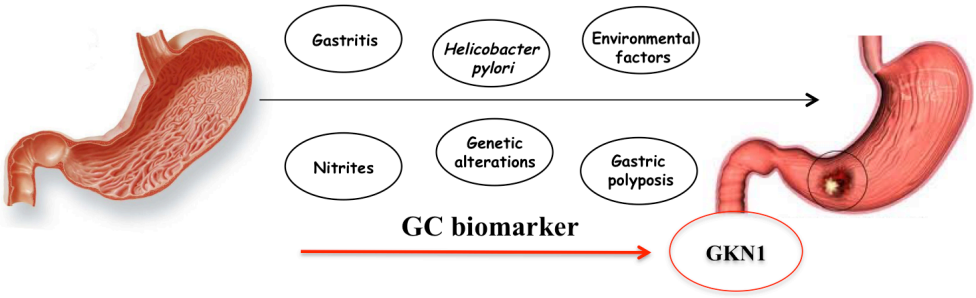
UNIVERSITA' DI NAPOLI "FEDERICO II"

DOTTORATO DI RICERCA
BIOCHIMICA E BIOLOGIA MOLECOLARE E CELLULARE
XXVIII CICLO

Searching for informative biomarkers for Gastric Cancer

Candidate
Valentina Villano



Academic Year 2014/2015

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Summary

Gastric cancer (GC) is still one of the prevalent leading causes of cancer-related deaths worldwide and high mortality rate is mainly due to late-stage diagnosis. GC is characterized by two distinct histological type of adenocarcinomas each having different epidemiological and pathophysiological features. The intestinal-type generally evolves through a relatively well-defined sequence of histological lesions. The diffuse-type has instead a poorer prognosis and develops through unknown genetic and morphological events from normal gastric epithelium. The pathogenesis of GC remains poorly understood however several environmental factors, such as *Helicobacter pylori* (*H. pylori*) infection can be the cause leading to this disease. This risk is probably the result of a combination of genetic and environmental factors in which the infection by *H. pylori* is of particular relevance. To search for gastric cancer biomarkers, we have analyzed the protein profile of malignant and normal gastric tissues and identified a novel stomach specific protein gastrokine 1 (GKN1) whose expression is reduced in *H. pylori* infected gastric mucosa and down-regulated or completely absent in GC tissues and precancerous lesions so this protein might play an important role as biomarker in carcinogenic process. On the basis of these evidences, we are investigating on the possible utility of GKN1 as an informative biomarker. To do this, we have analyzed by Western blot the expression profile of GKN1 in several biological fluids of normal individual in order to highlight the presence of GKN1 at the protein level. However, no GKN1 expression was possible to detect even if the search was performed by ELISA assay on serum of healthy individuals. We then have searched for the presence of GKN1 mRNA in the serum of healthy subjects by RT-PCR using several primers spanning over the entire GKN1 cDNA. This result suggests that qRT-PCR might be a possible media by which to follow the GKN1 expression profile in normal patients and in patients with gastric lesions in order to assess its possible use as preventive gastric cancer biomarker. The second part of my work is focused on identification of potential GKN1 interactors with the aim to add knowledge on its functional role within gastric cell lines and tissues. To this purpose we used a proteomic strategy and the GKN1 interactors were identified by peptide mass fingerprinting using MALDI-TOF mass spectrometry. The results show that GKN1 interacts whit SLC26A3, a protein expressed in apical membrane of intestinal epithelial cells. Therefore, we propose in future, the evalutation of SLC26A3 in human sera as a potential innovative biomarker in CG.

Riassunto

Il cancro gastrico (GC) è ancora oggi in tutto il mondo una delle principali cause di decessi correlati al cancro, e l'alto tasso di mortalità è dovuto principalmente alla diagnosi in fase avanzata. Il GC è caratterizzato da due tipi istologici distinti di adenocarcinomi, ciascuno con caratteristiche epidemiologiche e fisiopatologiche differenti. Il tipo intestinale evolve generalmente attraverso una sequenza relativamente ben definita di lesioni istologiche. Il tipo diffuso ha invece una prognosi peggiore e si sviluppa dal normale epitelio gastrico attraverso eventi genetici e morfologici sconosciuti. La patogenesi della GC rimane poco compresa tuttavia diversi fattori ambientali, come *l'Helicobacter pylori* (*H. pylori*) rappresenta la principale causa della malattia. Questo rischio è probabilmente il risultato di una combinazione di fattori genetici e ambientali in cui l'infezione da *H. pylori* è di particolare rilevanza. Per ricercare biomarcatori del cancro gastrico, abbiamo analizzato il profilo proteico di tessuti gastrici maligni e normali. E' stata individuata una proteina specifica dello stomaco, gastrokina 1 (GKN1), la cui espressione è ridotta nella mucosa gastrica con infezionw da *H. pylori* e down-regolata o completamente assente in tessuti GC e lesioni precancerose. Pertanto GKN1 potrebbe svolgere un ruolo importante come biomarker nel processo di cancerogenesi. Sulla base di queste evidenze, e stata valutata la possibile utilità della GKN1 come biomarker informativo. A tale scopo, è stato analizzato mediante Western blot il profilo di espressione della GKN1 in diversi liquidi biologici di individui normali al fine di evidenziare la presenza della GKN1 a livello proteico. Tuttavia, nessuna espressione di GKN1 è stato possibile rilevare anche quando la ricerca è stata effettuata mediante saggio ELISA. E' stato poi cercato di evidenziare la presenza dell'mRNA di GKN1 nel siero di soggetti sani mediante RT-PCR utilizzando diverse coppie di primers all'interno del cDNA di GKN1. Il risultato ottenuto ha evidenziato che la qRT-PCR potrebbe essere un possibile metodo per confrontare il profilo di espressione di GKN1 in pazienti normali e in pazienti con lesioni gastriche e quindi valutarne il possibile uso come biomarker cancro gastrico. La seconda parte della tesi si concentra sulla identificazione di potenziali interattori della GKN1, con l'obiettivo di aggiungere nuove

conoscenza sul ruolo funzionale della proteina in cellule e tessuti gastrici. A questo scopo è stata utilizzata una strategia proteomica e gli interattori della GKN1 sono stati identificati mediante MALDI-TOF. I risultati mostrano che GKN1 interagisce SLC26A3, una proteina espressa in membrana apicale delle cellule epiteliali intestinali. Pertanto, ci proponiamo in futuro, di valutare i livelli di espressione di SLC26A3 e di utilizzarlo come possibile innovativo biomarker del CG.

List of Tables and Figures

1. Introduction

1.1 Gastric cancer

Although the prevalence of gastric cancer is declining and varying geographically, it remains one of the most common cancers in Asian countries and is the fourth most commonly occurring cancer (9% of all cancers) worldwide. It is also the fourth leading cause of cancer death in both sexes worldwide (737,000 deaths, 9.7% of the total). Five-year survival rates have ranged from 90% to less than 5 percent, mainly depending on the stage of diagnosis. If gastric cancer can be detected and treated in early stages, the five-year survival rate is better than 90%; however, there is no apparent or specific symptom in early-stage gastric cancer. Thus, early detection of gastric cancer becomes more difficult. Essentially, endoscopy has been the promising tool with 2.7 to 4.6-times higher detection rate than barium studies. Early gastric cancer diagnosis by endoscopy depends on professional skill. However, there is no reliable biomarker for gastric cancer (Wu et al., 2014).

Two distinct histological types of adenocarcinoma showing different epidemiological and pathophysiological features often characterize gastric cancer. 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 pathogenesis remains poorly understood, but it's evident that several environmental and infective factors, such as *Helicobacter pylory* infection, can be the leading cause to this disease. In fact *H. pylory* infection is associated with risk of gastric adenocarcinoma, both intestinal and diffuses type, supported by the link between this infection and precancerous lesions, including chronic atrophy gastritis and dysplasia. The positive correlation between *H. pylory* infection and development of gastric cancer is well established and proven in prospective controlled studies. Exposure of gastric epithelial cells to the bacterium determines the release of cytokines and reactive oxygen species (ROS) leading to the infiammatory and immune response of the stomac epithelium, that in turn may cause genetic alterations and an increased risk of developing gastric

cancer (Altieri, F., Arcari, P & Rippa E. et al. 2013).

1.2 GKN1 as gastric cancer biomarker

Biomarkers are efficient diagnostic agents for detection and even early detection of diseases especially malignant diseases. Biomarkers are diverse biochemical components such as DNA sequence, RNAs, proteins, and metabolites. The final goal of biomarker application is to introduce a sensitive and specific indicator for a certain kind of disease (Zamanian-Azodi et al., 2015) unfortunately, there is no specific biomarker for gastric cancer. Previous studies aimed to disclose putative biomarkers by comparing the differential proteins between matched cancer and normal tissues. Many putative biomarkers have been identified including GRP78, GSTpi, Apo A1, A1AT and GKN1. Also the down-regulation of proteins may play an important role in carcinogenesis; Gastrokine1 (GKN1) is down-regulated in gastric cancer tissue compared to normal gastric tissue (Wu et al., 2014).The cells of the antral gastric mucosa synthesize GKN1. Previously known as AMP-18, CA11, FOVEOLIN, and TFIZ, was formally named 'GKN1' by the HUGO Gene Nomenclature Committee for its gastric specific expression and its highly conserved presence in the gastric mucosa of many mammalian species. (Martin et al., 2003; Oien et al., 3003) GKN1 belongs to a family of genes encoding stomach-specific proteins formed by 3 known members: GKN1, GKN2 and GKN3. The GKN1 gene (*CA11*) is located on the chromosome 2p13 and contains 6 exons separated by relatively short introns that encode a small protein of about 185 amino acids 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 GKN paralogs and the Hydrophobic NH_2-terminal signal peptide, that acts as a transmembrane anchor and/or signal peptide.

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) (Walsh-Reitz M.M et al. 2005); (Sánchez-Pulido et al., 2002). The BRICHOS domain shows several motifs of functional significance (Figure 1).

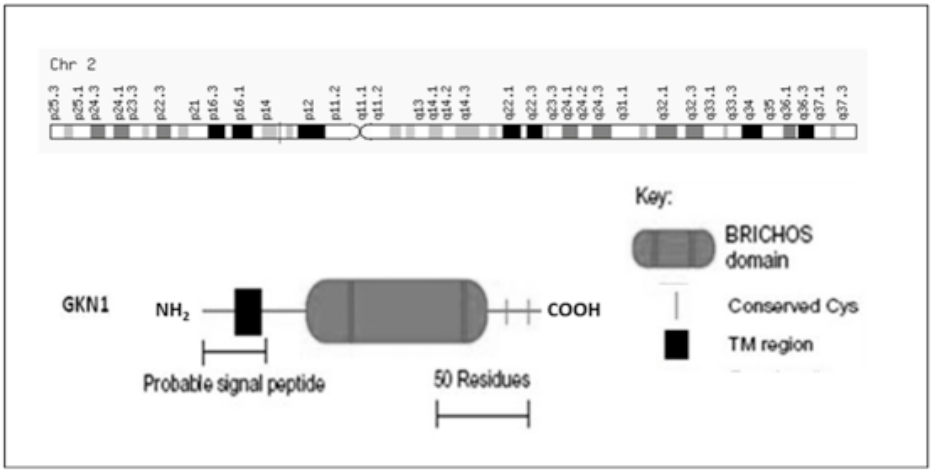
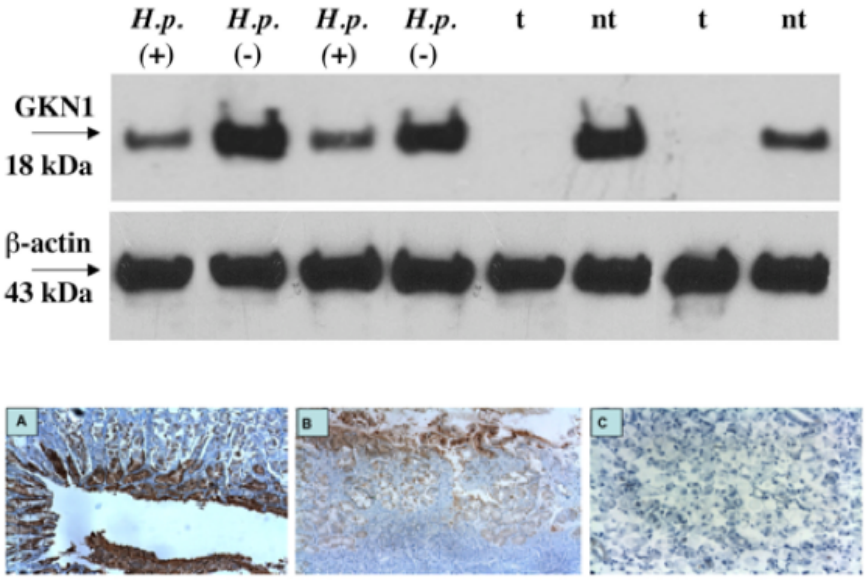


Figure 1. GKN1 genomic location and GKN1 human transcript. (upper panel) Chromosome bands according to Ensembl and genomic location according to GeneLoc. **(lower panel)** GKN11 protein structural prediction.

Immunoelectron microscopy indicated that GKN1 protein is localized within the granules just under the apical plasma membrane, suggesting that it is a secreted rather than an integral membrane protein acting primarly in the extracellular and luminal environment. The human protein expression is confined to the gastric epithelium, in particular in the surface mucous cells, except for trace levels in the uterus and placenta and it is richly expressed in the stomach of healthy individuals, but it is absent in gastric cancer tissues (Martin et al., 2003; Oien et al., 3003) (Figure.2). 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. Early work reported 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 (Lacy et al, 1993; Podolsky et al., 1997) . After gastric mucosa injury, restoration may occur very rapidly in the presence of GKN1 (Rippa et al., 2007) In contrast, if the protein is down regulated, the repair process may be

obstructed. 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 (Walsh-Reitz et al., 2005; Sánchez-Pulido et al., 2002) (Figure 3).



Figuure 2. Expression of GKN1 by Western blot analysis. Upper panel. Analysis of cell extracts from H.p (+) and H.p. (-) biopsies and from tumoral (t) and non-tumoral (nt) tissues. Lower panel. Immunohistochemistry of normal gastric tissue; B) Intestinal metaplasia; C) Intestinal type gastric cancer.

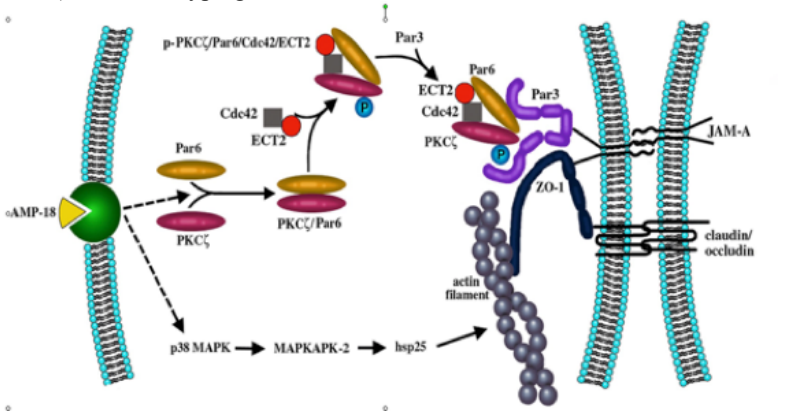


Figure 3. Proposed pathways by which AMP-18 protects the mucosal barrier and facilitates assembly of tight junction proteins.

Because a disruption in gastric homeostasis may result in the transformation of normal epithelial cells into cancer cells and permit cancer cells to proliferate and invade, there is no doubt that inactivation of GKN1 may render the gastric mucosa vulnerable to carcinogens or gastric injury, and eventually trigger genetic alterations in cancer-related genes, including oncogenes and tumor suppressor genes. Even if the biological function of GKN1 is still unclear, the protein may act as a functional tumor suppressor in gastric epithelial homeostasis and its loss of expression could make possible gastric carcinogenesis.

1.3 Biological activities of Gastrokine 1

Gkn1 belongs to the BRICHOS superfamily whose members are all associated clinically with dementia, respiratory distress syndrome and cancer (Sánchez-Pulido et al., 2002). 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 gastric cancer cell growth, reduce cell viability, proliferation and colony formation in AGS cells. It might be the main functional domain for the tumor suppressor activity (Yoon et al., 2013). Furthermore our group reported that GKN1 has anti-amyloidogenic properties thus functioning as a chaperone directed against unfolded segments and with the ability to recognize amyloidogenic polypeptides and prevent their aggregation (Altieri et al., 2014). Numerous studies have described frequent loss of GKN1 expression in gastric cancer (Oien et al., 2003), demonstrated that GKN1 mRNA was abundant only in normal human stomach, in all areas (cardia, body and antrum), but absent in gastric adenocarcinomas and gastro-esophageal adenocarcinomas cell line. Infact seems that the loss of expression of GKN1 may increase the risk of developing gastric disease. To understand the biological function of the protein in the normal physiology of the gastric mucosa many studies were carried out but no one has clarified its role. (Toback et *al*., 2002), proposed that GKN1 protein could have mitogenic impacts on intestinal epithelial cells. In contradiction to its protective and mitogenic activity whereas (Shiozaki et *al*., 2001), found that GKN1 was capable of

inhibiting cell proliferation after transfection, by reducing colony formation in MKN-28 gastric carcinomas cells. In order to characterize the structural and functional properties of the protein, our group produced a human recombinant GKN1 (hrGKN1) (Pavone et al., 2013) and it was tested on normal and tumors cell through a MTT assay which showed that the protein reduced cells proliferation of gastric cancers cells compared to human embryonic kidney cells (HEK293) and non-gastric cancer cells (H1355).

GKN1 can also induce apoptosis and senescence. (Xing et al., 2012; Rippa et al., 2011) 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. Furthermore our group investigated the effect of GKN1 on gastric cell line (Figure 4).

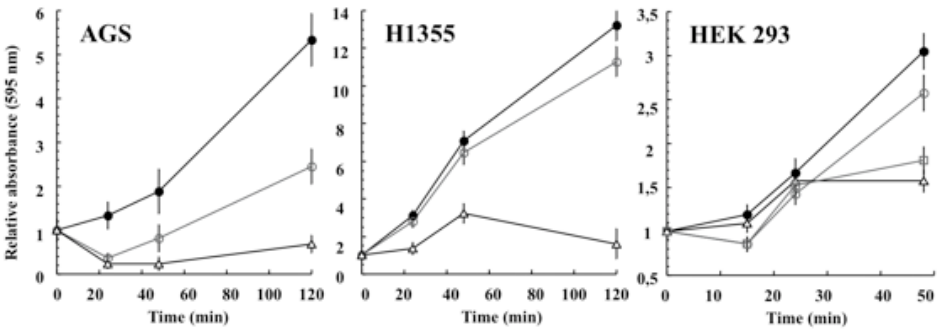


Figure 4. Effect of recombinant GKN1 on cell growth. The effect of GKN1 on cell growth was evaluated by the MTT assay after incubation of the cells with GKN1 at different times and concentrations. AGS cell growth in the absence (●) or in the presence of 0.5 μM (○) or 3 μM (△) GKN1. H1355 cells growth in the absence (●) or in the presence of 0.5 μM (○) or 3 μM (△) GKN1. HEK 293 cells growth in the absence (●) or in the presence of 5.5 μM (○), 11 μM (△) and 18 μM (□) GKN1, respectively.

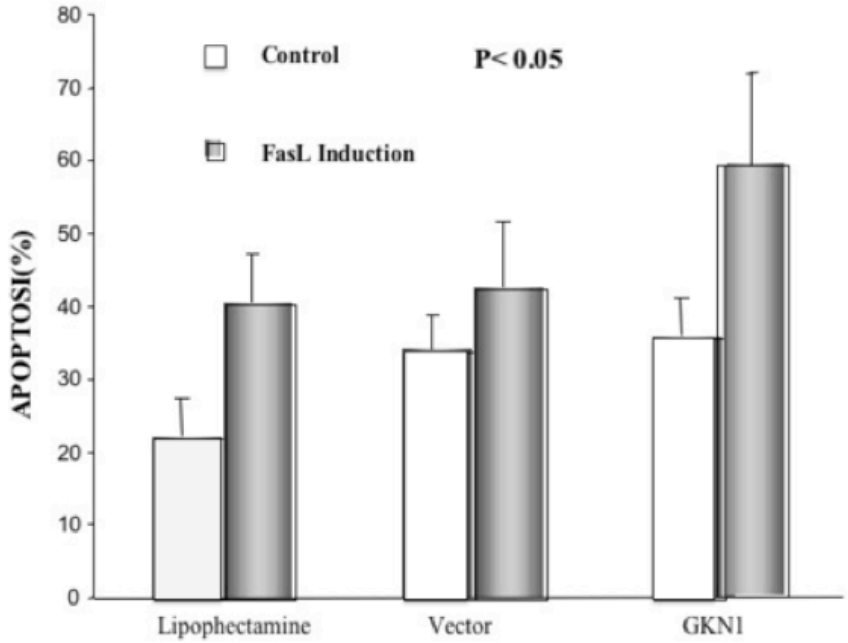


Figure 5. Effects of transfected GKN1 on cell death. Cell death was measured after transfection by TUNEL assay. Representative flow cytometry of cells treated with lipophectamine, cells transfected with empty pCDNA 3.1, cells transfected with GKN1 before and after incubation with antibody anti-FasL. Value from five independent experiments with similar results.

The overexpression of the protein in AGS and MKN28 cells stimulated higher expression of Fas receptor and sensitivity to Fas-ligand- induced apoptosis (Rippa et al., 2011) (Figure 5).

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 inhibit 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 (Yoon et al., 2013). Subsequent study found that GKN1 is involved also in epithelial mesenchyme transition, a process observed in response to injury, organ fibrosis and cancer. In particular the recovery of GKN1 expression induces the conversion of spindle-shape cells with abundant cytoplasm to circular-shaped cells, and suppresses cell migration and invasiveness abrogating the expression of PI3K/akt pathway proteins, concomitant with the re-expression of E-

cadherin (Yoon et al., 2013). All these data taken together show that GKN1 has a very important role in maintenance of gastric mucosal homeostasis and its early loss of function may facilitate gastric carcinogenesis.

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 GKN1 was a possible informative gastric cancer biomarker.

The second part of the work aimed highlight molecular interactors of GKN1 and to analyze if these interactors could represent also new possible biomarkers for gastric cancer.

2. Materials and Methods

2.1 Biological samples preparation

Fecal sample. 1 g of sample was freeze and thawed at -80 ° C (x 2 times). Subsequently, the sample was suspended in RIPA buffer containing 0.1% SDS at 2: 1 ratio (w/v), hold on ice for 30 min, centrifuged at 4 ° C for 15 'at 14,000 rpm and the resulting supernatant was used for Western blotting analysis.

Gastric juice sample. Two samples of gastric juice were collected from two healthy subjects at Cardarelli Hospital (GJ1 and GJ2). 5 mL of GJ were precipitated according the acetone method above described. After centrifugation, samples were washed once and re-suspended in rehydration buffer and analyzed by Western blotting.

Serum sample for western blot analysis. Generally, 1 ml of serum was diluted 1:1 with H_2O and centrifuged at 4,000 rpm in Centricon 30 in order to separate BSA. Filtered sample was then lyophilized overnight, re-suspended in 50 μL H_2O and analyzed by Western blotting of dot blot.

2.2 Serum collection

A total of 50 serum samples will be obtained from patients with GC and from healthy individuals were collected in collaboration with the Clinical Chemistry Lab of University of Naples Federico II and used in a frist approch to detected GKN1 protein levels using the ELISA assay (Cloud Clone Corp.). Blood samples will be collected in tubes with no anticoagulants and will be centrifuged at 3000 rpm for 10 min at 4 °C to completely remove cellular components. The collected serum will be stored at -80 °C. Written informed consents has been provided by all patients and healthy volunteers.

2.3 Western blot analysis

Samples were separated by SDS-PAGE, electrotransferred to PVDF membrane and reacted with the specific mouse anti-GKN1 antibody (detection limit: 0,1ng/mL) to analyze the levels of protein expression in biological samples. Immunoblots were visualized using HRP-conjugated secondary antibody and ECL Western blot detection kit (GE Healthcare).

2.4 RNA purification and cDNA synthesis

Serum or plasma samples will be lyzed in QIAzol Lysis Reagent (Qiagen). Total RNA from 1 mL of serum sample was prepared using miRNeasy Serum/Plasma Kit (Qiagen). RNA concentration was measured using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). One microgram of total RNA was retro-transcribed with the Quantitect Reverse Transcription kit (Qiagen, Germany).

2.5 Semiquantitative RT-PCR

Four µl of cDNA were amplified with 1unit of Taq DNA Polymerase (Invitrogen) in the buffer provided by the manufacturer which contains no $MgCl_{2.}$. Reactions were carried out in the PTC-0150 Mini-Cycler (Biorad). A first cycle of 5minutes at 95°C followed by 30 cycles (30 seconds at 95°C, 40 seconds at 58°C, 30 seconds at 72°C) and 10 minutes at 72 °C. To set up the procedure, we first selected 4 different pairs of primers designed spread over the sequence of GKN1 cDNA. In addition, already well known pairs of primers for GAPDH were used as a control.
Primers: F1-cctctgtccactgctttcgt, R1-tggttgcagcaaagccattt;
F2-gcttcagggtaagggaccag, R2-cttgcctcttgcatctcctca;
F3-ctttctagctcctgccctagc, R3-gttgcagcaaagccatttcc;
F4-caacaatgctggaagtgggc, R4-tcccttaccctgaagcttcttt.

2.6 Construction of the plasmid DNA standard

Results on serum samples were evaluated by absolute quantitation using GKN1 cDNA cloned in the pCDNA3.1 expression vector (pCDNA3.1-flGKN1) (Rippa et al., 2012). The content of positive recombinant plasmid was quantified using spectrophotometer. qRT-PCR standard curve was constructed using dilution from a standard plasmid solution of about 2.5 µg/µL.

2.7 Real-time PCR

Real-time PCR quantification will be performed using a CFX 96 thermocycler (Bio-Rad) with SYBR green PCR Master Mix (Bio-Rad) to detect the presence of GKN1 in serum samples. The following
primers for GKN1 cDNA sequence will be used:

Fw gcttcagggtaagggaccag, Rev cttgcctcttgcatctcctca.

2.8 Elisa kit Cloud-Clone Corp for detection of GKN1

For detection of GKN1 levels in serum it's been used a specific ELISA kit, the kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of GKN1 in human serum, plasma, tissue homogenates and other biological fluids. The assay is conducted according to the protocol. Briefly: the microliter plate provided in this kit has been pre-coated with an antibody specific to GKN1. Standards or samples are then added to the appropriate microliter plate wells with a biotin-conjugated antibody specific to GKN1 (detection limit 0,057 ng/mL). Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain GKN1biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is mesaured spectrophotometrically at a waveleangth of 450nm ± 10 nm. The concentration of GKN1 in the samples is then determined by comparing the O.D. of the samples to the standard curve. ELISA kit for GKN1 detection was calibrated using as standard the recombinant protein (rhGKN1) that allowed to determine a concentration range of GKN1 of 0.5 - 10 ng/ml of serum (Fig.3). The assay was subsequently performed on about 30 fresh sera from healthy subjects in different analysis conditions: any serum samples are treated at 56 °C for 20 minuts to remove the high PM proteins, others are analyzed whitout preliminary treatment.

2.9 AMP18biotinylation, affinity pull-down and mass spectrometry analysis

The biotinylation of recombinant AMP18 (rAMP18) (Pavone et al.2013) was performed by incubating 1.4 mg of protein with 20 mM NHS-biotin (N-hydroxysuccinimide biotin) (Thermo Scientific) for 30 min at room temperature. To eliminate the excess of biotin, the sample was then subjected to gel filtration chromatography on a Sephadex G-25 column. The biotinylated rAMP18 was quantified with the Bradford method. Biotinylated

rAMP18 (650 μg) was then incu- bated with 9 mg of Streptavidin MagneSphere Paramagnetic par- ticles/beads (Promega, Madison, USA) for 1 h at room temperature under stirring in order to promote the bond between the bio- tinylated rAMP18 and streptavidin. The same volume of buffer without protein was loaded as negative control. Magnetic beads were washed 3 times with 1 PBS according to manufacturer's instructions. The rAMP18-bounded beads were then incubated overnight at 4°C with 1.8 mg of protein extract from human non- tumoral gastric mucosa. Following beads washing with 1 PBS, elution was performed with 2 mM D-biotin; the eluted samples were freeze-dried and stored at 20 until mass spectrometry analysis.

Proteins from pooled fractions were precipitated with trichloro acetic acid (TCA) and dissolved in 100 μL of 50 mM ammonium bicarbonate. Disulphide reduction was performed with 2.5 mM DTT at 55 °C for 15 min, followed by carbamidomethylation with 7.5 mM iodoacetamide at room temperature in the dark for 15 min. Protein digestions were performed at 37 °C for 3 h by adding 5 μL of a 70 ng/ mL Tosylamido-2-Phenylethyl Chloromethyl Ketone (TPCK) porcine trypsin solution. Mass spectrometry analysis was performed using a quadrupole time of flight (ESI-Q-q-TOF) mass spectrometer equipped with an electrospray ionization source. Tryptic peptides were separated by means of a modular CapLC system (Waters, Manchester, UK) as reported elsewhere (A.Chambery et al. 2009). Samples were loaded onto a C-18 precolumn (5 mm length x 300 mm ID) at a flow rate of 20 mL/min and desalted for 5 min with a solution of 0.1% formic acid. Peptides were then directed onto a symmetry-C18 analytical column (10 cm 300 mm ID) using 5% CH3CN, containing 0.1% formic acid at a flow rate of 5 mL/min. The elution was obtained by increasing the CH3CN/0.1% formic acid concentration from 5% to 55% over 60 min. The precursor ion masses and associated fragment ion spectra of the tryptic peptides were mass measured with the mass spectrometer directly coupled to the chromatographic sys- tem. Electrospray mass spectra and tandem MS/MS data were ac- quired on the Q-TOF mass spectrometer operating in the positive ion mode with a source temperature of 80 °C and with a potential of 3500 V applied to the capillary probe. MS/MS data on tryptic peptides

were acquired in the data directed analysis (DDA) MS/MS mode. A maximum of three precursor masses was defined for concurrent MS/MS acquisition from a single MS survey scan. MS/ MS fragmentation spectra were collected from m/z 50 to m/z 1600. The MS/MS data were centroided, deisotoped, and charge- state- reduced to produce a single, accurately measured monoisotopic mass for each peptide and the associated fragment ions. For protein identifications, processed spectra were searched against Swiss-Prot database (release 2012_06; 20238 entries) using the Mascot soft- ware (version 2.2 from Matrix Science) applying the Homo sapiens taxonomy restriction. Fixed (Cys as S-carbamidomethyl derivate) and variable (Met as oxidized methionine) modifications were considered, allowing one trypsin missed cleavage site and a mass tolerance of 100 ppm. Identifications were accepted as positive when probability scores were significant at $p < 0.05$. To increase the SLC26A3 coverage identification was also performed by using the Protein Lynx Global Server 2.3 software (Waters) to search monoisotopic masses against an in house- developed database of SLC26A3 sequence.

2.10 Cell culture, transfection and western blotting

Human gastric adenocarcinoma cell line (AGS) was grown in DMEM-F12 (Dulbecco's modified Eagle medium-Cambrex) sup- plemented with heat inactivated FBS, 100 U/ml penicillin, 100 mg/ mL streptomycin, 1% L-glutamine at 37 °C in a 5% CO2 atmosphere and transfected with 4 mg of empty vector pcDNA 3.1 or pcDNA3.1- flAMP18. The efficiency of transfection of gastric cancer cells with flAMP18 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. Proteins from cell extracts were analyzed by Western blotting using mouse anti-SLC26A3 antibody at a dilution of 1:1000 and mouse anti-AMP18 at 1:500. Detection was per- formed using the enhanced chemiluminescence detection kit (SuperSignal West Pico) following manufacturer's instructions. All films were analyzed by using the Image J software.

2.11 Ni-NTA agarose pull-down

Transfected AGS cell extracts (500 mg) were incubated with 50 mL of Ni-NTA Agarose (Qiagen) pre-equilibrated with binding buffer (50 mM NaH2PO4, 300 mM NaCl pH 8.0) for 16 h at 4 C°. After incubation, the resin was washed 4 times with binding buffer containing 10 mM imidazole to reduce aspecific protein binding, resuspended in 30 mL of SDS loading buffer, heated to 95 C°for 5 min and subjected to Western blot analysis.

2.12 RNA isolation and RT- PCR

RNA was extracted with the TRIZol reagent (Invitrogen, UK). Total RNA amount was quantified by using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, UK). One microgram of total RNA was retro-transcribed with the Quantitect Reverse Transcription kit (Qiagen, Germany) and cDNA was diluted for semi-quantitative real-time PCR analyses on LightCycler 480 Real-Time PCR System (Roche, Germany) with Taqman probe chemis- try. Additional information are reported in Supplementary data. Results were normalized on the housekeeping glyceraldheyde-3-phosphate dehydrogenase (GAPDH) gene. A comparison of SLC26A3 expression levels was performed before and after the transfection with the pcDNA3.1-flAMP18 as previously described (E.Rippa et al. 2011) and in non-tumoral (N) versus tumoral (T) tissues. Relative gene expression was calculated according to (Pfaffl et al., 2011) by using the DDCt method (where DDCt corresponds to the increase in the threshold cycle of the target gene with respect to the increase in the threshold cycle of the housekeeping gene, GAPDH). Hence, the final quantification value for each condition indicated the relative change of gene expression of the target gene in non-tumoral (N) tissues (or non-transfected AGS cells) compared to the paired control tumoral (T) stomachs from the same patients (or from the same AGS cells transfected with pcDNA3.1-flAMP18). SLC26A3 mRNA integrity of was verified by RT PCR on total RNA prepared from transfected and non-transfected AGS cells. The following primers were used: forward tggcagctagtgtggcattt, reverse ctttgttgcgcttgcgtaga,

corresponding to the amino acid regions A489-F494 and L568-K573 of SLC26A3, respectively. The amplified PCR product length was 258 bp.

2.13 Population

The study population comprised 27 patients with GC recruited at Hospital A. Cardarelli, Naples, Italy. All patients were interviewed regarding smoking habit, alcohol intake and chronic use of drugs. Pathologist G. de Dominicis performed the macro dissection of tumor and non-tumor tissues of GC patients during surgery. Gastric cancer was classified according to Lauren criteria. 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 Universita Federico II). The assigned protocol number of the study was 34/15.

2.14 Immunohistochemistry

Four-micrometer thick, formalin-fixed, paraffin-embedded tis- sue sections were stained with hema- toxylineeosin for light microscopic examination. Consecutive sections were stained for immunohistochemistry using polyclonal rabbit (Sigma) anti- SLC26A3 antiserum (dilution 1:500).

2.15 Statistical analyses

Statistical analyses were performed by two-tailed paired Stu- dent's t-test using KaleidaGraph 4.1.1 software. Data were reported as means ± standard deviation (SD). The significance was accepted at the level of $p < 0.05$.

3. Results

Part A. Evaluation of the expression of gastrokine 1 in body fluids

To assess if GKN1 could represent a possible biomarker for gastric cancer, the first approach was to ascertain its presence in most important human biological fluids taken from healthy subject. This approach was necessary before to follow its presence in samples from patients with gastric cancer. Therefore, we analyzed the following human fluids: fecale, gastric juice and blood serum.

3.1Body fluid analyses: Western blotting on fecal and gastric juice.

We first analyzed easy withdrawal samples such as fecal. Subsequently, we tested fecal sample. Protein extract on freeze samples were analyzed by western blotting using mouse anti-GKN1 antibody. (Figure 6) shows the result of this analysis using as control the protein extract from human gastric mucosa.

Moreover, we have avaluated GKN1 espression levels in gastric juice of two healthy subjects: the samples were precipitated according the aceton method as described in Materials and Methods and analyzed by western blotting using mouse anti-GKN1 antibody. (Figure 7) show the result of the protein expression using as a control the protein extract from human gastric mucosa.

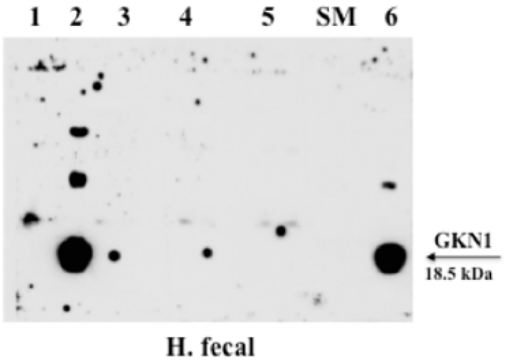


Figure 6. Analysis of GKN1 expression in human faecal. Samples from normal individual were collected and analyzed by Western blots using mouse anti-GKN1 antibody. Lanes: 1, h.fecale 1 (45 ug, 32 μL); 2, human gastric mucosa (30 μg); 3, h.fecale 2 (45 ug, 32 μL); 4, h.fecale 3 (45 ug, 32 μL); 5, h.fecale 4 (45 ug, 32 μL); SM, size marker; 6, human gastric mucosa (30 μg).

Using western blotting to detect the presence of GKN1 in urine, fecal and gstric juice semples, unfortunalely, we did not found the protein in the examined samples.

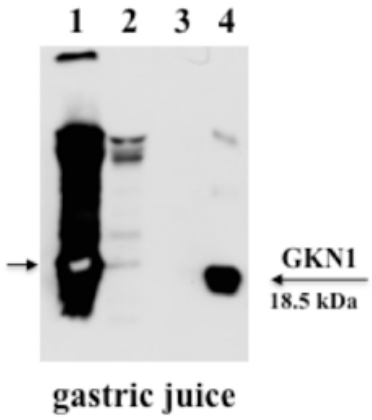


Figure 7. Analysis of GKN1 expression in human gastric juices. Lanes: 1 and 2, gastric juice sample 1 and 2 (32 μL), respectively; 3, gastric juice sample 2 after wash (32 μL); 4, human gastric mucosa (30 μg).

3.2 Body fluid analyses: Western blotting on Human serum

Because human serum is a rich source of biochemical products that can act as indicators of the physiological or clinical status of a patient, we subsequently exploited the potentiality of GKN1 as gastric cancer biomarker in this biological fluid. We first tried to use Western blotting technique to analyze human sera of healthy individuals. Because of the unusually high abundance of human serum albumin (HSA) in serum that can interfere with the resolution and sensitivity of several proteome techniques, samples were partially purified by Centricon 30 to remove high molecular weight proteins and then analyzed by Western blotting comparing the intensity of the signals with that of human gastric mucosal extract.

As show (Figure 8-A) show the result of the protein expression on intact serum of healthy and pathological samples instead, (Figure 8-B) show the result of protein expression on serum after protein concentration

using as a positive control the protein extract from human gastric mucosa and as negative control BSA to be sure of have removed the proteins that could have been interfere with analysis. Also in this case, detection with monoclonal mouse anti-GKN1 antibody did not show any positive signals compared to that shown by human gastric mucosal extract.

This assess if the results obtained by Western blotting would have been comparable with that of another methods, serum samples from healthy subject were also analyzed with ELISA kit for GKN1. As reported in (Figure 9), the calibration curve obtained using as control sample recombinant GKN1 showed a lower detection limit for the ELISA method of about 0.1 ng/mL. Nevertheless, the analyses performed on human sera of healthy individuals did not give any positive spectrophotometric answer at 450 nm.

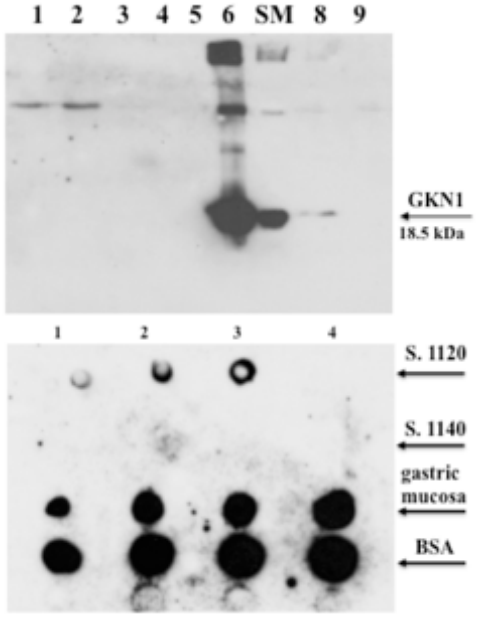


Fig. 8. Analysis of GKN1 expression in human sera. A. Lanes: 1, 2 and 3, intact serum samples (32 μL) from normal subjects; 4 and 5, intact serum samples from subjects with GC (32 μL); 6, human gastric mucosa (30 μg); SM, size marker; 8 and 9, intact serum samples (32 μL) from normal subjects. **B.** Samples of human serum from healthy subjects, after concentration in Centricon 30, were analyzed by dot blot using mouse anti-GKN1 antibody. Lanes 1, 2, 3 and 4: 1, 2, 5 and 10 μL, respectively of concentrared serum sample 1120 and 1140; 1, 2, 5 and 10 μg, respectively of gastric mucosa and BSA.

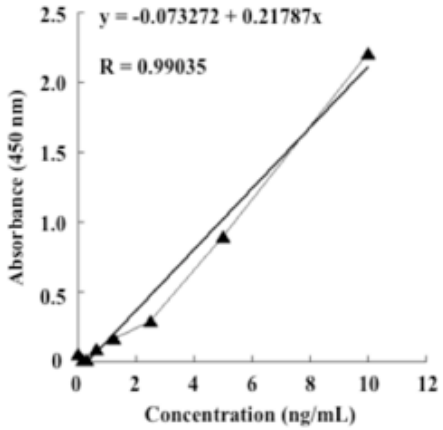


Figure 9. Analysis of GKN1 expression in human sera by ELISA method. GKN1 ELISA detection standard curve constructed according the kit instructions protocol. GKN1 recombinant protein standard (▲); curve fit (-).

3.3 Body fluid analyses: qRT-PCR on Human serum

Subsequently, it was tried to detect GKN1 mRNA in sera of healthy volunteers. Four sets of PCR primers, spanning over GKN1 coding sequence, were defined by using PrimerBlast Server (Figure. 10) and used to perform semi-quantitative PCR on fresh human sera. As reported in (Figure. 11) (lanes a), all primer couples were able to amplify the corresponding GKN1 cDNA region giving PCR products of the expected length. This finding suggested the presence of a full GKN1 mRNA in the samples analyzed. To check if the four PCR products were corresponding to GKN1 cDNA, PCR sequencing analyses were performed. The results confirmed that the nucleotide sequences of the four PCR products were encoding GKN1 (not shown). Therefore, we decided to use the qRT-PCR strategy to detect GKN1 mRNA in the serum obtained from a population of wealthy subject and in that from patients with diagnosis of gastric cancer. We took advantage from a collection of sera withdrawn from patients with a diagnosis of gastric cancer that underwent surgical treatment. The clinicopathologic characteristics of the gastric cancer patients are outlined in Table 1. The intestinal type was well differentiated in one, moderately differentiated in three and poorly differentiated in the remaining cases, while as far as concerns stage, these

were all advanced. Diffuse type GC was poorly differentiated and advanced in all cases. The non-tumoral areas of intestinal type GC showed a variable degree of gastric atrophy with diffuse IM, instead, the peritumoral areas of diffuse type GC showed a variable degree of non dysplastic inflammation. Characterization of non-tumoral gastric mucosa (N) from tumoral one was based on the macroscopic aspect of the normal tissue compared to the tumoral one, as evaluated from the hospital pathologist, and from the analysis by Western blotting of GKN1 expression level in non-tumoral and tumoral tissues of each patient (data not shown) (Di Stadio et al., 2015). qRT-PCR was performed by absolute quantification by a standard curve method constructed using as reference DNA a plasmid containing flGKN1 cDNA (Rippa et al., 2011). We used serial 10-fold dilutions of plasmids to construct a standard curve by plotting the logarithm of the plasmid copy number against the measured cycle values. The standard curve had a wide range of DNA copies/μl (from $3.78x10^{1}$ up $3.78x10^{8}$) with a linear correlation (R^2) of 0.99351, and a slope of -3.909 (Figure. 12-A). The level of circulating GKN1 mRNA of all 23 healthy samples showed a median cycle number value of 29.6673 (range 27.702–31.380). GC patients gave a median Ct value of 30.1269 (range 28.713–31.279). The statistical evaluation of the results using Student t-Test of unpaired data with unequal variance, indicated a non-significant difference among the two groups ($p = 0.1138$) (Fig.12-B). From the standard curve, the mean circulating copies of GKN1 mRNA were $1.81x10^{5}$ and $2.38x10^{5}$ copies/ml in patients and healthy individuals, respectively.

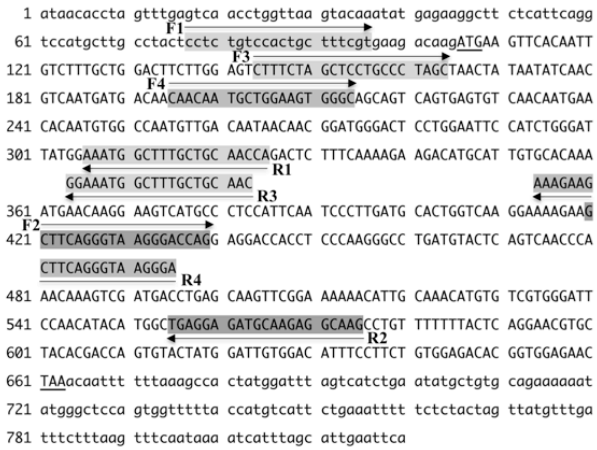


Figure 10. PCR primers for GKN1 mRNA detection in Human sera. Nucleotide sequence of GKN1 cDNA. The coding region is represented in capital letters, start and stop codons are underlined. PCR primers pairs F1-R1, F2-R2, F3-R3 and F4-R4 are highlighted in gray, respectively.

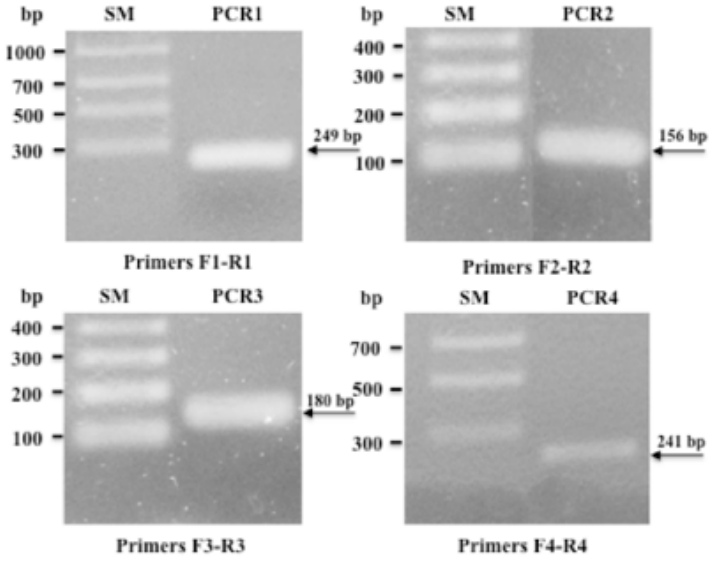


Figure 11. Evaluation of the expression of GKN1 mRNA in human sera. Representative semi-quantitative PCR performed on a serum of a healthy subject performed before (lanes a) and after (lanes b) storage of the sample at 4°C for 24 h. PCR reactions were carried out using the primers pairs F1-R1, F2-R2, F3-R3 and F4-R4 as indicated in Figure 10.

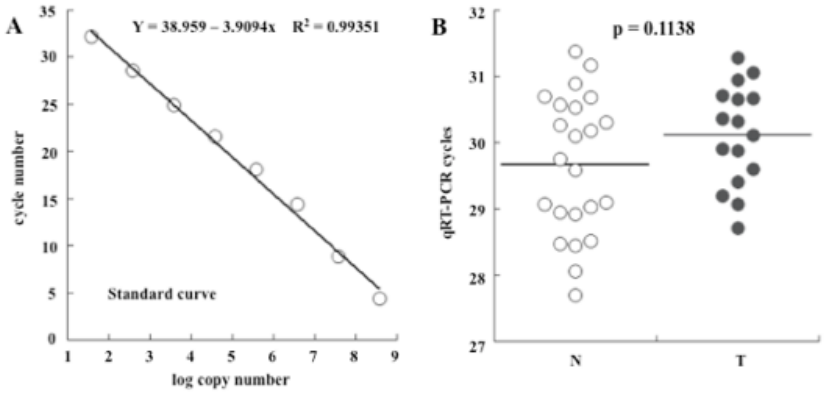


Figure 12 A. standard curve had a wide range of DNA copies/μl (from $3.78x10^1$ up $3.78x10^8$) with a linear correlation (R^2) of 0.99351, and a slope of -3.909 **B**. statistical evaluation of the results using Student t-Test.

Part B. Search for other possible biomarker for gastric cancer

3.1 Identification of SLC26A3 as an interaction partner of rAMP18

Functional proteomic strategy was subsequently exploited with the purpose to identify other possible markers for gastric cancer. Tacking advantage from the availability of recombinant GKN1 (rGKN1), a functional proteomic strategy was setteled. Biotynilated GKN1 bound to streptavidin beads, was incubated in the presence of non-tumoral gastric mucosal cell extract as described in the Methods section. After appropriate washing, beads were eluted with 2 mM D-biotin and the resulting fractions were pooled and analyzed by mass spectrometry. Only in the sample treated with rGKN1, besides the presence of AMP18, it was interestingly identified the membrane channel protein SLC26A3, also known as Down-Regulated in Adenoma (DRA). Such protein belongs to the family of solute-linked carrier (SLC26) that code for proteins capable of carrying a variety of monovalent and divalent anions.

3.2. Validation of the interaction by pull-down and western blot analyses

The interaction between GKN1 and SLC26A3 was first confirmed by applying a traditional pull-down strategy. Taking advantage of the $(His)_6$-tag sequence in the pCDNA3.1-flAMP18 vector (Rippa E. et al. 2007) , a pull-down was performed following transfection of the AGS cells with

the pCDNA3.1-flAMP18 construct encoding for the full length AMP18 protein (Rippa E. et al. 2007).As reported in (Figure 13), in cells transfected with flAMP18, SLC26A3 was specifically pulled-down toghether with flAMP18 as detected by anti-SLC26A3 mouse antibody, thus confirming its interaction with AMP18. In this experimental setting, a band with a molecular weight of about 32 kDa was detected for SLC26A3. The specificity of the interaction was verified by the absence of similar bands in the negative control samples transfected with pcDNA3.1 empty vector (Figure 13, lane 2) and in the absence of cell extract (Figure 13, lane 3).

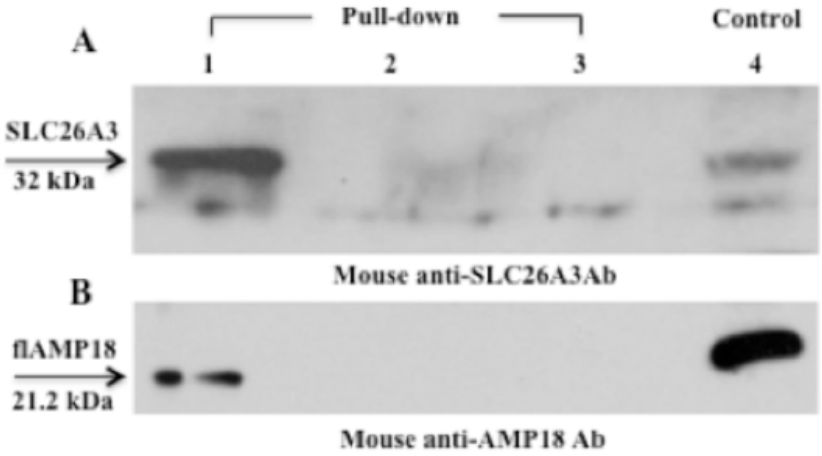


Fig.13 Pull-down experiment. AGS cells extracts after 24 h after transfection with pcDNA3.1-flAMP18 were pulled-down with Ni-NTA agarose and washed with 10 mM imidazole. The Ni-NTA resin was directly loaded on SDS-PAGE and analyzed by western blot by using mouse anti-SLC26A3 (A) and mouse anti-AMP18 antibody (Ab) (B). (Lane 1) AGS cells transfected with pCDNA3.1-flAMP18 encoding for full length AMP18 (flAMP18); (lane 2) AGS cells transfected with pCDNA3.1 as negative control; (lane 3) Ni-NTA alone; (lane 4) control AMP18 expression in AGS cells transfected with pCDNA3.1-flAMP18 encoding flAMP18.

3.3 Expression of SLC26A3 in gastric tissues and gastric cancer cells

On the bases of results obtained, we analyzed the expression profile of SLC26A3 in gastric tissues and in gastric adenocarcinoma cell lines. As reported in Figure. (14 A), a protein of about 86 kDa was detected in gastric tumoral (T) and non-tumoral (N) tissues whereas, as found in the

pull-down experiment (Fig. 13A), a band of about 32 kDa was mainly detected within the AGS adenocarcinoma cells, together with a weaker band of 86 kDa. To better investigate the expression of SLC26A3 in the AGS gastric cancer cell line, both cell lysates and comparable amounts of whole membrane pellets of control and flAMP18 transfected AGS cells re-suspended in 5x Laemmli buffer were analyzed by western blotting. As reported in Fig. 14B, the presence of the 32 kDa band was detected in AGS cell lysates whereas, in the whole membrane pellet, the 86 kDa band was also present with a lower intensity with respect to the 32 kDa band. The 86 kDa protein was also found to be highly expressed in non-tumoral gastric antrum mucosa (Fig. 14B).

3.4 Evaluation of SLC26A3 expression levels in gastric cancer tissues

Taking advantage from our collection of surgical specimens, we then analyzed the expression level of SLC26A3 in human non-tumoral and tumoral gastric tissues. Samples were obtained from patients with a diagnosis of gastric cancer that underwent surgical treatment. 27 paired samples of gastric tissues from same subjects were dissected after intervention in non-tumoral (N) and tumoral (T) area. The clinicopathologic characteristics of the gastric cancer patients are outlined in Table 1. The intestinal type was well differentiated in one, moderately differentiated in four and poorly differentiated in the remaining twentytwo cases, while as far as concerns stage, these were all advanced. Diffuse type GC was poorly differentiated and advanced in all cases. The non-tumoral areas of intestinal type GC showed a variable degree of gastric atrophy with diffuse IM, instead, the peritumoral areas of diffuse type GC showed a variable degree of non dysplastic inflammation. Characterization of non-tumoral gastric mucosa (N) was based on the macroscopic aspect of the normal tissue

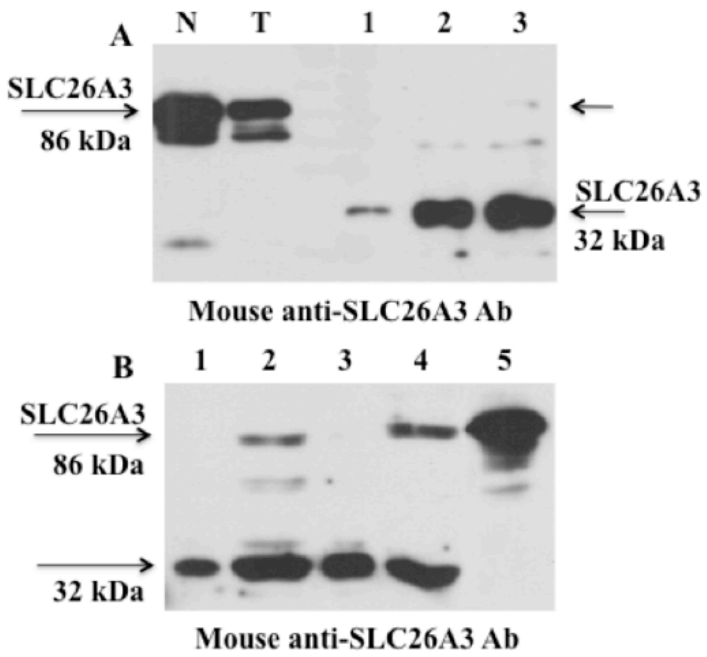


Fig.14 Expression of SLC26A3 in gastric tissues and gastric cancer cells. A. Expression of SLC26A3 in human gastric tissues and AGS cell line using mouse anti- SLC26A3 antibody. Lanes: T, tumoral tissue (20 mg); N, non-tumoral tissue (20 mg); 1e3, AGS cell extracts 10, 20 and 30 mg, respectively. B. Expression of SLC26A3 in non- transfected and transfected AGS cell line with pcDNA3.1-flAMP18. Lanes: 1 and 2, cell lysate (20 mg) and whole membrane pellet, respectively of control AGS cells; 3 and 4, cell lysate (20 mg) and whole membrane pellet of transfected AGS cells, respectively; 5, gastric antrum mucosal extract (20 mg).

compared to the tumoral one, evaluated from the hospital pathologist, and from our previous work showing that AMP18 was highly expressed in gastric non-tumoral tissues but down-regulated or totally absent both at transcription and translation level in GC tissues (Nardone G. et al2008) . Therefore, we first analyzed all the collected tissues for AMP18 expression levels. Fig. 15Aa shows the expression profiles of AMP18 in representative paired non-tumoral (N) and tumoral (T) tissues as evaluated by Western blot analysis on cellular extracts using mouse anti-AMP18 antibody. In all tumoral samples analyzed, it was observed almost total absence of AMP18. Subsequently, the same samples were analyzed by Western blot for SLC26A3 expression levels using mouse anti-SLC26A3 antibody. As reported in Fig. 15Ab, in almost all paired non-tumoral

and tumoral samples analyzed it was observed a reduction of the band intensity of the tumoral sample compared to its paired tumoral one. Statistical evaluation of the expression levels of SLC26A3 in all paired non-tumoral (N) and tumoral (T) samples, evaluated from SLC26A3 bands densitometry and normalized to the corresponding GAPDH level (Fig. 15Ac), showed an average reduced expression level of SLC26A3 in tumoral areas of about 36% (Fig. 15B).

To assess whether the SLC26A3 down-regulation occurred also at mRNA level, real-time PCR analyses were performed on total RNA extracted from three paired non-tumoral and tumoral gastric tissues in which SLC26A3 was down-regulated at protein level. As reported in Fig. 16C, compared to non-tumoral tissues, the statistical evaluation of qRT-PCR results showed a similar decrease of SLC26A3 mRNA levels in tumoral tissues. GAPDH was used as control for qRT-PCR.

Table 1 Clinical and histopathological characteristics of gastric cancer patients

Variable	**n. 27**
Age at surgery (yrs)	
Mean ± SD	64 ± 13
Range	32 - 81
Sex M/F	16/11
Tumor type	
Intestinal	16
Diffuse	11
Stomach region	
Antrum	10
Corpus	9
Fundus	5
Not defined	3
Grade of differentiation	
Well	1 (3.7%)
Moderate	4 (14.8%)
Poor	22 (81.4%)
Stage	
Early	0 (0%)
Advanced	27 (100%)

Immunohistochemical evaluation of SLC26A3 (Fig 12) showed strong

immunostaining for SLC26A3 protein in the cytoplasm of cells from the superficial epithelium to the upper third of the glandular epithelium of colonic and gastric mucosa (Fig. 16A and 16B, respectively). The SLC26A3 immunopositivity was instead lower in intestinal metaplasia (Fig. 16C) and more reduced in the tumoral areas of GC, both in the superficial and glandular epithelium (Fig. 16D). Higher magnification of non-tumoral gastric tissue is reported in Fig. 17.

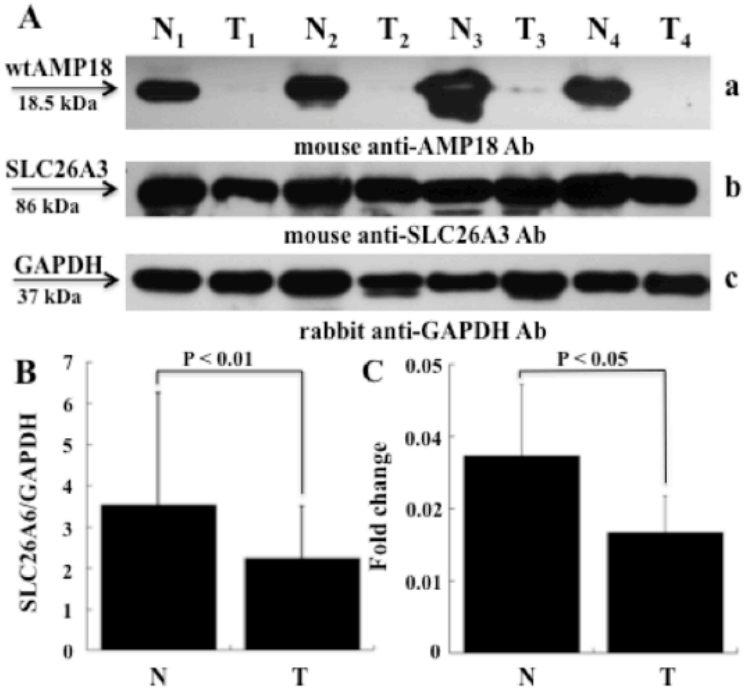


Fig.15 Expression levels of SLC26A3 in human gastric tissues. A. Representative Western blot of equal amounts of cell extracts (20 mg) analyzed in 27 paired non-tumoral (N) and tumoral (T) human gastric samples using mouse anti-AMP18 (a), mouse anti-SLC26A3 (b) and rabbit antieGAPDH (c) antibodies (Ab). B. Densitometric analysis. Statistical eval- uation of the expression levels of SLC26A3 protein in all non-tumoral (N) and tumoral (T) paired samples was calculated from the densitometry of SLC26A3 bands normalized towards the corresponding densitometry of GAPDH bands. C. qRT-PCR analysis. Total RNA was prepared from gastric tissues and analyzed by qRT-PCR for SLC26A3 mRNA level compared to GAPDH mRNA as reference sample. Samples: N and T, non-tumoral and tumoral gastric tissues, respectively. Data from three experiments are reported as mean values ± SD.

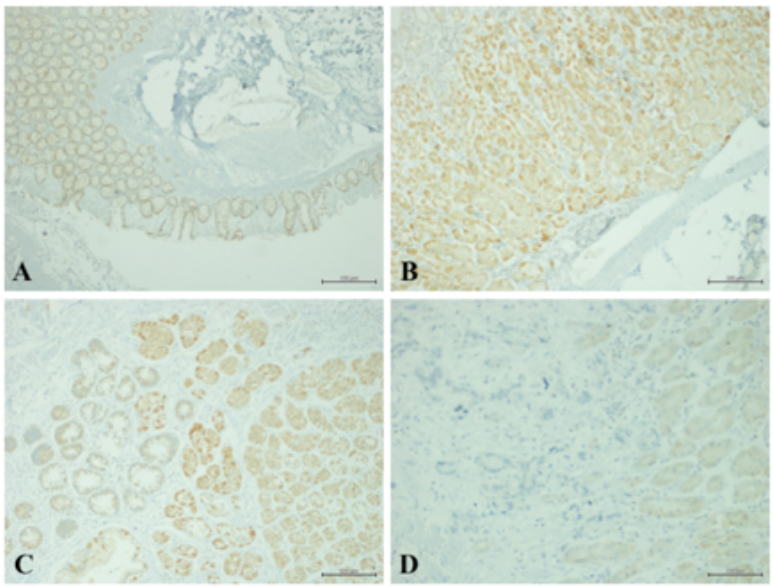


Fig. 16. Immunohistochemical evaluation of SLC26A3 expression. A. Non-tumoral colonic mucosa (positive control) (IHC 20). B. Non-tumoral gastric mucosa. The submucosa shows no positivity (IHC 20). C. Intestinal metaplasia. D. Tumor gastric mucosa (IHC 20 x)

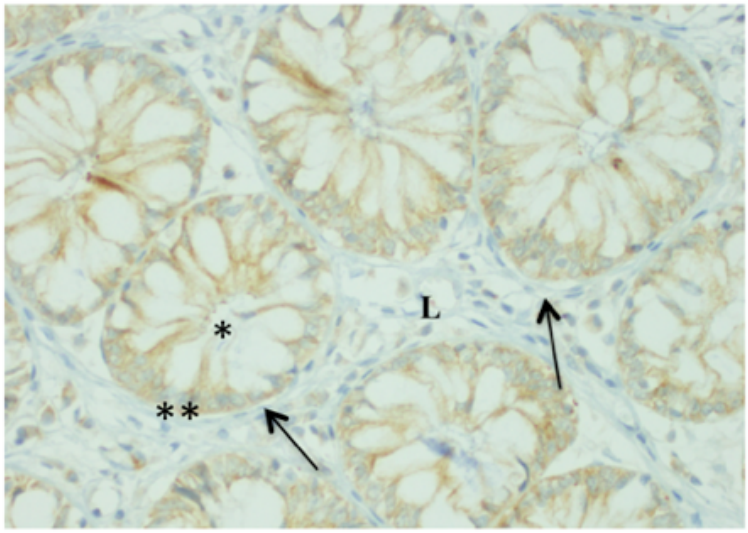


Figure 17. SLC26A3 immunostaining of non-tumoral gastric cell. Cross-section of non-tumoral gastric glands showing the presence of parietal cells and principal cells and typical tubular architecture of the gastric pits. (*) Luminal part of the gastric gland. (**) Basolateral part of the gastric gland. Arrow indicates the basal membrane. L is the lamina propria (connective tissue).

Discussion

Cancer is the third foremost cause of death worldwide; there is no proper early detection and treatment methods available relative to various cancers. Gastrointestinal cancers are among the most rampant malignancies and are fatal if remained uncured. Common treatments are chemotherapy, radiotherapy, and surgery. For this reason, the possibility to dispose of a biomarker for the early detection of the disease is of relevant importance. In fact, biomarker tests started to demonstrate their novel features in the detection and management of patients with different malignancies. Sensitivity and specificity in determining biomarkers are crucial due to clinical cancer detection, surveillance after treatment and therapy selection. A protein marker could be useful in distinguishing malignant from benign, differentiate from other diseases, and identifying each stages with high specificity and sensitivity. Biomarkers are efficient diagnostic agents for detection and even early detection of diseases, are diverse biochemical components such as DNA sequence, RNAs, proteins and metabolites; the final goal of biomarker application is to introduce a sensitive and specific indicator for certain kind of disease. Unfortunately, there is no specific biomarker for many kinds of malignant disease as Gastric cancer. Under this regards, it was a challenge to search for possible informative biomarkers. Being commercially available a sensitive anti-GKN1 antibody, several biological fluids like urine, fecal, gastric juice and serum were first analyze using Western blotting a in the latter case, also specific ELISA Kit for quantitative measurement of GKN1 in serum samples. No GKN1 was possible detect in the analyzed samples. In particular, for ELISA assay, about 30 fresh sera from normal subject were analyzed. Because the given detection limit of both GKN1 antibody and Elisa kit is about 0.15 ng/mL (using as control recombinant GST tagged GKN1), the lack of detection of GKN1 in human sera, unless totally absent, might depend by a far lower concentration of the protein. Therefore, more sensitive detection methods for the protein in the serum (actually not yet available) must be used in order to ascertain this possibility.

The above results prompted us to search for GKN1 at transcription level. It is well known that mRNA in body fluid such as blood, has been

proved as a novel resource to replace conventional tools for disease identification (Juusola J and Ballantyne J, 2003; Li et al., 2006; Miura et al., 2008), and successfully used as cancer-related biomarker (Anker et al., 1999; Rieger-Christ et al., 2003; Wong et al., 2003). In fact, mRNA markers have been the targets for identifying patients with colorectal, breast, lung, and thyroid cancers, and malignant melanoma (Kopreski et al., 2001; Bunn PJ Jr, 2003; Wong et al., 2004; Fugazzola et al., 2002; Kopreski et al., 1999). All these published studies were performed for testing a single mRNA marker. In this scheme, we tried to assess if GKN1 circulating mRNA in serum might represent a biomarker for gastric cancer detection. The expectation was that in the sera of patients with GC, a lower or undetectable level of GKN1 mRNA should have been observed. Despite the strong down-regulation of GKN1 protein levels in gastric cancer, the quite comparable levels of GKN1 mRNA strongly indicated that this down-regulation is not observed at transcription levels thus suggesting translation regulation mechanism for GKN1 expression. The result obtained suggested to direct the search towards other non-invasive gastric cancer biomarker such as long noncoding RNAs (lncRNAs), a recently discovered class of non-coding RNAs (ncRNA) that are emerging as a promising new class of biomarkers for tumour diagnosis (Xu et al., 2014).

By using an affinity pull-down strategy, we then observed in human non-tumoral gastric mucosa the interaction between GKN1 and SLC26A3, a protein expressed in the apical membrane of intestinal epithelial cells, that is supposed to play a critical role in Cl^- absorption and fluid homeostasis together with Na^+/H^+ exchange mediated by Na^+/H^+ exchanger 3 (H. Hayashi et al. 2002, N.C. Zachos et al. 2005).SLC26A3 has also been shown to function as a Cl^-/HCO_3^- exchanger in heterologous expression systems (M.N. Chernova et al. 2003, H. Hayashi et al. 2001, J.E. Melvin et al.1999) and deletion of SLC26A3 by gene targeting produces chloride-losing diarrhea (CLD) in mice, a phenotype similar to CLD (congenital chloride diarrhea first called CCD) in humans (S. Wedenoja et al. 2011). The pull-down strategy used in our study was based on GKN1 biotinylation and MS analysis of affinity-purified proteins on streptavidin magnetic beads. The observed interaction was

confirmed by pull-down experiments using flGKN1 bound to Ni-NTA resin from AGS cell extracts transfected with flAMP18. In this instance, an intense immunoreactive band of about 32 kDa was detected with mouse anti-SLC26A3 antibody. Interestingly, the occurrence of a major SLC26A3 immunoreactive band of 86 kDa was instead evidenced in tumoral and non-tumoral tissues, whereas in the gastric cancer cell line AGS both bands were detected, although the 32 kDa band was found to be much more intense. The 86 kDa band corresponded to the full length protein as predicted on the basis of the theoretical molecular mass deduced from the amino acid sequence of SLC26A3 (84.5 kDa) (C.W. Schweinfest et al.1993, M.K. Byeon et al.1996).

The membrane topology model of SLC26A3 (S. Makela et al. 2002, R.H. Moseley et al. 1999) predicts the existence of a NH_2-terminal domain of about 500 amino acids containing 12 transmembrane segments (A. Bairoch et al. 2005) and an intracellular COOH-terminal region of 250 amino acids containing two protein-protein interaction domains: a STAS (sulfate transporters and anti-sigma factors) domain and a PDZ-interacting domain probably playing a regulatory role (M.R. Dorwart et al. 2008, S.B. Ko, W. Zeng et al. 2004, G. Lamprecht et al. 2002, G. Lamprecht et al 2004, S. Lissner et al. 2010, S. Lissner et al. 2010, H. Rossman et al.2005). Experimental evidence supports the hypothesis that the N- and C-terminal regions are located in the cytosol (M.R. Dorwart et al. 2008). It has been proposed that *N*-glycosylation protects SLC26A3 from proteolytic enzymes in the intestine under physiological conditions, while deglycosylation of SLC26A3 may contribute to the pathogenesis of diarrhea associated with CCD. Trypsin cleavage sites of SLC26A3 are located in extracellular loops of the trans-membrane domain. These sites might be protected from trypsin digestion when HASLC26A3 is glycosylated (H. Hayashi et al.2012, M.K. Byeon et al. 1998). Therefore, the 32 kDa band observed. Therefore, the 32 kDa band observed by Western blotting in AGS cells might result from proteolytic processing of SLC26A3. This band might correspond to the C-terminal region of SLC26A3 probably cleaved at the level of Arg or Lys present in the last extra-cellular loop of the 12 trans-membrane domain model.

It is known that SLC26A3 is expressed in colon tissue and its down-regulation correlates with colon tumor progression (T.M. Antalis et al.1998). In contrast, SLC26A3 expression in gastric tissue was not reported. One of the first publications in this field from Byeon et al. (M.K. Byeon et al.1996), described that there was no expression of DRA in stomach. Wang et al., 2002, further showed mild expression levels of DRA in the small intestine but emphasized its major role as Cl^-/HCO_3^- exchanger in the colon (Z. Wang et al. 2002). Rat DRA-specific mRNA expression was detected only in specific segments of the digestive tract (duodenum, ileum, cecum, proximal colon and distal colon) but not in the stomach (C. Barmeyeret al.,2007). It must be noted however, that SLC26A3 protein expression was detected in the fundic glands region and the pyloric glands region of the stomach in mice (H. Xu, J. Li et al.2001). In addition, the function of SLC26A3 in human gastric tissue was reported by Seidler and Sjoblom, 2012(U. Seidler et al.), and linked to HCO_3^- secretion that originated from the surface cells, since a similar type of secretion was also observed in antral mucosal. Moreover, they also reported that although the expression levels of HCO_3^- in murine stomach has been evaluated in about 50% and 10% of that of the duodenum and distal colon, respectively, these levels should be still sufficient to explain luminal Cl^--dependent HCO_3^- secretory rate important for mucosal defence against very low luminal pH (U. Seidler et al., 2012). In this work, we showed for the first time that SLC26A3 was expressed in human gastric tissue and was also down-regulated in human gastric cancer tissues. In fact, compared to non-tumoral area, the evaluation of SLC26A3 exspression level in the tumoral area showed a statistical significative reduction (about 36%) eventhough no SLC26A3 reduction was observed in 6/27 paired samples. Moreover, the standard deviation values observed for both non-tumoral (N) and tumoral (T) groups clearly indicate a great inter-individual variability among the specimens analyzed. Regarding the Clinicopathological characteristics of the gastric cancer patients (Table 2), it appeared that there was no correlation among tumor type and SLC26A3 down-regulation neverthless, we can postulate that the low SLC26A3 expression might appear in an advanced tumor stage and thus correlated to the tumor prognosis. However, to better

underline the clinical importance of the observed SLC26A3 reduction, a larger population of gastric cancer patients should be analyzed.

Conclusion

In conclusion, although we have shown for the first time the presence of GKN1 mRNA in human serum of healthy subjects as well as in that of patients with diagnosis of gastric cancer, when we analyzed by qRT-PCR these sera, we did not observe significant statistical differences among the two population. Therefore, we could not confirm our hypothesis that serum GKN1 mRNA could serve as non-invasive marker for GC. Moreover, our results suggested that SLC26A3, a new molecular interactor of GKN1, seems to be a potential biomarker of cell tansformation since it was down-regulated during tumor progression both at protein and mRNA levels. Therefore, evaluation of SLC26A3 in human sera might end-up as a successful potential GC biomarker.

5. References

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