference between Ct_{Input} and Ct_{IP}. NoIP (IgGs control) have been subtracted from IP. All quantitative ChIP data were derived from at least three independent experiments, and for each experiment, qPCR was performed in triplicate. Error bars indicate the maximum deviation from the mean value of two independent experiments. Asterisks indicate statistically significant differences at a $p < 0.001$ level.

DISCUSSION/CONCLUSIONS

Gastrokine 1 (GKN1) is a stomach-specific protein important for maintaining the physiological function of the gastric mucosa, being involved in the replenishment of the surface lumen epithelial cell layer and in maintaining mucosal integrity. Individuals with a lower expression of the protein have an increased risk of developing gastric diseases. The protein, in fact, is downregulated in *H. pylori*-infected gastric tissues and a loss of the protein expression is detected in gastric cancer tissues and precancerous lesions (13-15). So it is thus clear that the expression of GKN1 decreased throughout the progressive stages of neoplastic transformation, from gastritis to intestinal metaplasia, and it plays an important role in the process of formation and development of gastric cancer. It has also been demonstrated that GKN1 expression induces apoptosis in gastric cancer cells (10). This finding was also confirmed by the ability of recombinant human GKN1 (rhGKN1) to exert a higher antiproliferative effect on gastric cancer cells (AGS) compared to normal human embryonic kidney cells (HEK 293) and non-gastric cancer cells (H1355) (11). The higher sensitivity of AGS cells to GKN1 exposure was likely linked to the role played by GKN1 in maintaining gastric mucosal integrity and to its function as a gastric tumor suppressor.

For these reasons, it becomes very important to identify and characterize the molecular mechanisms that involve GKN1 to better identify its biological role in gastric tissue and in the process of carcinogenesis. To this aim, this thesis was initially aimed at verifying whether the GKN1 possess a role of chaperone against amyloidogenic peptides. The results obtained in the first part of the study showed the ability of rhGKN1 in reducing the fibrils aggregation of β-amyloid peptide (1-40), one of the main components of senile plaques in Alzheimer' s disease that is also ubiquitary. Further investigations have confirmed the formation of a GKN1• Aβ complex characterised by a K_D of 34 μM. These data highlighted a chaperon-like property of GKN1 (or its BRICHOS domain) in binding and preventing the formation of amyloidogenic b-sheet folding. In prevention or treatment of amyloid diseases, it appears essential to identify new chaperones with some level of specificity for Aβ and/or with its precursor protein APP and to understand

at which stage of APP processing and/or Aβ aggregation these specific chaperones might interfere. To give a biological interpretation of the results observed *in vitro,* it was hypothesized that GKN1 could interct with APP in gastric cells. This hypothesis was experimentally confirmed by pull-down assay in gastric cancer cell lines transfected with GKN1. Moreover, it was also explored the intervention of GKN1 in the processing of the Aβ precursor. What interestingly emerged was an activity of GKN1 as inhibitor of proteolytic γ-cleveage. Literature indicates that γ-secretase inhibitors (GSIs) are emerging as a new class of anticancer agents for the treatment of solid and hematological malignancies, but little is known about their effects on gastric cancer. GSIs are the indirect inhibitors of Notch, a single-pass transmembrane receptor protein whose pathway is overexpressed in several tumors (30). Thus it is probable that the apoptotic activity of GKN1 against gastric cancer cells was correlated indirectly with the inhibition of γ-secretase observed. Further investigations are necessary to clarify this aspect.

Regarding the mechanism by which *GKN1* gene is down-regulated in gastric cancer, this remains still an open question so the second section of my work was focused to clarify the causes of *GKN*1 gene inactivation and to determine if epigenetic mechanisms could be involved in this process. The data obtained showed for the first time a possible epigenetic mechanism of histone modifications that could lead to disregulate *GKN1* gene transcription in gastric cancer. In fact, we have observed the massive presence of the repressive histone modification H3K9triMe and the recruitment of the specific histone methyltrasnferase SUV39H1 on the *GKN1* gene promoter in human tumor tissues with respect to normal tissues. These results are much more relevant and reliable if considered that it was obtained *in vivo* on human gastric tissue samples. These findings represent the first step in the characterization of the epigenetic machinery that leads to gene silencing in gastric cancer and there will be further studies aimed at this purpose. Next goal will be to identify the specific transcription factor that is able to bind *GKN1* promoter and to recruit a corepressor complex including SUV39H1 and/or other histone modifier enzymes in gastric cancer tissues. Understanding whether *GKN1* gene inactivation can play a key role in the switch from normal gastric

tissue to gastric carcinogenesis may be important to identify the early stages of the disease and to make efficient screening of risk patients. Under this aspect, to understand the molecular epigenetic mechanisms that could lead to gene silencing and to determine whether this event might be involved in the development and progression of gastric cancer would provide an essential starting point for the development of new therapeutic strategies based on epigenetic targets for alternatives gene.

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