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CHAPTER 9

Role of Gastrokine 1 in Gastric Cancer: A Potential Diagnostic Marker and Antitumor Drug

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Abstract: Gastric cancer is still one of the prevalent leading causes of cancer-related deaths worldwide. The high mortality rate is mainly due to late-stage diagnosis, which has a very poor prognosis (five-year survival rates are as little as 3-10%). By contrast, early stage gastric cancer is highly curable with five-year survival rates exceeding 90%. Early detection is therefore the single most important factor influencing the outcome for gastric cancer patients. However, there are currently no biomarkers of acceptable sensitivity and specificity to detect early stage gastric cancer. Gastric infection with *Helicobacter pylori* (*H. pylori*) seems to be a risk factor for gastric cancer. Epidemiological studies have shown that patients that test serum positive to *H. pylori* infection are associated with a three- to six-fold increase in the risk of gastric cancer, findings that are compatible with pathological links between *H. pylori*-associated gastritis, pre-cancerous lesions, and subsequent cancer of the stomach. In a previous study, moving from genomic to proteomic investigation, we have identified a protein, gastrokine 1 (GKN1), a stomach-secreted protein previously named AMP-18 (18kDa Antrum Mucosa Protein) that was highly expressed in normal tissues and significantly down-regulated in *H. pylori* positive patients with respect to *H. pylori* negative subjects. In addition, we have also found that GKN1 is normally expressed in gastric mucosa but not in primary tumours, both at the transcriptional and translational levels. On the basis of these findings, we propose that GKN1 can be used as a new molecular marker that could be useful to predict early cell transformation and might be a potential novel target for gastric cancer therapeutics. Moreover, we have also found that recombinant GKN1 affect cancer cell growth and that the transient expression of GKN1 in human gastric cancer also inhibits cell growth and induces apoptosis, thus suggesting the importance of GKN1 in preventing cancer development in gastric tissues. Finally, we hypothesize that GKN1 might act as a tumor suppressor and thus foresee its importance as an antitumor drug.

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INTRODUCTION

The molecular mechanisms of neoplastic transformation include genetic alterations (point mutations, deletions and gene rearrangements) that lead to the production of altered proteins with respect to their wild-type counterpart (Rosenberg, 2001). These proteins might be specific tumour markers and, as such, they can be considered useful targets for the development of therapeutic and molecular diagnostic strategies. In the past years, a great effort has been devoted to the possibility of performing a detailed genetic analysis of tumour tissues by means of DNA microarray. These techniques include differential display, cDNA membrane arrays (Clontech), cDNA microarrays and serial analysis of gene expression (Oien, *et al*., 2003; Hippo, *et al*., 2002; Mori, *et al*., 2002; El-Rifai, *et al*., 2001; Yoshikawa, *et al*., 2000). However, RNA-based expression strategies are limited by the poor correlation between RNA and protein expression. Moreover, measurements made at the mRNA level will fail to detect posttranslational modifications of proteins and protein isoforms (Orrù, *et al*., 2003; Griffin, *et al*., 2002; Gygi, *et al*., 1999). Gene expression at the mRNA level is not necessarily correlated to the expression of the protein that particular gene codes for. Therefore, we cannot always predict whether it is directly responsible for the phenotypic behavior of the neoplasia. For example, in eukaryotic cells, a different transcriptional and translational activity of a given gene may exist; the expression of this gene can be also post-translationally regulated by its degradation. More recently, the method of global analysis of cellular proteins, known as proteomics, has shown great promise in identifying novel diagnostic and prognostic biomarkers for human cancers (Li, *et al*., 2008; Vlahou and Fountoulakis, 2005; Michener, *et al*., 2002; Petricoin, *et al*., 2002).

Tumour markers are often characterized by the presence of mutations in the amino acid sequence and/or by post-translational modifications (phosphorylation, acetylation) that modify some molecular properties of the protein such as the

isoelectric point and the molecular mass, thus allowing their separation by bidimensional electrophoresis on polyacrylamide gel, followed by their identification by mass spectrometry (2D-MS) (Baek, *et al*., 2004; Orrù, *et al*., 2003). Moreover, tumour associated proteins or peptides can be released in the blood and thus used as serum markers of the neoplasia (Anderson and Anderson, 2003), or they can induce specific serum antibody titers (Mintz, *et al*., 2003).

Gastric cancer, despite its declining incidence rate, remains as the fourth most common cancer and the second cause of cancer-related death worldwide (Ferlay *et al*. 2010) Gastric cancers are often characterized by two distinct histological types of adenocarcinomas, each having different epidemiological and pathophysiological features (Lauren, 1965). The intestinal-type generally evolves through a relatively well-defined sequence of histological lesions, namely non-atrophic gastritis, chronic atrophic gastritis, intestinal metaplasia, and dysplasia (Correa, 1995; Correa, 1992). On the contrary, the diffuse subtype is also preceded by years of chronic *H. pylori*-associated gastritis, although the molecular pathways and histologic changes involved in progression to cancer are less characterized. A multistage process in which several alteration of genetic and epigenetic nature accumulates characterizes the progress of gastric carcinogenesis. The chronic active gastritis is the primary condition related to *H. pylori* colonization, causing an elevated risk of gastric adenocarcinoma, both intestinal type and diffuses type. (Bornschein and Malfertheiner, 2011). Anyhow, the propensity to develop disease is an aspect that remains unclear but may depend on host characteristic, particular bacterial factors such as the virulence of the infecting strains or to the specific interactions between host and microbe, besides the environmental factors (Kabir, 2009).

GASTROKINES AND GASTRIC CANCER

The importance of *H. pylori* infection in promoting gastric cancer is already well known. Exposure of gastric epithelial cells to the bacterium determines a complex inflammatory and immune reactions associated to production of neutrophils, release of cytokines and reactive oxygen species (ROS) and induction of nitric oxide synthase (NO) that in turn may cause genetic alterations leading to cancer in a subset of subjects. *H. pylori* infection, a necessary but not sufficient cause, is

associated with a 5.9-fold increased risk of non-cardia gastric cancer in a combined analysis of nested case control studies (Helicobacter and Cancer Collaborative Group, 2001). A prospective cohort study in Japan highlighted that gastric cancer developed in 2.9 % of 1,246 *H. pylori*-infected persons over 7.8 years. In contrast, none of the 280 *H. pylori*-uninfected persons developed gastric cancer (Wu, *et al*. 2000). It is noteworthy that the prevalence of *H. pylori* infection and incidence of gastric cancer vary among different countries. This highlights the importance of host genetic factors and other factors beyond *H. pylori* infection in the carcinogenesis of gastric cancer (Leung, *et al*. 2008).

In order to search for possible informative biomarkers for gastric cancer, the protein profile of malignant and non-malignant gastric tissues was extensively analysed. From these analyses, a secreted protein, specifically expressed in gastric mucosa, gastrokine 1 (GKN1), formerly foveolin or AMP18 kDa (AMP18) was identified. The expression of this protein was found to be markedly reduced or completely absent in of stomach carcinomas. Gastrokine1 belongs to a family of genes encoding stomach-specific proteins formed by 3 known members: GKN1 (Martin, *et al*., 2003), GKN2 (Du, *et al*., 2003), and GKN3 (Menheniott, *et al*., 2010). Gastrokine expression is coordinately down-regulated in *H. pylori* infection and frequently is lost in gastric carcinoma (Moss, *et al*., 2008; Nardone, *et al*., 2007). Collectively, these studies argue that gastrokines participate at the *H. pylori* host susceptibility and function as gastric tumor-suppressor genes. In recent studies, TFF1, a small protein synthesized and secreted by the gastric epithelial cells, was demonstrated, in most gastric adenocarcinomas, to be down-regulated or absent (Kjellev *et al*., 2009; Machado *et al*., 1996). Moreover, a previous study showed that the TFF1-deficient mice developed antropyloric adenoma (Lefebvre *et al*., 1996) therefore TFF1 appears to be involved in gastric carcinogenesis. The recent demonstration of a GKN2/trefoil factor (TFF1) heterodimer in gastric mucus suggests that gastrokines may have an important role in the regulation of TFFs function (Westley, *et al*., 2005).

The human GKN1 gene has been localized in a region of chromosome 2p13 of about 6 kb and contains 6 exons 2 (Yoshikawa, *et al*., 2000). The protein is made of 185 amino acid residues and contains a N-terminal signal peptide. GKN1 is expressed only in normal human stomach, in all areas (cardia, body and antrum),

but is absent in gastric adenocarcinomas, gastro-oesophageal adenocarcinoma cell lines, and other normal and tumour gastro-intestinal tissues including placenta and ovary (Oien, *et al*., 2004). The protein is characterized by the presence of a central domain known as BRICHOS (Martin, *et al*., 2003; Shiozaki, *et al*., 2001). BRICHOS domain is made of 100 amino acid residues and contains several motifs of likely functional significance. It is present also in other proteins such as BRI2 (Sanchez-Pulido, *et al*., 2002), related to familial British dementia; chondromodulin-I (ChM-I), associated with chondrosarcoma; and lung surfactant protein C (SP-C), linked with Respiratory Distress Syndrome

CHARACTERIZATION OF GKN1 EXPRESSION IN NORMAL AND GASTRIC TUMOR TISSUES

Expression of GKN1 in normal gastric tissues and in pre-malignant and malignant gastric tissues.

Employing immunohistochemistry (IHC), western blot (WB), immunofluorescence (IF) and flow cytometry assay, we investigated on the expression level, biological activity and regulation mechanism of GKN1. The expression of GKN1 was examined in normal gastric tissue, pre-malignant (non-atrophic gastritis, chronic atrophic gastritis, intestinal metaplasia and gastric dysplasia) and different malignant histopathological types of gastric cancer (intestinal and diffuse). The goal of these experiments was to ascertain whether there is any relation between the different histopathological states and the levels of GKN1 expression.

Methodology. A large population of patients who underwent upper gastrointestinal endoscopy in our university hospitals were enrolled. Tissues of gastric tumors and their corresponding distant non-tumor tissues were collected from 28 subjects with gastric cancer (intestinal and diffuse types) who underwent surgery. Interview has been performed focusing on diet and lifestyle habits, family history and other toxic environmental factors. A positive gastric cancer family history was also carefully verified. All tissue specimens were diagnosed by pathologist and confirmed by an experienced pathologist. Specimens were also collected for culture test, histological and immunohistochemical tests and for molecular approach (RT-PCR, Western Blot).

The clinical and histological characteristics of the 65 dyspeptic patients (36 *H. pylori* negative and 29 *H. pylori* positive) are reported in Table **1A**. Only 5 of the *H. pyl*ori negative cases had minimal infiltration of lymphomonocytes in the lamina propria. *H. pylori* infection was present in 29/65 patients suffering from active gastritis (25 mild-moderate and 4 severe); moreover 10 patients showed mild or moderate atrophy that was associated in 4 cases with focal antral complete IM. Among the 29 subjects with infection 21 (72%) were positive for CagA strain. The characteristics of the 28 patients with Gastric Cancer (GC) (20 intestinal and 8 diffuse) are outlined in Table **1B**.

Table 1: Clinical and histopathological characteristics of the dyspeptic (A) and gastric cancer (B) patients

A	***H. pylori*-negative n. 36**	***H. pylori*-positive n. 29**
Age (yrs) Mean ± SD	44 ± 15	39 ± 20
Sex M/F	20/16	18/11
LMN (lymphocytes and monocytes)		
Absent	31 (86%)	0 (0%)
Mild	5 (14%)	8 (28%)
Moderate	0 (0%)	17 (58%)
Severe	0 (0%)	4 (14%)
PMN (polymorphonuclear cells)		
Absent	36 (100%)	0 (0%)
Mild	0 (0%)	10 (32%)
Moderate	0 (0%)	12 (41%)
Severe	0 (0%)	7 (27%)
Atrophy		
Absent	36 (100%)	19 (66%)
Mild	0 (0%)	6 (21%)
Moderate	0 (0%)	4 (14%)
Severe	0 (0%)	0 (0%)
IM (intestinal metaplasia)		
Absent	36 (100%)	25 (86%)
Present	0 (0%)	4 (14%)
Anti-*H. pylori* IgG	0 (0%)	29 (100%)
Anti-CagA IgG	0 (0%)	21 (72%)

A	***H. pylori*-negative** **n. 36**	***H. pylori*-positive** **n. 29**
B	**Intestinal type** **n. 20**	**Diffuse type** **n. 8**
Age (yrs) Mean ± SD	63 ± 11	54 ± 14
Sex M/F	12/8	4/4
Grade of Differentiation		
Well	4 (20%)	0 (0%)
Moderate	8 (40%)	0 (0%)
Poor	8 (40%)	8 (100%)
Stage		
Early	4 (25%)	0 (0%)
Advanced	16 (75%)	8 (100%)
Anti-*H. pylori* IgG	16 (80%)	6 (75%)
Anti-CagA IgG	14 (70%)	3 (38%)

The intestinal type was well differentiated in 4, moderately differentiated in 8 and poorly differentiated in the remaining 8 cases, while four were in the early stage and the remaining 16 in the advanced stage. Diffuse type of GC was poorly differentiated and advanced in all cases. The non-tumoural areas of intestinal type of GC showed a variable degree of atrophic gastritis with diffuse IM, instead, the peritumoural areas of diffuse type of GC showed a variable degree of non-dysplastic inflammation. *H. pylori* infection was not revealed in the tumoural areas in all cases. It is interesting to point out, however, that *H. pylori* infection was detected, in the peritumoral areas, in 6 cases (4 early and 2 advanced). Anti-*H. pylori* IgG antibodies were detected in 16/20 patients with intestinal histotype GC and in 6 out of 8 with diffuse type GC. Anti-CagA antibodies were detectable in 17/28 cases (14 intestinal and 3 diffuse histotype). Tissue distribution of GKN1 expression was evaluated by immunohistochemistry and by Western blots analysis on tissues and cell extracts obtained from normal and pathological human samples. For immunohistochemical studies, 7-micron sections of frozen unfixed gastric tissues were used. The percentages of immunoreactive cells were scored as follows: 0-5% = negative; 5-25% = low staining; 25-50% = moderate staining; >50% = intense staining. The study was carried out using a commercial anti-GKN1 monoclonal antibody from Abnova (Taiwan). As reported in Fig. **1**, the

immunohistochemical evaluation of the surgical specimens generally evinced a lack of GKN1 in cancer specimens.

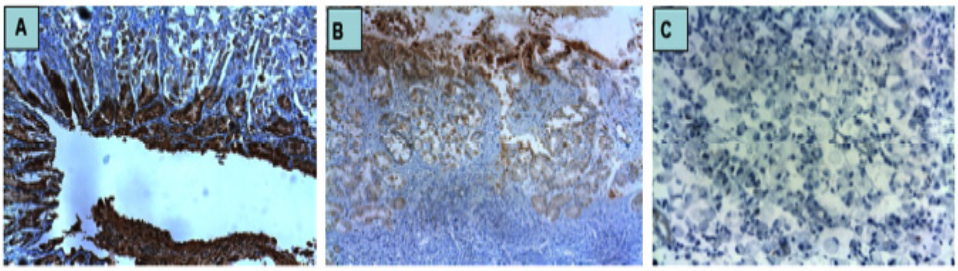

Figure 1: Immunohistochemical detection of GKN1 protein in surgical specimens from: A) Normal Gastric Mucosa; B) Intestinal metaplasia; C) Intestinal type gastric cancer; (Rippa *et al*. 2011).

GKN1 expression was also evaluated by Western blot (WB) analysis (Fig. **2**). GKN1 protein expression was detected in all *H. pylori*-negative subjects whereas GKN1 was dramatically down-regulated in *H. pylori*-positive gastritis. Densitometric evaluation of the bands showed a significantly lower level in *H. pylori*-positive patients compared to the *H. pylori*-negative subjects (0.19 ± 0.02 *vs.* 0.44 ± 0.07, $p < 0.005$). In contrast, GKN1 protein expression was not detectable in any of the tumoral areas but was expressed in non-tumoral areas. The hystopathological aspect of the tumor specimens correlated the GKN1 expression with tumor subtype and was a strong indication that GKN1 could be employed as a useful prognostic biomarker.

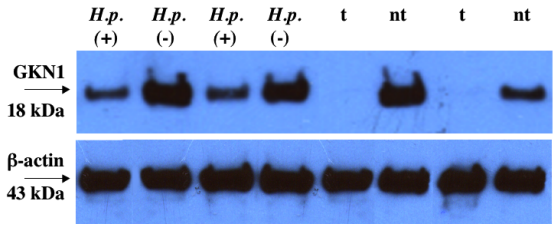

Figure 2: Expression of GKN1 in human specimens. *H.p.* (+), *H. pylori*-positive cases; *H.p.* (-), *H. pylori*-negative cases; t, tumoral area; nt, non-tumoral area.

This hypothesis could be enforced by the analysis of the expression of other new possible markers of gastric adenocarcinoma like the intestinal trefoil factor (ITF),

the enhancer of zeste homolog 2 (EZH2) or transgelin in order to correlate their expression with clinico-pathological factors (Huang, *et al*., 2008; Moss, *et al*., 2008; Mattioli, *et al*., 2007; Dhar, *et al*., 2005; Yamachika, *et al*., 2002).

CHARACTERIZATION OF THE MOLECULAR AND FUNCTIONAL ROLE OF GKN1

Effect of GKN1 Over-Expression in Gastric Cancer Cell Lines

A lthough a detailed function remains to be identified, GKN1 appears to be important in maintaining mucosal integrity and could play a role in cell proliferation and differentiation. GKN1 protects the intestinal mucosal barrier by acting on specific tight junction proteins and stabilizing peri-junctional actin by increasing accumulation of specific tight and adherens junction proteins and also promotes healing by facilitating restitution and proliferation following injuries (Walsh-Reitz *et al*., 2005; Toback, *et al*., 2003. The protective function of GKN1 might be ascribed to the presence of the BRICHOS domain. In fact, it has been found that the BRICHOS domain of another gastric protein, GKN2, originally named GDDR, related to GKN1, is also down-regulated in gastric cancer. This protein was described as TFZ1 due to its binding to tumor suppressor proteins such as the trefoil protein 1 (TFF1). Taken together, this remarks suggested that this interaction seems to be important in the regulation of the integrity of the mucosa (Baus-Loncar *et al*., 2007; Otto *et al*., 2006; Bruce *et al*., 2005). The down-regulation of GKN1 observed in *H. pylori*-infected gastric mucosal epithelial cells (Nardone, *et al*., 2007), the loss of its expression in gastric cancer in precancerous lesion (Moss *et al*., 2008; Nardone *et al*., 2008) and its absence in human gastric cancer cell lines (AGS and MKN28) (Oien *et al*., 2004; Segal *et al*., 1996; Motoyama, *et al*. 1986) indicated that GKN1 participates in the host response to *H. pylori* and might exert a gastric tumor suppressor function (Du *et al*., 2003). It is plausible that GKN1 acts as a tumor suppressor though the regulation of epigenetic modifications, cell cycle or loss of heterozygosis, as it was observed for other tumor suppressor genes such as TFF1. (Carvalho, *et al*., 2002). Moreover, conflicting results have been reported: Martin *et al*., (2003) have proposed that GKN1 protein exerts mitogenic effects on IEC-6 cells when compared with EGF, whereas Shiozaki, *et al*., (2001) have found that this protein can inhibit growth in MKN28 cells after transfection. Therefore, if GKN1 shows

the ability to stimulate or inhibit the proliferation of gastric cells, it is important to define its role in inflammatory bowel disease (IBD) or in gastric carcinogenesis. To this aim, we initiated a systematic study to define the molecular mechanism of the protein in the processes of proliferation and signalling transduction. Thus, besides the analysis of GKN1 expression in tissues specimens at normal, premalignant and malignant gastric mucosa level, we investigated on the effects of GKN1 on gastric cancer cells either by treating the cell with exogenous recombinant GKN1 (rGKN1) or by its cDNA-mediated overexpression.

Methodology. Recombinant GKN1 (rGKN1) was expressed in *P. pastoris* and purified according to the procedure reported by Pavone *et al*., 2012. Cell proliferation was assessed using a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma). Cell proliferation was expressed as the percentage of formazan formation in the treated samples, compared to the negative control treated only with solvent (Pavone *et al*., 2012). For transient expression of GKN1 in gastric cancer cells, the coding sequence of full-length human GKN1 cDNA was cloned into the eukaryotic expression vectors pcDNA 3.1 (Invitrogen USA). As experimental systems, human gastric carcinoma epithelial cell lines AGS and MKN28 (Motoyama, *et al*., 1986) were used. Cells were cultured in DMEM F12 (Dulbecco's modified Eagle'smedium) in a humidified incubator with 5% CO_2 at 37° C and transfected in 60 mm-diameter dishes with expression plasmids (construct and void vector; 4μ ((Rippa *et al*., 2011).

The ability of rGKN1 to affect cell proliferation *in vitro* using different cell cultures was evaluated. The results reported in Fig. **3** showed that rGKN1 possessed an anti-proliferative activity after 24 h of treatment of the AGS cell line at a concentration of 0.5 μM. It was also observed that the anti-proliferative action was dose dependent. In fact, the inhibition of cell growth increased at increasing rGKN1 concentration. This activity resulted instead less marked using a non-gastric cancer cell line (H1355) or a normal cell line (HEK 293), although for the latter higher concentrations of rGKN1 were required. These results strongly indicated that rGKN1 was able to exert its function specifically on gastric cancer cells.

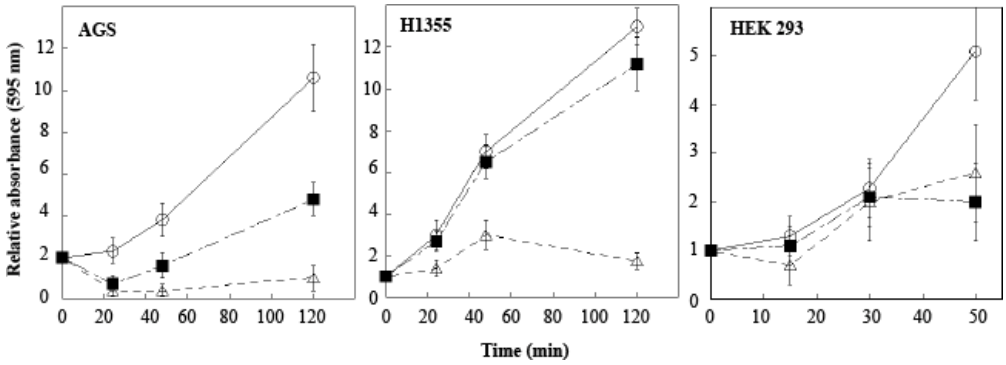

Figure 3: Effect of recombinant GKN1 on cell growth. The effect of GKN1 on cell growth was evaluated by the MTT assay after incubation of the cells with GKN1 at different times and concentrations. AGS cell growth in the absence (○) or in the presence of 0.5 μM (■) or 3 μM (△) GKN1. H1355 cells growth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

The effects of the transient expression of GKN1 on cell growth and apoptosis were evaluated by cytofluorimetry (Fig. **4**). It was found that the expression of GKN1 in MKN28 cells, compared to control, reduced cell growth (Rippa, *et al*., 2011). In agreement with our results, Shiozaky *et al*., 2001 reported that transfection with CA11 gene reduced colony formation of MKN-28 gastric cancer cells. Also a novel mammal gastrokine, GKN3, reduced the growth of GKN3-overexpressing MKN28 cells (Menheniott, *et al*., 2010). Because tissue repair is linked to many signals coming from the local environment it is important to understand why gastric cells are addressed to undergo apoptosis, proliferation or survive, following the inflammatory process. Therefore, gastric epithelial cell apoptosis could be influenced by GKN1 during inflammation. The Fas-ligand (FasL) system has been recognized as the major pathway for the induction of apoptosis in a variety of human normal and neoplastic cells (Nagata, 1996; Itoh and Nagata, 1993; Suda, *et al*., 1993). The CD95 receptor (APO-1/Fas) belongs to TNFR superfamily and is a type I transmembrane protein (Itoh, *et al*., 1991). Therefore, restoration of GKN1 could increase the level of expression of Fas, normally extremely low, in MKN28 cells (Osaki, *et al*., 2001). We observed that restoration of GKN1 expression in negative GC cells (MKN28) induces a significant increase of Fas expression and this appears to be specific as also demonstrated by the enhancement of the Fas mRNA transcription. CD95 receptor

and ligand system was also described in infection by *H. pylori*, however in this case other apoptotic factors are involved as well (Rudi *et al*., 1998). These include TRIAL and its receptor subtypes (Martin, *et al*., 2004; Yang, *et al*., 2003). Because *H. pylori*-associated chronic gastritis involves apoptosis of gastric epithelial cells by activation of the CD95 receptor and ligand system, cells overexpressing GKN1 showed an increase in apoptosis that is mainly due to the exposure of the MKN28 cells to FasL compared to that observed in the control cells. In addition, when FasL binds to Fas, intracellular death caspases are activated, resulting in apoptotic demise of the cell (Chen, *et al*., 1999). Also, a time-dependent increase in apoptotic cells was observed in GKN1 transfected cells and, as expected, cleaved forms of caspase-3, normally present as a 32 kDa inactive precursor (Zou, *et al*., 1997), were found. This finding, evaluated by Western blot and fluorimetric assay suggest that GKN1-transfected cells were signalled to die (Schlegel, *et al*., 1996; Wang, *et al*., 1996; Nicholson, *et al*., 1995). However, GKN1- induced apoptosis is suppressed when caspase-3 and caspase-8 are inhibited (Yoon, *et al*., 2011).

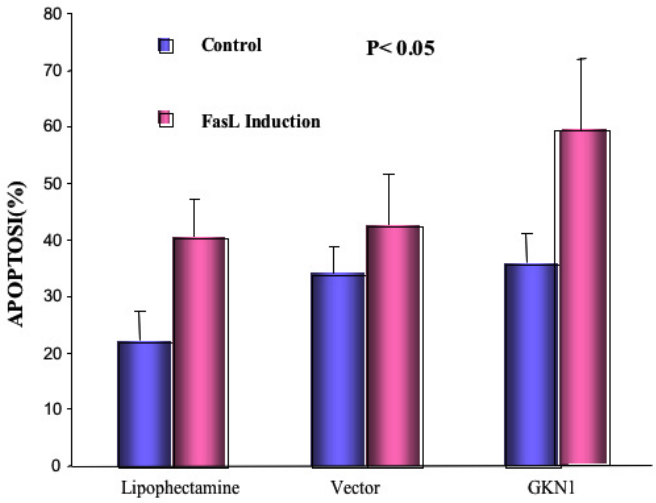

Figure 4: Effects of transfected GKN1 on cell death. Cell death was measured after transfection by TUNEL assay. Representative flow cytometry of cells treated with lipophectamine, cells transfected with empty pcDNA 3.1, cells transfected with GKN1 before and after incubation with antibody anti-FasL. Value from five independent experiments with similar results.

CONCLUSION

In the present study we have investigated expression of GKN1, mRNA and protein, in tissues specimen from normal and *H. pylory* infected gastric mucosa, atrophic gastritis, intestinal metaplasia, dysplasia, gastric cancer cell line and gastric cancer. We found that GKN1 is down-regulated and lost progressively in *H. pylori*-infected gastric mucosa and GC tissues but is abundant present in the adjacent non-cancer tissues as well as in normal gastric mucosa in agreement with other previous studies. The immunohistochemical analysis of human specimens showed a progressive decrease of GKN1 expression thus suggesting that the decrease of GKN1 is an early event in gastric precancer or cancer, and the presence of GKN1 may protect gastric mucosa from neoplasia. Thus GKN1 may be a potential biomarker candidate for early detection of gastric cancer. Moreover, the effects of GKN1 on gastric cancer cells were investigated; our data indicated that restoration of protein in cancer cells induced Fas-pathway mediated apoptosis and suggest that GKN1 may play an important role as a tumor suppressor gene. In conclusion, GKN1 appears as a modulator of apoptosis during the early stages of acute gastric injury and may influence tissue repair and be instrumental in determining the individual host response.

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CONFLICT OF INTEREST

The author(s) confirm that this chapter content has no conflict of interest.

REFERENCES

Anderson, N.L. and Anderson, N.G. 2002. The human plasma proteome: history, character, and diagnostic prospects. *Molecular and Cellular Proteomics*, 1, pp. 845-67. Erratum in: *Molecular and Cellular Proteomics*; 2003, 2, pp.50.

Baek, H.Y., Lim, J.W., Kim, H., Kim, J.M., Kim, J.S., Jung, H.C. and Kim, K.H. 2004. Oxidative stressrelated proteome changes in *Hilicobacter pylori*-infected human gastric mucosa. *Biochemical Journal*, 15, pp. 291-9.

Baus-Loncar, M., Lubka, M., Pusch, C.M., Otto, W.R., Poulsom, R. and Blin, N. 2007. Cytokine Regulation of the Trefoil Factor Family Binding Protein GKN2 (GDDR/TFIZ1/blottin) in Human Gastrointestinal Epithelial Cells. *Cellular Physiology and Biochemistry*, 20, pp. 193-20.

Bornschein, J. and Malfertheiner, P. 2011. Gastric carcinogenesis. *Langenbecks Arch Surg*, 396, pp. 729-42.

Bruce, R., Westley, S., Griffin, M. and May, F.E.B. 2005. Interaction between TFF1, a Gastric Tumor Suppressor Trefoil Protein, and TFIZ1, a Brichos Domain Containing Protein with Homology to SP-C. *Biochemistry*, 44, pp. 7967-75.

Carvalho, R., Kayademir, T., Soares, P., Canedo, P., Sousa, S., Oliveira, C., Leistenschneider, P., Seruca, R., Gött, P., Blin, N., Carneiro, F. and Machado, J.C. 2002. Loss of heterozygosity and promoter methylation, but not mutation, may underlie loss of TFF1 in gastric carcinoma. *Laboratory Investigation*, 82, pp. 1319-26.

Chen, M.K., Strande, L.F., Beierle, E.A., Kain, M.S., Geldziler, B.D. and Doolin, E.J. 1999. Fas-Mediated Induction of Hepatocyte Apoptosis in a Neuroblastoma and Hepatocyte Coculture Model. *Journal of Surgical Research*, 84, pp. 82-7.

Correa, P. and Piazuelo, M,B. 2008. Natural history of *Helicobacter pylori* infection. *Digestive and Liver Disease*, 40, pp. 490-6.

Correa, P. 1992. Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Research*, 52, pp. 6735-40.

Correa, P. 1995. *Helicobacter pylori* and gastric carcinogenesis. *American Journal of Surgical Pathology*, 19 pp. S37-43.

Dhar, D.K., Wang, T.C., Tabara, H., Tonamoto, Y., Maruyama, R., Tachibana, M., Kubota, H. and Nagasue, N. 2005. Expression of Trefoil Factor family members correlates with patients prognosis and neoangiogenesis. *Human Cancer Biology*, 11, pp. 6472-78.

Du, J.J., Dou, K.F., Peng, S.Y., Wang, W.Z., Wang, Z.H., Xiao, H.S., Guan, W.X., Liu, Y.B. and Gao, Z,Q. 2003. Down-regulated full-length novel gene GDDR and its effect on gastric cancer. *Zhonghua Yi Xue Za Zhi*, 83, pp. 1166-68.

El-Omar, E.M., Rabkin, C.S., Gammon, M.D., Vaughan, T.L., Risch, H.A., Schoenberg, J.B., Stanford, J.L., Mayne, S.T., Goedert, J., Blot, W.J., Fraumeni, J.F. Jr and Chow, W.H. 2003. Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. *Gastroenterology*, 124, pp. 1193-201.

El-Rifai, W., Frierson, H.F., Harper, J.C., Powell, S.M. and Knuutila, S. 2001. Expression profiling of gastric adenocarcinoma using cDNA array. *International Journal of Cancer*, 92, pp. 832–38.

Ferlay, J., Parkin, D.M. and Steliarova-Foucher, E. 2010. Estimates of cancer incidence and mortality in Europe in 2008. *Eur J Cancer*, 46, pp. 765-81.

Forman, D., Newell, D.G. and Fullerton, F. 1991. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from prospective investigation. *BMJ*, 302, pp. 1302-05.

Goldstone, A.R., Quirke, P. and Dixon, M,F. 1996. *Helicobacter pylori* infection and gastric cancer. Journal of Pathology, 179, pp. 129-37.

Griffin, T.J., Gygi, S.P., Ideker, T., Rist, B., Eng, J., Hood, L. and Aebersold, R. 2002. Complementary profiling of gene expression at the transcriptome and proteome levels in *Saccharomyces cerevisiae*. *Molecular and Cellular Proteomics*, 1, pp. 323-33.

Gygi, S.P., Rochon, Y., Franza, B.R. and Aebersold, R. 1999. Correlation between protein and mRNA abundance in yeast. Molecular and Cellular Biology, 19, pp. 1720-30.

Helicobacter and Cancer Collaborative Group. 2001. Gastric cancer and Helicobacter pylori: a combined analysis of 12 case control studies nested within prospective cohorts. *Gut*, 49, pp. 347-53.

Hippo, Y., Taniguchi, H., Tsutsumi, S., Machida, N., Chong, J.M., Fukayama, M., Kodama, T. and Aburatani, H. 2002. Global gene expression analysis of gastric cancer by oligonucleotide microarrays. *Cancer Research*, 62, pp. 233-40.

Holcombe, C. 1992. *Helicobacter pylori*: the African enigma. *Gut*, 33, pp. 429-431.

Huang, Q., Huang, Q., Chenn, W., Wang, L., Lin, W., Lin, J. and Lin, X. 2008. Identification of transgelin as a potential novel biomarker for gastric adenocarcinoma based on proteomics technology. *Journal of Cancer Research and Clinical Oncology*, 134, pp. 1219-27.

Itoh, N. and Nagata, S. 1993. A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. *Journal of Biological Chemistry*, 268, pp. 10932-7.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y. and Nagata, S. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell*, 66, pp. 233-43.

Kabir, S. 2009. Effect of Helicobacter pylori eradication on incidence of gastric cancer in human and animal models: underlying biochemical and molecular events. *Helicobacter*, 14, pp. 159-171.

Kjellev, S. 2009. The trefoil factor family – small peptides with multiple functionalities. *Cell Mol Life Sc*, 66, pp. 1350-1369.

Lauren, P. 1965. The Two Histological Main Types of Gastric Carcinoma: Diffuse and So-Called Intestinal-Type Carcinoma. An Attempt at a Histo-Clinical Classification. *Acta Pathologica et Microbiologica Scandinavica*, 64, pp. 31-49.

Lefebvre, O., Chenard, M.P., Masson, R. Linares J, Dierich A, LeMeur M, Wendling C, Tomasetto C, Chambon P. and Rio MC. 1996. Gastric mucosa abnormalities and tumorigenesis in mice lacking the pS2 trefoil protein. *Science*, 274, pp. 259-262.

Leung, W.K., Wu, M.S., Kakugawa, Y., Kim, J.J., Yeoh, K.G., Goh, K.L., Wu, K.C., Wu, D.C., Sollano, J., Kachintorn, U., Gotoda, T., Lin, J.T., You, W.C., Ng, E.K., Sung, J.J. and Asia Pacific Working Group on Gastric Cancer. 2008. Screening for gastric cancer in Asia: current evidence and practice. *Lancet Oncol*, 9, pp. 279-87.

Li, W., Li, J.F., Qu, Y., Chen, X.H., Qin, J.M., Gu, Q.L., Yan, M., Zhu, Z.G. and Liu, B.Y. 2008. Comparative proteomic analysis of human gastric cancer. *World Journal of Gastroenterology*, 14, pp. 5657-64.

Machado, J.C., Carneiro, F., Blin, N. and Sobrinho-Simoes M. 1996 Pattern of pS2 protein expression in premalignant and malignant lesion of gastric mucosa. *Eur J Cancer Prev*, 5, pp. 169-79.

Martin, J.H., Potthoff, A., Ledig, S., Cornberg, M., Jandl, O., Manns, M.P., Kubicka, S., Flemming, P., Athmann, C., Beil, W. and Wagner, S. 2004. Effect of *H. pylori* on the Expression of TRAIL, FasL and their Receptor Subtypes in Human Gastric Epithelial Cells and their Role in Apoptosis. *Helicobacter*, 9, pp. 371-86.

Martin, T.E., Powell, C.T., Wang, Z., Bhattacharyya, S., Walsh-Reitz, M.M., Agarwal, K. and Toback, F.G. 2003. A novel mitogenic protein that is highly expressed in cells of the gastric antrum mucosa. *American Journal of Physiology Gastrointestinal and Liver Physiology*, 285, pp. G332-43.

Mattioli, E., Vogiatzi, P., Sun, A., Abbaddessa, G., Angeloni, G., D'Ugo, D., Trani, D., Gaughan, J.P., Vecchio, F.M., Cevenini, G., Persiani, R., Giordano, A. and Claudio, P.P. 2007. Immunohistochemical analysis of pRb2/p130, VEGF, EZH2, p53, p16(INK4A), p27(KIP1), p21(WAF1), Ki-67 expression patterns in gastric cancer. *Journal of Cellular Physiology*, 210, pp. 183-191.

Menheniott, T.R., Peterson, A.J., O'connor, L., Lee, K.S., Kalantzis, A., Kondova, I., Bontrop, R.E., Bell, K.M. and Giraud, AS. 2010. A Novel Gastrokine in Mammals Marks Gastric Atrophy and shows evidence of adaptive gene loss in human. *Gastroenterology*, 138, pp. 1823-35.

Michener, C.M., Ardekani, A.M,. Petricoin, E.F., Liotta, L.A. and Kohn, E.C. 2002. Genomics and proteomics: application of novel technology to early detection and prevention of cancer. *Cancer Detection and Prevention*, 26, pp. 249-55.

Mintz, J.P., Kim, H., Kim-Anh, D., Wang, W., Zinner, R.G., Cristofanilli, M., Arap, M.A., Hong, W.K., Troncoso, P., Logothetis, C.H., Pasqualini, R., and Arap, W. 2003. Fingerprinting the circulating repertoire of antibodies from cancer patients. *Nature Biotechnology*, 21, pp. 57-63.

Mori, M., Mimori, K., Yoshikawa, Y., Shibuta, K., Utsunomiya, T., Sadanaga, N., Tanaka, F., Matsuyama, A., Inoue, H. and Sugimachi, K. 2002. Analysis of the gene-expression profile regarding the progression of human gastric carcinoma. *Surgery*, 131, pp. S39-47.

Moss, S.F., Lee, J.W., Sabo, E., Rubin, A.K., Rommel, J., Westley, B.R., May, F.E., Gao, J., Meitner, P.A., Tavares, R. and Resnick, M.B. 2008. Decreased expression of gastrokine 1 and trefoil Factor Interacting Protein TFIZ1/GKN2 in gastric cancer: influence of tumor histology and relationship to prognosis. *Clinical Cancer Research*, 14, pp. 4161-7.

Motoyama, T., Hojo, H., and Watanabe, H. 1986. Comparison of seven cell lines derived from human gastric carcinomas. *Acta Pathologica Japonica*, 36, pp. 65-83.

Nabewera, H.M. and Logan, R.P. 1999. Epidemiology of *Helicobacter pylori* infection: transmission, translocation and extragastric reservoirs. *Journal of Physiology and Pharmacology*, 50, pp. 711-22.

Nagata, S. 1996. Apoptosis by death factor. *Cell*, 88, pp. 355-65.

Nardone, G, Martin, G., Rocco, A., Rippa, E., La Monica, G., Caruso, F. and Arcari, P. 2008. Molecular expression of Gastrokine 1 in normal mucosa and in *Helicobacter pylori*-related preneoplastic and neoplastic gastric lesions. *Cancer Biology and Therapy*, 7, pp. 1890-95

Nardone, G., Rippa, E., Martin, G., Rocco, A, Siciliano, R.A., Fiengo, A. Cacace, G., Malorni, A., Budillon, G. and Arcari, P. 2007. Gastrokine 1 expression in patients with and without *Helicobacter pylori* infection. *Digestive and Liver Disease*, 39, pp. 122-29.

Nicholson, W.D., Ali, A., Thornberry, N.A., Vailancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., Munday, N.A., Raji, S.M., Smulson, M.E., Yamin, T.T., Yu, V.L. and Miller, D.K. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature*, 376, pp. 37-43.

Oien, K.A., McGregor, F., Butler, S., Ferrier, R.K., Downie, I., Bryce, S., Burns, S. and Keith, W.N. 2004. Gastrokine 1 is abundantly and specifically expressed in superficial gastric epithelium, down-regulated in gastric carcinoma, and shows high evolutionary conservation. *Journal of Pathology*, 203, pp. 789-97.

Oien, K.A., Vass, J.K., Downie, I., Fullarton, G. and Keith, W.N. 2003. Profiling, comparison and validation of gene expression in gastric carcinoma and normal stomach. *Oncogene*, 22, pp. 4287-300.

Orrù, S., Caputo, I., D'Amato, A., Ruoppolo, M. and Esposito, C. 2003. Proteomics Identification of Acylacceptor and Acyl-donor Substrates for Transglutaminase in a Human Intestinal Epithelial Cell Line: implications for celiac disease. *Journal of Biological Chemistry*, 278, pp. 31766-73.

Osaki, M., Kase, S., Kodani, I., Watanabe, M., Adachi, H. and Ito, H. 2001. Expression of Fas and Fas ligand in human gastric adenomas and intestinal-type carcinomas: correlation with proliferation and apoptosis. *Gastric Cancer*, 4, pp. 198-205.

Otto, W.R., Patel, K., McKinnell, I., Evans, M.D., Lee, C.Y., Frith, D., Hanrahan, S., Blight, K., Blin, N., Kayademir, T., Poulsom, R., Jeffery, R., Hunt, T., Wright, N.A., McGregor, F. and Oien, K.A. 2006. Identification of blottin: A novel gastric trefoil factor family-2 binding protein. *Proteomics*, 6, pp. 4235-45.

Parsonnet, J., Friedman, G.D., Oremtreich, N. and Vogelman, H. 1997. Risk for gastric cancer in people with CagA positive or CagA negative *Helicobacter pylori* infection. *Gut*, 40, pp. 297-301.

Pavone, L.M., Del Vecchio, P., Mallardo, P., Altieri, F., De Pasquale, V., Rea, S., Martucci, N.M., Di Stadio, C.S., Pucci, P., Flagiello, A., Masullo, M., Arcari, P. and Rippa, E. 2013. Structural characterization and biological properties of human gastrokine 1. *Mol Biosyst*, 9, pp. 412-21.

Petricoin, E.F., Ardekani, A.M., Hitt, B.A., Levine, P.J., Fusaro, V.A., Steinberg, S.M., Mills, G.B., Krieg, R.C., Paweletz, C.P., Liotta, L.A. and Petricoin, E.F. 2002. Clinical proteomics for cancer biomarker discovery and therapeutic targeting. *Technology in Cancer Research and Treatment*, 4, pp. 263-72.

Pisani, P., Parkin, D.M., Bray, F. and Ferlay, J. 1999. Estimates of the worldwide mortality from 25 cancers in 1990. *International Journal of Cancer*, 83. pp. 18-29.

Rippa, E., La Monica, G., Allocca, R., Romano, M.F., De Palma, M. and Arcari, P. 2011. Overexpression of gastrokine 1 in gastric cancer cells induces Fas-mediated apoptosis. *Journal of Cellular Physiology*, 226, pp. 2571-8.

Rosenberg, S.A. 2001. Progress in human tumour immunology and immunotherapy. *Nature*, 411, pp. 380-4.

Rudi, J., Kuck, D., Strand, S., von Herbay, A., Mariani, S.M. Krammer, P.H., Galle, P.R. and Stremmel, W. 1998. Involvement of the CD95 (APO-1/Fas) receptor and ligand system in *Helicobacter pylori*-induced gastric epithelial apoptosis. *J Clin Invest*, 102, pp. 1506–1514.

Sanchez-Pulido, L., Devos, D. and Valencia, A. 2002. BRICHOS: a conserved domain in proteins associated with dementia, respiratory distress and cancer. *Trends in Biochemical Science*, 27, pp. 329-32.

Schlegel, J., Peters, I., Orrenius, S., Miller, D.K., Thornberry, N.A., Yamin, T.T. and Nicholson, D.W. 1996. CPP32/apopain is a key interleukin 1 beta converting enzyme-like protease involved in Fasmediated apoptosis. *Journal of Clinical Investigation*, 271, pp. 1841-4.

Segal, E.D., Falkow, S. and Tompkinset, L.S. 1996. *Helicobacter pylori* attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins. *Proceedings of the National Academy of Sciences USA*, 93, pp. 1259-64.

Shiozaki, K., Nakamori, S., Tsujie, M., Okami, J., Yamamoto, H., Nagano, H., Dono, K., Umeshita, K., Sakon, M., Furukawa, H., Hiratsuka, M., Kasugai, T., Ishiguro, S. and Monden, M. 2001. Human stomach-specific gene, CA11, is down-regulated in gastric cancer. *International Journal of Oncology*, 19, pp. 701-7.

Suda, T., Takahashi, T., Golstein, P. and Nagata, S. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell*, 75, pp. 1169-78.

Toback, F.G., Walsh-Reitz, M.M., Musch, M.W., Chang, E.B., Del Valle, J., Ren, H., Huang, E. and Martin, T.E. 2003. Peptide fragments of AMP-18, a novel secreted gastric antrum mucosal protein, are mitogenic and motogenic. *American Journal of Physiology Gastrointestinal and Liver Physiology*, 285, pp. G344-53.

Vlahou, A. and Fountoulakis, M. 2005. Proteomic approaches in the search for disease biomarkers. *Journal of Chromatogr B Analytical Technologies in the Biomedical Life Sciences*, 814, pp. 11-9.

Walsh-Reitz, M.M., Huang, E.F., Musch. M.W., Chang, E.B., Martin, T.E., Kartha, S. and Toback, F.G. 2005. AMP18 protects barrier function of colonic epithelial cells: role of tight junction proteins. *American Journal of Physiology Gastrointestinal and Liver Physiology*, 289, pp. G163-71.

Wang, X., Zelenski, N.G., Yang, J., Sakai, J., Brown, M.S. and Goldstein, J.L. 1996. Cleavage of sterol regulatory element binding proteins (SREBPs) by cpp32 during apoptosis. *EMBO Journal*, 15, pp. 1012–20.

Watanabe, T., Tada, M. and Nagai, H. 1998. *Helicobacter pylori* infection induces gastric cancer in Mongolian gerbils. *Gastroenterology*, 115, pp. 642-48.

Westley, B.R., Griffin, S.M. and May, F.E. 2005. Interaction between TFF1, a gastric tumor suppressor trefoil protein, and TFIZ1, a brichos domain-containing protein with homology to SP-C. *Biochemistry*, 44, pp. 7967-75.

Whary, M.T., Sundina, N., Bravo, L.E., Correa, P., Quinones, F., Caro, F. and Fox, J.G. 2005. Intestinal helminthiasis in Colombian children promotes a Th2 response to Helicobacter pylori: possible implications for gastric carcinogenesis. *Cancer Epidemiology, Biomarkers and Prevention*, 14, pp. 1464-9.

Wu, C.Y., Tseng, J.J., Chou, M.M., Lin, S.K., Poon, S.K. and Chen, G.H. 2000. Correlation between Helicobacter pylori infection and gastrointestinal symptoms in pregnancy. *Adv Ther*, 17, pp. 152-8.

Yamachika, T., Werther, J.L., Bodian, C., Babyatsky, M., Tatematsu, M., Yamamura, Y., Chen, A. and Itzkowitz, S. 2002. Intestina Trefoil Factor: a marker of poor prognosisi in gastric carcinoma. *Clinical Cancer Research*, 8, pp. 1092-99.

Yang, Y., Deng, C.S., Peng, J.Z., Wong, B.C.Y., Lam, S.K. and Xia, H.H.X. 2003. Effect of *Helicobacter pylori* on apoptosis and apoptosis related genes in gastric cancer cells. *Molecular Pathology*, 56, pp. 19-24.

Yoon, J.H., Song, J.H., Zhang, C., Jin, M., Kang, Y.H., Nam, S.W., Lee, J.Y. and Park, W.S. 2011. Inactivation of Gastrokine 1 gene in gastric adenomas and carcinomas. *Journal of Pathology*, published on line in Wiley Online Library.

Yoshikawa, Y., Mukai, H., Hino, F., Asada, K. and Kato, I. 2000. Isolation of two novel genes, own-regulated in gastric cancer. *Japanese Journal of Cancer Research*, 91, pp. 459-63.

Zou, H., Henzel, W.J., Liu, X., Lutschg, A. and Wang, X. 1997. Apaf-1, a Human Protein Homologous to C. elegans CED-4, Participates in Cytochrome c–Dependent Activation of Caspase-3. *Cell*, 90, pp. 405-13.