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Down-Regulated Gene Expression of *GKN1* and *GKN2* as Diagnostic Markers for Gastric Cancer

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***Abstract*—**Gastric Cancer (GC) has high morbidity and fatality rate in various countries. It is still one of the most frequent and deadly diseases. Gastrokine1 (*GKN1*) and gastrokine2 (*GKN2*) genes are highly expressed in the normal stomach epithelium and play important roles in maintaining the integrity and homeostasis of stomach mucosal epithelial cells. In this study, 47 paired samples that were grouped according to the types of gastric cancer and the clinical characteristics of the patients, including gender and average of age. They were investigated with gene expression analysis and mutation screening by monitoring RT-PCR, SSCP and nucleotide sequencing techniques. Both *GKN1* and *GKN2* genes were observed significantly reduced found by (Wilcoxon signed rank test; $p<0.05$). As a result of gene screening, no mutation (no different genotype) was detected. It is considered that gene mutations are not the cause of gastrokines inactivation. In conclusion, the mRNA expression level of *GKN1* and *GKN2* genes statistically was decreased regardless the gender, age, or cancer type of patients. Reduced of gastrokine genes seem to occur at the initial steps of gastric cancer development.

***Keywords*—**Diagnostic biomarker, gastric cancer, nucleotide sequencing, semi-quantitative RT-PCR.

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I. Background

GASTRIC cancer is the fourth most prevalent malignancy in worldwide, it is second leading cause of cancer mortality and affecting about one million people per year [1]. In the United States, an estimated 21,320 cases of gastric cancer (13,020 men and 8,300 women) reported and 10,540 patients were dead from this disease in 2012 [2]. There is a geographic diversification in the occurrence of gastric cancer [2]. Most cases are recorded in South America, China, Japan and significantly less in the Western Europe [3]. Gastric cancer consists of two pathological variants [3]. The development of intestinal tumors is characterized by progression of several sequential steps that start with gastritis and then progresses to mucosal atrophy, intestinal metaplasia, dysplasia, and carcinoma with subsequent metastatic dissemination [4]. Adenocarcinomas arising from gastric epithelium are the most common malignancies of the stomach (90% of cases) [4]. Malignancies arising from connective tissue (sarcoma) and from lymphatic (lymphoma) are less common [5]. Genetic and environmental factors are two important risks in gastric carcinogenesis [6]. Gastric cancer is associated with a number of risk factors; such as Helicobacter pylori infection, age, family history, smoking, alcohol consumption, obesity, and diet [5].

The *GKN1* and *GKN2* are abundant and specifically expressed proteins in the superficial gastric epithelium; they have been isolated from the cells of the gastric mucosa in several mammalian species [6]. Both of them are expressed in normal mucosal epithelial of the whole stomach to protect and maintaining the integrity of gastric [7].

The human *GKN1* gene (AMP18), a member of BRICHOS superfamily has been localized in a region of chromosome 2p13, the gene spans about 6 KB in size and contains 6 exons [8]. It is expressed only in normal human stomach, in all areas (antrum, body and cardia), but it is absent or consistently down-regulated from gastric adenocarcinoma, gastro-esophageal adenocarcinoma cell lines and other gastrointestinal tumors [9]. *GKN2* is a secretory peptide of human gastric surface mucous cells (SMCs) [9]. It forms disulfide-linked heterodimers with the trefoil factor family (TFF) peptide, binding to TFF2 was also reported [9] [10]. GKN1 and GKN2 have high homology of 26% of the amino acid residues [10]. The *GKN1* and *GKN2* genes are located in close proximity on the same chromosomes in the genomes of both mice and humans [11]. Gastrokines have major clinical significance; they likely participate in the host mucosal response to H. Pylori and via anti-proliferative or epithelial homeostatic activity, may act as a stomach-specific tumor suppressors [6], [9], [10]. The influence of *GKN1* on cell growth was evaluated, demonstrating that the *GKN1* down-expression reduced colony formation, inhibited cell growth, and induced G2/M arrest of gastric cancer cells [10]. The down-expression of the other member in gastrokines family, such as *GKN3* gene, it has been also inhibit cell proliferation in gastric epithelial cell lines [11].

In the present study, we aimed to determine the possible relationship between *GKN1* and *GKN2* genes, and gastric cancer by monitoring the mRNA expression analysis and mutation screening, in order to investigate the role of *GKN1* and *GKN2* genes in gastric cancer.

II. Materials and Methods

A. Tissue Specimen

Normal and cancerous gastric tissue specimens were obtained from a total 47 patients (47 normal controls and 47 tumors). The samples were collected from the Department of General Surgery of Gaziantep University. Thirty one pairs

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male and five pairs females (57-68 years) gastric adenocarcinoma, nine pairs male and two pairs female (4-58 years) gastric diffuse cancer were enrolled in this study. Biopsies were taken from the tumor and the tumor free corpus, the tissue samples of the gastric stored at -80˚C until further analysis. This study was approved by the local ethical committee, in accordance with the declaration of Helsinki.

B. RNA Extraction and RT- PCR

Total RNA was extracted by Rneasy Total RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sample with (A260- A320) / (A280- A320) ratios less than 1.7 and/or yields less than 0.5 µg total RNA were excluded from subsequent analysis. RNA was converted into cDNA using ProtoScript First Strand cDNA Synthesis Kit (BioLabs, England). The newly synthesized cDNA was amplified by conventional PCR with five pairs of specific primers, were designed by SDSC workbench online primer design program (Table I) and a pair of primer were designed for *GAPDH* (housekeeping gene). PCR reaction mixture was performed by using MJ Research, AB Applied Biosystem thermal cyclers. Agarose gel electrophoresis (2% w/v) is used to check the efficiency of PCR reactions and used to expression measurements, and stained with ethidium bromide (EtBr) to make the DNA visible under UV light.

C. Agarose Gel and Expression Analysis

Our cDNA samples were electrophoresed in 2% agarose gel to separate and measure mRNA expression level of *GKN1* and *GKN2* genes and normalized with *GAPDH* expression level. In this study, the image of agarose gel was captured and quantitated expression level of mRNA by ImageJ software program (version 1.46r, downloaded from http://imagej.nih.gov/ij) [12]. The statistical analysis of mRNA expression was carried out using Wilcoxon signed rank test. Significance was assumed for values $p \leq 0.05$.

TABLE I
PRIMER SEQUENCES, PCR PRODUCT SIZE OF FIVE REGIONS OF *GKN1* AND *GKN2* GENES AND OPTIMAL ANNEALING TEMPERATURE

Primers	Sequence 5'to 3'	Optimal Annealing Temperature	PCR Product Size
GKN1-1			
F.Primer	CTCATTCAGGTCCATGCTTGC	57.4°C	350 bp
R.Primer	CATCAAGGGATTGAATGGAGGG		
GKN1-2			
F.Primer	CCTCCATTCAATCCCTTGATGC	51°C	360 bp
R.Primer	AAAAACCACTGGAGCCCATA		
GKN2-1			
F.Primer	AGAGATATCCACATCTTCAAGCCC	58.5°C	253 bp
R.Primer	GCATGGATGTTAATGATGGCGG		
GKN2-2			
F.Primer	CAATGGTGGCAATGTTCAGGAG	57°C	278 bp
R.Primer	CCAGAGGGTTGTACTTGACCC		
GKN2-3			
F.Primer	AGAAACAGGCTCTGGACAACA	61°C	312 bp
R.Primer	TGACTTTTGAGTGTAAACCAAGG		

D. Mutation Analysis

PCR-Single strand Conformation Polymorphism (SSCP) and nucleotide sequence analysis were applied for both *GKN1* and *GKN2* genes to screen and monitor the probable variations in the sequence. The fragments which display different electrical mobility were assessed via nucleotide sequence analysis. In this study, ABI 3130X nucleotide sequence analyzer (Singapore) was used. The PCR fragments of the *GKN1* and *GKN2* were excised from the agarose gel and utilized as a source of DNA template for sequence specific PCR amplification.

III. RESULTS

A. Gene Expression Result

The quantitative mRNA expression level of *GKN1* and *GKN2* tumor samples was decreased compared to expression of normal samples (*GKN1*; P= 0.00/ *GKN2*; P= 0.00). The results were statistically high significant (Figs 1 and 2).

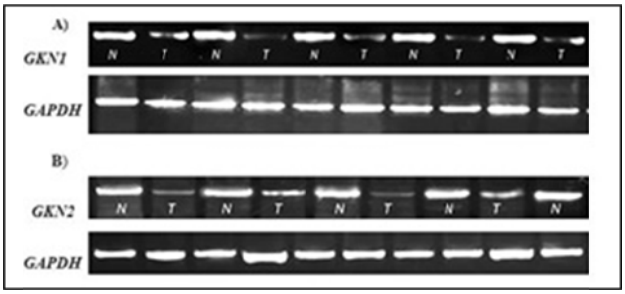


Fig. 1 The result of mRNA expression level of *GKN1* and *GKN2* by 2% agarose gel electrophoresis. A) *GKN1* expression in normal controls and tumors of gastric cancer. B) *GKN2* expression in normal controls and tumors of gastric cancer

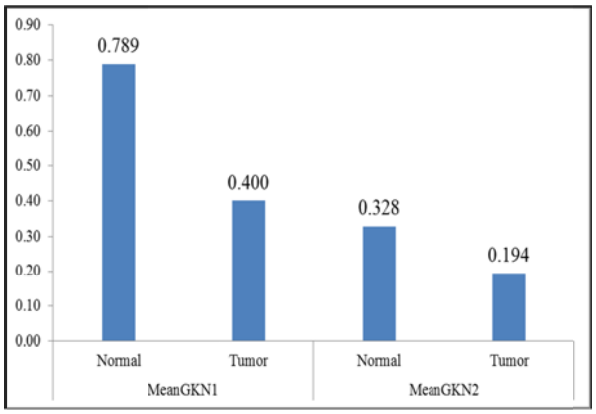


Fig. 2 The mRNA expression level of *GKN1/GAPDH*, and *GKN2/GAPDH* genes in both normal and tumor samples

The mRNA of *GKN1/GABDH* was weakly expressed in tumor samples than the normal controls. In addition, mRNA of *GKN2/GABDH* expression in gastric tissue was significantly and progressively down regulated in tumor samples according to normal controls (Fig. 2).

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B. Mutation Result

In the present study, the mRNA sequences of *GKN1* and *GKN2* genes were screened. It was tried to find different genotypes (different bands). All PCR products of the whole *GKN1* and *GKN2* sequences, were electrophoresed on a 7% polyacrylamide gel. The SSCP results were showed no different bands. In order to confirm SSCP results, the fragments which showed no variation were excised from the gel and analyzed by nucleotide sequencer. The DNA sequence of *GKN1* and *GKN2* gene was obtained from the NCBI website, to compare the resulting DNA sequences of patient samples (Query Sequence) with the reference sequence. No variation was found in the sequence of PCR template for our target region after comparing with the reference sequence (Fig. 3).

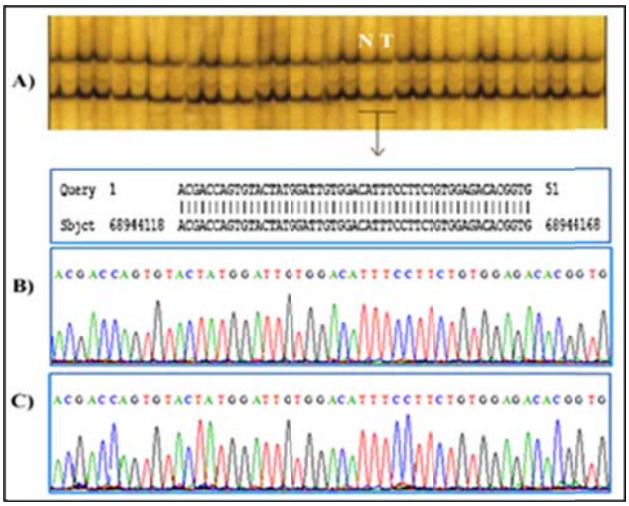


Fig. 3 The mutation screening result of *GKN1* gene. A) The result of 7% polyacrylamide gel image after staining with silver nitrate for *GKN1* as a result of SSCP, no different band (genotypes) was observed. B) The normal sample of the *GKN1* gene was analyzed by sequencer ABI 3130. C) The patient sample of the *GKN1* gene was analyzed by sequencing, no variation found

IV. Discussion

In the present study, we investigate probable mutations and determine mRNA expression level of *GKN1* and *GKN2* genes in gastric cancer patients. Gastrokines are stomach mucus cell-secreted proteins are specifically expressed in gastric mucosa to protection and may be involved in maintaining the integrity of the gastric mucosal epithelium and promoting epithelial restoration of gastrointestinal cells, mediating injury reparation of gastric mucosa [9]. GKN1, a stomach-specific protein also named as 18 kDa antrum mucosa protein (AMP-18) or foveolin belongs to the gastrokine family of gastric mucus cell-secreted proteins [9].

GKN1 may act as a tumor suppressor gene in gastric carcinogenesis because of the reduced mRNA expression level of *GKN1* in patients infected by *H. pylori* and the most complete absence of its expression in gastric cancer, and precancerous lesions [13]. *GKN1* has a protective effect by addition aggregation of specific tight and adherent junction proteins and also protecting their loss after injury [13]. The presence in GKN1 of the BRICHOS domain might explain its protective role [13]. In addition, the BRICHOS domain is present in another gastric protein GKN2, known as TFIZ1, because of its homology to GKN1 [16]. This protein is involved in the binding of tumor suppressor proteins such as the trefoil protein 1 (TFF1) (16). Such interaction could be important in the regulation of the integrity of the mucosa [16].

GKN2 is a secretory peptide of human gastric surface mucous cells (SMCs) [16], [18]. It forms disulfide-linked heterodimers with the trefoil factor family (TFF) consisting of 60 amino acid residues. It acts as gastric tumor suppressor, that protects gastric epithelial cells from damage but can promote invasive properties of tumor cells [11]. *GKN1* and *GKN2* show pronounced similarity 26% of the amino acid residues in the mature proteins are identical and 56% are semi-conserved genes [10]. Both peptides are synthesized via precursors typical of secretory proteins [10]. GKN1 and GKN2 differ remarkably by an additional cysteine residue in GKN2 at position 38 which is linked via a disulfide bond to Cys-58 of TFF1 [6]. Thus, mature GKN2 (consisting of 164 amino acid residues and TFF1 form a disulfide-linked heterodimer whereas GKN1 does not form such heterodimers [6].

Significant loss of *GKNs* expression in gastric adenomas cancer was detected by [10]. Although genetic and epigenetic alterations of *GKN1* were rarely detected in gastric adenocarcenomas, loss of copy number and mRNA transcript of *GKN1* were frequently observed in gastric carcinomas. In addition, transfection of wild-type *GKN1* resulted in inhibition of cell proliferation and increasing in apoptosis [14]. Oien et al. [9], Moss et al. [17] and previous study showed that loss of *GKN1* and *GKN2* expression are found at both the mRNA and protein levels in gastric cancer, particularly of the diffuse type. *GKN1* is one of the most abundant transcripts in normal stomach, and is down regulated in gastric carcinoma [15]. *GKN1* is likely to be important in the normal gastric function and may play a role in gastric mucosal protection [17].

In our study, the mRNA expression level of *GKN1* and *GKN2* genes was significantly lost (down-regulated) (Fig. 1). Yoshikawa et al. [8] reported the *GKN1* and *GKN2* expression down regulated, is compatible with our results were down regulated.

Previous studies have demonstrated and carried out mutational analysis of the *GKN1* and *GKN2* gene in 40 gastric adenomas and 81 cancers. Unexpectedly, there were no somatic mutations of the gene in gastric adenomas and cancers, reporting that somatic mutations may not be a cause of *GKN1* and *GKN2* inactivation [14]. Our result compatible with above, we were applied mutation analysis technique (SSCP) for 47 pair of variable type of gastric cancer. The study is unable to detect any different genotypic band, subsequently nucleotide sequence analysis did not detect any mutation (Fig. 3).

A recent study showed association between *GKN2* and *GKN1* expression, a close correlation between the expression of *GKN1* and *GKN2* (P=0. 0074) a novel function of *GKN2* inhibition of

the effects of *GKN1* on cell proliferation, viability and apoptosis in the maintenance of gastric mucosal homeostasis [19]. Interestingly, ectopic *GKN2* expression significantly suppressed *GKN1* induced anti-growth signaling by inhibiting miR-185 expression, and inducing epigenetic modification [19]. Furthermore, *GKN2* expression was regulated in a *GKN1*-dependent manner by inactivation of the NF-κB signaling pathway [19].

In conclusion, a statistical significant association was constituted between the reduced level of both *GKNs* and gastric cancer pathogenesis relied on normal gastric tissue samples. In order to understand the investigation between gastric cancer and biomarker; further analysis is necessary.

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