Original article:

The expression of BCL-G in leukemia and gastrointestinal tissues

Alenzi FQ[1], Al-bukhari TA[2], Al-Rabea MW[3], Alenazi RG[4], Alenizy WO[5]

Abstract:

Background & Objective:- BCL-G is a novel protein of Bax (BCL-G associated protein X) that induces caspase-mediated apoptosis. These proteins play important roles in regulating apoptosis in both normal or neoplastic cells, however their cellular and tissue distribution remains to be determined. Thus, the current study objective was to elucidate the distribution and expression pattern of BCL-G in normal and malignant gastrointestinal human tissues. ***Methods:-*** The distribution and expression of BCL-G was measured by immunohistochemistry using a rabbit monoclonal antibody against BCL-G in formalin-fixed, paraffin embedded, benign or malignant human tissue. ***Results:-*** A variable pattern of positive expression of BCL-G was observed within all the tissues studied. BCL-G expression was found to be localized to the cytoplasmic paranuclear granules of the epithelialium in the majority of organs examined. Intensity of BCL-G staining was associated withthe maturation state of benign tissue. ***Conclusion:-*** Here we demonstrate that BCL-G exhibits a specific tissue distribution pattern that appears to correlate with cellular differentiation. While such distribution patterns are complex they provide an intriguing insight into overall function that would require further investigation to fully elucidate their physiological/pathological significance.

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Introduction

BCL-G has been shown to be highly conserved in humans and many mammals being responsible for encoding BCL-G_L and BCL-G_S, where the former has a wide tissue distribution while, the latter has been detected only in the human testis using PCR.

BCL-G_S was found to contain only the BH3 domain and therefore capable of inducing apoptosis, in contrast, BCL-G_L, does not induce apoptosis (REF1). More importantly, BCL-G is found on human chromosome 12 with decreased expression observed in human prostate or breast cancer samples, suggesting that it functions as an anti-cancer gene (REF2-3). However, sequence analysis of BCL-G in several human cancers including urinary bladder or sequamous cell carcinoma did not reveal any mutation thereby excluding its role as a tumor suppressor gene (REF4-5). Interestingly, BCL-G has some structures in common with BFK (Bcl-2 Family Kia), which contains BH2 and BH3 that confer pro-apoptotic activity (REF6). BCL-G interacts with a transport protein complex facilitating protein trafficking inside the cell rather than inducing apoptosis (REF7). BCL-G has been shown to be expressed in many mouse tissues including spleen, lungs and testis (REF8).

The aim of this study was to determine the levels and cellular localization of BCL-G using immune histochemical (IHC) techniques in human gastrointestinal tissues.

1. Faris Q. Alenzi, College of Appl.Med.Sci, Prince Sattam bin Abdulaziz University, Al-Kharj, Saudi Arabia
2. Talat A. Al-bukhari, Dept. of Immunology, College of Medicine, UQU, Makkah, Saudi Arabia
3. Mohamed W.Al-Rabea, Dept. of Immunology, College of Medicine, KAU, Jeddah, Saudi Arabia
4. Reda G. Alenazi, Dept. of Public Health, MOH, Arar, Saudi Arabia
5. & Wail O. Alenizy, Dept. of Ophthalmology, KKH, Harf Albatin, Saudi Arabia

Correspondence to: Faris Q. Alenzi, Professor of Immunology College of Applied Medical Sciences, Prince Sattam bin Abdulaziz University (PSAU), Saudi Arabia, Email: fqalenzi@ksu.edu.sa

Materials and methods

Tissue Specimens & Reagents

Formalin-fixed, 3mm sections were cut from paraffin-embedded normal or malignant human tissue and placed on saline-coated slides. In this archival retrospective study, and in order to test a range of antibody titers, we examined an average of 4 sections from each specimen. Tumors were taken from patients during surgery between January 2014 and January 2016, tissue samples obtained from many the hospitals, KAMC, KFU, DAS, and PSAU. Normal tissues were resected adjacent to the tumor site as part of the normal surgical procedure. In all cases both Research Ethics Committee approvals and patient written informed consent, were given in all cases.

Immunohistochemistry (IHC)

Specific secondary antibody (peroxidase labeled polymer) conjugated with goat anti-mouse immunoglobulins were incubated in Tris-HCl buffer containing carrier protein and an anti-microbial agent (DAKO; K4006)., Tissue sections were then diluted 1:50 for 1 h, and incubated at room temperature. Slides were removed from the Sequenza for addition of substrate-chromogen. Where present (such as in liver), endogenous biotin was blocked and, test sections containing malignant tissue and controls were de-waxed in three changes of xylene, hydrated in three changes of absolute alcohol and one of 70% alcohol then finally into water. These sections were washed in tap water for 10 min and then dehydrated. Nuclei were stained with hematoxylin; and sections were mounted in DAKO paramount. All dilutions and the extensive washes between steps were carried out at room temperature using phosphate buffered saline unless otherwise specified.

Sections from kidney (which are known to be sensitive to the antibody BCL-G) were included as a positive control in every experiement. Negative controls were duplicates of the test sections where the primary antibody was substituted by the matched isotype Ab control Antigen sensitivity to fixation and processing of the tissue was tested in lung sections (used in this way as positive control) immunostained with monoclonal anti E-cadherin. E-cadherin is known to be sensitive to fixation methods and processing duration. Thus, it was assumed that matching of, staining patterns, indicated that any variable staining pattern with Mab was due to differences in fixation and processing times of the original tissue specimens. BCL-G Mab was sourced from (Abcam, Cambridge, MA, USA). Based on earlier preliminary work to determine the appropriate concentrations and dilutions to use, BCL-G Mab was diluted as 1:100 with PBS antibody diluents.

Heat-Mediated Antigen Retrieval using Microwave

Since some epitopes in formalin fixed sections are not available to the antibody, pre-treatment was required by heating in citrate buffer (other calcium-chelating buffers can also be used). We found it essential to mount the sections on saline coated slides. These slides were microwaved at 800W for 15 min in 3 min cycles.

Analysis of immunoreactivity staining was as follows

Five visual fields at x200 magnification were counted by two pathologists, blinded as to the identity of each slide. . The mean intra-observer coefficient of variation for repeat counts was less than 4%. Intensity of staining was assessed by a visual scoring system which defined this staining as either negative (-), weakly positive (1+), positive (2+), or strongly positive (3+). The distribution of staining was further classified in terms of whether expression was membranous or cytoplasmic. If the latter, it was further subdivided into whether it was diffuse or granular. The presence of the degree of background staining was also recorded.

We considered positive control tissues as indicative of correctly prepared tissue and appropriate staining techniques. A positive control section was included in every experiement for each antibody used. The positive control tissue was examined first in order to ascertain that all the reagents were functioning properly. Observation of the presence of a brown colored end product at the site of the target antigen was taken as indicative of positive reactivity if negative staining was appearent, these test specimens were considered invalid.

A negative control was included in every experiment to confirm the specificity of the primary antibody. The negative control tissue was examined after the positive control tissue to verify the specific labeling of the target by the primary antibody. The absence of specific staining in the negative control tissue was taken as confirmation of the lack of antibody cross-reactivity to cells/cellular components. If specific staining occurred in any negative control tissue, these test specimens were regarded as invalid.

On a technical level, and as noted in the results section, the positive controls required a long duration of microwaving for successful antigen retrieval Extended microwaving meant that it was important to ensure that tissue sections adequately adhered to the slides. This was achieved by the use

of 3-(Triethoxysilyl) propylamine (saline) coated slides. Some tissues were more fragile and tended to be destroyed by this duration of heating. Other researchers have found the use of autoclaving, or pressure cooking, to be less destructive.

Expression of BCL-G in Leukemic patients:

Seven Saudi patients were diagnosed with chronic phase of chronic myeloid leukaemia (CP-CML) at our hospital who were scheduled to receive imatinib therapy. Peripheral blood and bone marrow samples obtained from these patients. Mononuclear cells (MNCs) were obtained by density gradient centrifugation over Ficoll-Hypaque (1.077 g/ml) (Nyegaard) as previously described. Human $CD34^+$ cells were purified using MiniMACS magnetic immune-affinity columns as directed by the manufacturer (Milteny Biotec). The purity was about 95% that was confirmed by flow cytometry. Direct immunofluorescence was used to confirm the presence of BCL-G. 10^5 cells were deposited on slides using a cytocentrifuge and stained with an anti BcL-G FITC conjugated antibody (PharMingen at 1:100 dilution at RT for 1 hour. Slides were washed three times with PBS for 5 min, mounted with glycerol and visualized under an Olympus fluorescence microscope. Matched isotype antibody control was used at 1:100 at RT for 1 hour.

Results

Initial staining demonstrated that the optimal conditions for BCL-G expression occurred after an antigen heat mediated retrieval time of 20 min. The results were consistent in that all epithelia of all tissues studied, positive staining was observed in the endothelial lining of capillaries, mast cells, plasma cells, neutrophils, pneumocytes, paneth/ enteroendocrine cells, deep gastric chief cells, and the mesenteric plexus ganglia throughout the gut. Typically, we found that all cancerous areas within tissues stained as positive.

Digestive system

Pancreas

The majority of pancreatic tissue had islet cells that were negative for BCL-G staining except for one section where strongly positive staining was observed in a coarse granular paranuclear pattern (**FIGURE-1**). Pancreatic sections mostly had weak diffuse cytoplasm staining of the acini (table-1). Cancerous areas of the pancreas were the most part positive with weakly stained cytoplasm. Two sections had discrete paranuclear staining of acini, both these sections exhibited positive staining of poorly differentiated adenocarcinoma. The ducts had positive staining in the majority of sections.

Table-1. Staining in pancreatic tissues was defined as strong (+3), moderate (+2), weak (+1), or negative (NEG).

Type of tissue	Number of tissues	Islets	Acini	Ducts
Normal	15	+1	+1	+1
Pancreatic cancer	15	NEG	+2	+2

Liver

Hepatocytes were found to have weak and diffuse staining throughout the cytoplasm in normal liver tissue, while portal structures were consistently negative (hepatic artery, portal vein, bile ducts and lymphatics). In areas of infiltrating adenocarcinoma, the cytoplasm of the hepatic cells had positively diffused staining, as did the cancer cells, with those bile ducts contained within the cancerous areas showing granular staining (7 of these cases were related to an obstructed biliary system). In one section with infiltrating gastric carcinoma there was diffuse weak cytoplasm staining of hepatocytes and granules (+2) in groups of 3-4 paranuclear within cytoplasm and the portal structures were positive (**TABLE-2**).

Table-2. Staining in liver tissues was defined as strong (+3), moderate (+2), weak (+1), or negative (NEG).

Type of tissue	Number of tissues	Hepatic artery	Portal vein	Bile ducts	Lymphatics	Hepatocytes
Normal	8	NEG	NEG	+1	+3	+3
Hepatocellular carcinoma (HCC)	11	NEG	NEG	NEG	NEG	+1

Gallbladder

Sections of gallbladders showed only weak diffuse staining of the columnar epithelium. The remainder of the structures (lamina propria, smooth muscle, connective tissue, capillaries and lymphoid aggregates) were all negative.

Stomach

Epithelia of the surface glands of the stomach stained negative. In the deeper (1/3) part of the mucosal

layer of the gastric glands (chief cells) only diffuse granular staining was seen with continuous staining along the horizontal zone (**FIGURE-2**). The lamina propria, smooth muscle and lymphoid aggregates all stained negative, while the cancerous areas together with inflammatory or plasma cells were all positive..

Small intestines

In duodenal biopsies (**TABLE-3**), the Brunners glands were for the most part negative. Similar to the muscle layers and smooth muscle of the muscularis, the lymphoid aggregates throughout the small intestine (MALT) were also negative. The lamina propria exhibited diffuse positive cells (possibly plasma or mast cells). The parasympathetic ganglion of the mesenteric plexus (Auerbach's plexus) demonstrated positively staining granules throughout the entire small bowel. In most sections, nuclear granules in the cytoplasm showed positive staining in the columnar cells of the epithelium and in the crypts. Positive granular staining of the paneth (enteroendocrine) cells was associated with this staining pattern. In contrast, only weak staining was observed in cancerous tissue (**FIGURE-3**).

TABLE-3. Staining pattern of BCL-G in the small intestine was defined as strong. (+3), moderate (+2), weak (+1), or negative (NEG).

Type of tissue	Number of tissues	Epithelium & crypts	Paneth & enteroendocrine	Lamina propria	Muscularis	Mesenteric plexus	Brunners glands	MALT
Normal	39	+3	+3	+3	+1	+1	+2	+3
Cancer	26	NEG	+1	+1	NEG	NEG	NEG	NEG

Large bowel

The majority of sections obtained from the lamina propria, muscularis, MALT or smooth muscle exhibited negative staining. Similar to our findings in the small bowel, the parasympathetic ganglia of the mesenteric plexus (Auerbach›s plexus) demonstrated granular staining in all sections studied. The epithelium showed moderate positive staining in the granule located in the paranuclear areas within the cytoplasm. The glands of the large bowel showed granular staining within the cytoplasm of the enteroendocrine cells (**TABLE-4)(FIGURE-4**). The cancerous areas typically exhibiting strong diffuse positive cytoplasmic staining.

Table-4. Staining in tissue from the large bowel was defined as strong (+3), moderate (+2), weak (+1), or negative (NEG).

Type of tissue	Number of tissues	Epithelium	Gland	Lamina propria	muscularis	MALT	Smooth muscle	Ganglia
Normal	10	+3	+3	+3	+1	NEG	NEG	+1
Colon cancer	10	+1	NEG	NEG	NEG	NEG	NEG	+1

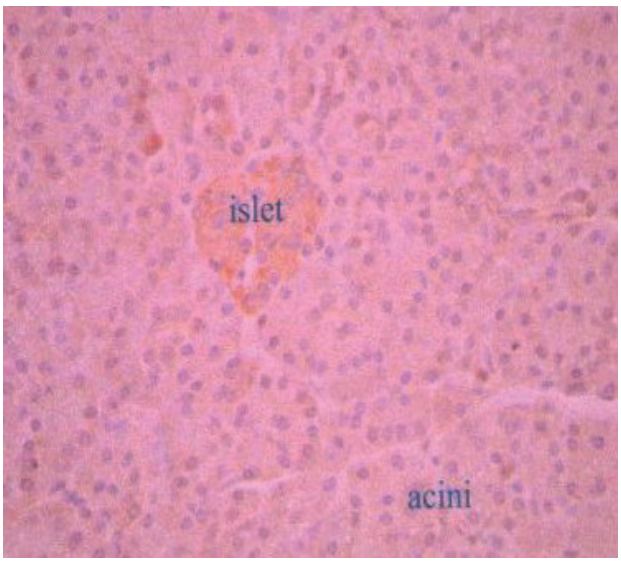

Figure-1: Pancreas

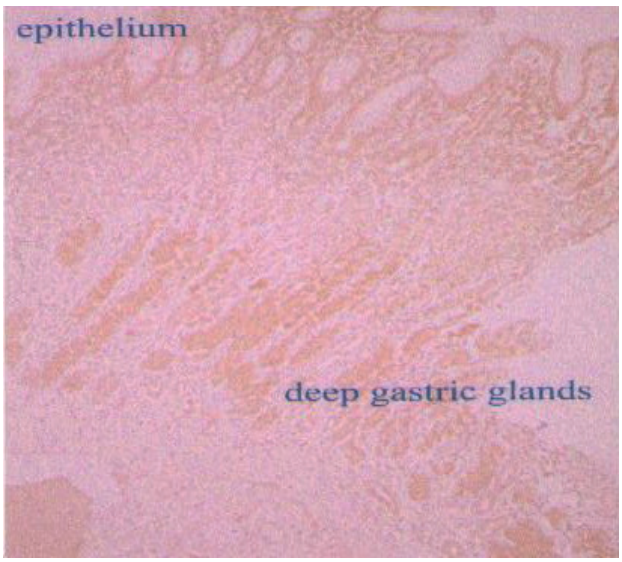

Figure-2: Stomach

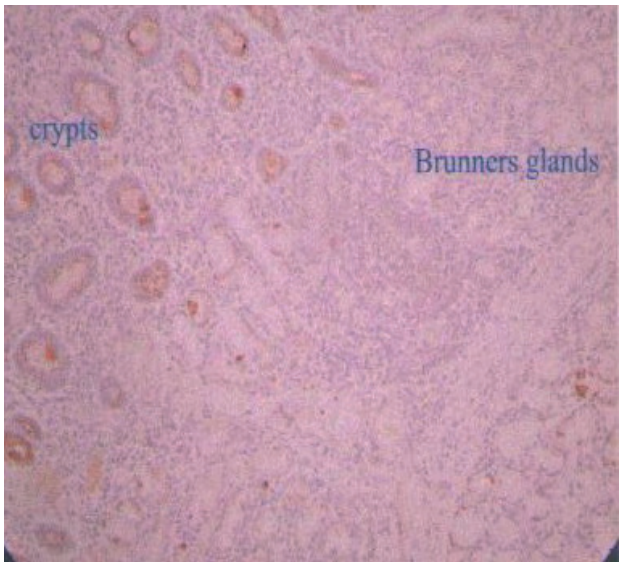

Figure-3: Small intestines

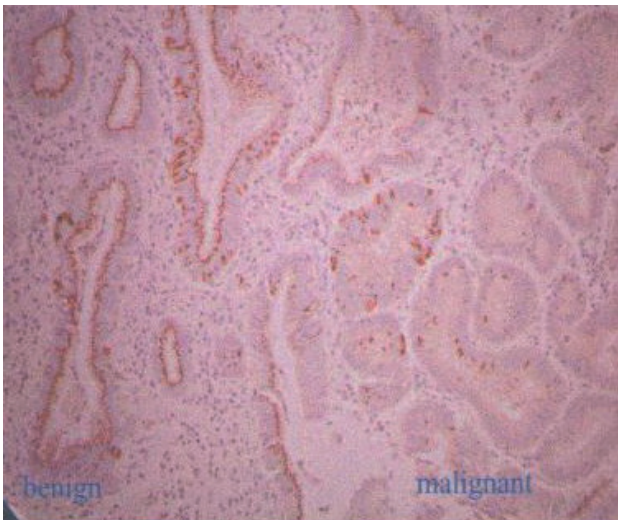

Figure-4: Large bowel

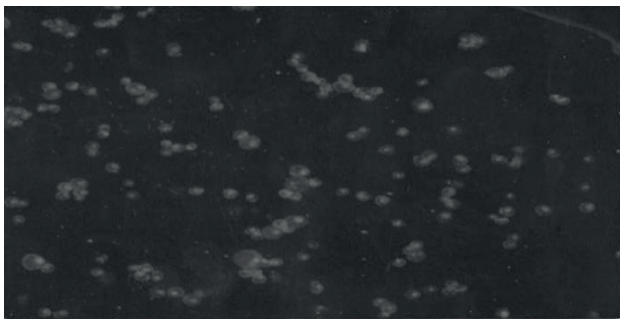

Figure-5: CD34 CML cells stained positive for BCL-G.

Expression of BCL-G in Leukemic patients:

Firstly, experiments were performed to establish that BCL-G was expressed by CD34+ and in day 14 CFU-GM cells. BCL-G was present in approximately 90 percent of the tested cells (Figure-5), compared with the normal FITC-Ig G negative control (data not shown).

Discussion

This study demonstrates for the first time the distribution and staining pattern of BCL-G in a large range of different types of normal and malignant tissues together with samples from leukemic patients. We observed that the majority of kidney sections demonstrated strong positive paranuclear staining in the cytoplasm of the distal tubules. In an extensive array of tissues we observed a consistently variable pattern of staining for BCL-G. Epithelia from all sources studied had a weakly positive pattern of cytoplasmic expression. This was confirmed as a reproducible response of the tissue as similar patterns were observed when the sections were re-examined on different days. In addition, most epithelia, taken from multiple sources, had positive apical paranuclear granules. These granules are very specific and stable in their location within the cytoplasm in relationship to the nucleus. This finding suggests that this protein may be involved in shuttling to and/or from the nucleus, or alternatively localized in lysosomes if undergoing fragmentation). It is also possible that the protein is positioned within organelles associated with the nucleus, such as the endoplasmic reticulum or the Golgi apparatus, or even located within the mitochondria.

To examine the nature of any potential association between cellular differentiation and expression levels of BCL-G, three malignancies with graded differentiation patterns were studied. The anatomical disparity of the localization within the epithelium indicated that staining levels of BCL-G intensified with increasing cancer differentiation.

One observation of note was that increased staining of cytoplasmic granules increased in intensity with differentiation, with the basal pair of layers being completely free of positive staining. In addition, we demonstrated the presence of positive granules in open nuclei that were predominantly located in the middle half of the epithelium.

In terms of differences between benign and malignant tissues, we found that cancerous areas tended to show a reduction in staining intensity, that was limited to a diffuse weakly positive staining of the cytoplasm that was often associated at a focus of positive granules. Rickards and colleagues demonstated downregulation of BCL-G in prostate cancers that was sufficient to reduce tumor sensitivity to UN-induced cytotoxicity (REF9). Similarly, the same workers also demonstrated reductions in BCL-G expression in breast cancer tissues that was not associated with rates of patient survival (REF10).

To date, there is insufficient data to establish a relationship between CML and BCL-G (REF11). However, we believe that increased expression of the BCL-G that acts as an anti-apoptotic gene might correlate with BC transformation, or be associated with poor treatment responses, or both. Therefore, the presence of fully functional genes regulating both cell cycle and apoptosis might maintain the balance between the in vivo rate of cell division and apoptosis of any cell population. Thus, any failure or loss of function of BCL-G or any one of the family members, may result in increased self-replication.

It is also possible that the BCL-G protein might have a yet to be described function that may account for such variability. We suggest that the variety of staining patterns of BCL-G expression in different cells exhibiting diverse distributions may have functional consequences beyond apoptosis, or that this distribution pattern is closely involved in the interaction with BCL-G and the apoptosis specific proteins. Consequently, future studies wouldcompare the staining pattern of BCL-G with other apoptotic markers which play an important role in tumor suppression.

Acknowledgement

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