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Research Article

The Expression of BCL-G in Leukemia and Gastrointestinal Tissues

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Abstract

Background & Objective: BCL-G is a novel protein of Bax (BCL-G associated protein X) inducing caspase-mediated apoptosis. These proteins are involved in regulating apoptosis both in normal, and in neoplastic cells although their cellular and tissue distribution is currently unknown. Accordingly, the objective of the current study was to ascertain and define the pattern of distribution and expression of BCL-G in normal and malignant gastrointestinal human tissues.

Methods: Using a rabbit monoclonal antibody against BCL-G, the distribution and expression of BCL-G was assessed by immunohistochemistry in formalin-fixed, paraffin-embedded, benign and malignant human tissues

Results: We found a variable pattern of positive expression of BCL-G within all the tissues we studied. BCL-G expression was typically localized in the cytoplasmic paranuclear granules in the epithelial cells in most organs we examined. The intensity of BCL-G staining related to the maturation state in benign tissue.

Conclusion: We have demonstrated that BCL-G exhibits a specific distribution pattern that appears to correlate with cellular differentiation. However, while these distribution patterns are complex they do give a tantalizing insight into function, and consequently need further investigation to determine their physiological/pathological significance.

Keywords: Leukemia; Gastrointestinal tissues; BCL-G

Introduction

BCL-G is highly conserved in humans as well as many mammals encoding BCL-G_L and BCL-G_S, where the former is was displayed widely in many tissues, the latter detected only in testis by PCR.

BCL-G_S was found to contain only BH3 domain thus able to induce apoptosis, while, BCL-G_L, doesn't induce apoptosis [1]. More importantly, BCL-G is situated on human chromosome 12 and its expression was found decreased in human prostate and breast cancer samples, suggesting that its acts as anti-cancer gene [2,3]. However, sequence analysis of BCL-G in many human cancers including urinary bladder and squamous cell carcinoma did not show any mutation excluding its role as a tumor suppressor gene [4,5]. Interestingly, BCL-G shares some features with BFK (Bcl-2 Family Kia), which contain BH2 and BH3 with apoptotic activity [6]. BCL-G interacts with the transport protein complex, likely to work in protein trafficking inside the cell not inducing apoptosis [7]. BCL-G was expressed in many mouse tissues such as spleen, lungs and testis [8].

The aim of this study was to determine the levels and cellular localization of BCL-G in human tissue from gastrointestinal tissues by using Immunohistochemical (IHC) technique in human tissues.

Materials and Methods

Tissue Specimens and Reagents

Using formalin-fixed paraffin-embedded normal or malignant human tissue, 3mm sections were cut from and placed on saline-coated slides. In this archival retrospective study, and in order to test a range of antibody titers, we took an average of 4 sections from each specimen. Taken from patients during surgery for removal of various tumors between January 2014 and January 2015, these tissue samples were obtained from the University Hospital, Minufiya University, Minufiya, Egypt. Normal tissues were resected adjacent to the tumor site as part of the normal surgical procedure. Both Research Ethics Committee approvals, and written patient informed consent, were obtained in all cases.

Immunohistochemistry (IHC)

Following incubation with a specific secondary antibody (peroxidase labeled polymer) conjugated with goat anti-mouse immunoglobulins in Tris-HCl buffer containing carrier protein and an anti-microbial agent (DAKO; K4006), tissue sections were diluted 1:50 for 1 hr. and then incubated at room temperature. Slides were removed from the Sequenza for addition of substrate-chromogen. To block endogenous biotin where present (such as in liver), 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. Following this, the nuclei were stained with hematoxylin; and sections were mounted in DAKO paramount. All dilutions and the extensive washes between steps were performed at room temperature using phosphate buffered saline unless otherwise specified.

As positive controls, sections from kidney (which are known to be sensitive to the antibody BCL-G) were included for every run. Negative controls were duplicates of the test sections, with only the primary

antibody substituted with the matched isotype Ab control To test antigen sensitivity to fixation and processing of the tissue, lung sections (used in this way as positive control) were immunostained with monoclonal anti E-cadherin.

Since E-cadherin is known to be sensitive to fixation methods and the duration of processing, if the staining patterns matched, it was extrapolated that the variable staining pattern with Mab was due to the 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 diluent.

Heat-mediated antigen retrieval using microwave

Since some epitope 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

All slides were counted by two pathologists, blinded as to the provenance of each slide. They studied five visual fields at x 200 magnification. 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 defined in terms of whether expression was membranous or cytoplasmic. If it was cytoplasmic, it was further subdivided into whether it was diffuse or granular. The presence of background staining was also noted.

We regarded positive control tissues as indicative of correctly prepared tissue and proper staining techniques. Included in every run was a positive control section 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. In cases where the positive control tissues did not demonstrate positive staining, these test specimens were considered invalid.

A negative control was included in every run to verify the specificity of the primary antibody. The negative control tissue was examined after the positive control tissue, and was to verify the specific labeling of the target by the primary antibody. We took the absence of specific staining in the negative control tissue 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. Problems encountered with such a long microwaving duration 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 diagnosed as chronic phase of chronic myeloid leukemia (CP-CML) at our hospital. Peripheral blood and bone marrow samples obtained from these patients in CP-CML who were scheduled to receive imatinib therapy. Mononuclear cells (MNCs) were obtained by density gradient centrifugation over Ficoll-Hypaque (1.077 g/ml) (Nyegaard). After washing with HBSS (GibcoBRL), cells were resuspended in HBSS. Purification of human $CD34^+$ cells were separated using MiniMACS magnetic immuno affinity columns as directed by the manufacturer (Milteny Biotech). The purity is about 95%, confirmed by flow cytometry. Direct immunofluorescence was used to confirm the presence of BCL-G. 105 cells were deposited on slides using a cytocentrifuge and stained with an anti BCL-G FITC conjugate (PharMingen). The cells were incubated with the antibody at 1:100 dilutions at RT for 1 hour. Slides were washed three times with PBS for 5 min, mounted with glycerol mountant and checked under an Olympus fluorescence microscope. Matched isotype antibody control was used at 1:100 at RT for 1 hour.

Results

Using a 1:100 dilution of the antibody, the 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 seen in the endothelial lining of capillaries, mast cells, plasma cells, neutrophils, pneumocystis, 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: Most pancreatic tissue demonstrated that islet cells were negative for BCL-G staining except for one section where it was strongly positive in a coarse granular paranuclear pattern (Figure 1). Pancreatic sections nearly all showed weak diffuse cytoplasm staining of the acini (Table 1). Cancerous areas of the pancreas were mostly positive and showed weakly stained cytoplasm. Two sections showed discrete paranuclear staining of acini and, in both these sections containing poorly differentiated adenocarcinoma, the cancer was positive. The ducts stained positively in the majority of sections.

Liver: In normal liver tissue, hepatocytes showed weak and diffuse staining throughout the cytoplasm. The portal structures were consistently negative (hepatic artery, portal vein, bile ducts and lymphatics).

In areas of infiltrating adenocarcinoma, cytoplasm of the hepatic cells were positively diffused stained, as were the cancer cells, with those bile ducts that were contained within the cancerous areas showing granular staining (7 of these cases were related to an obstructed biliary system).

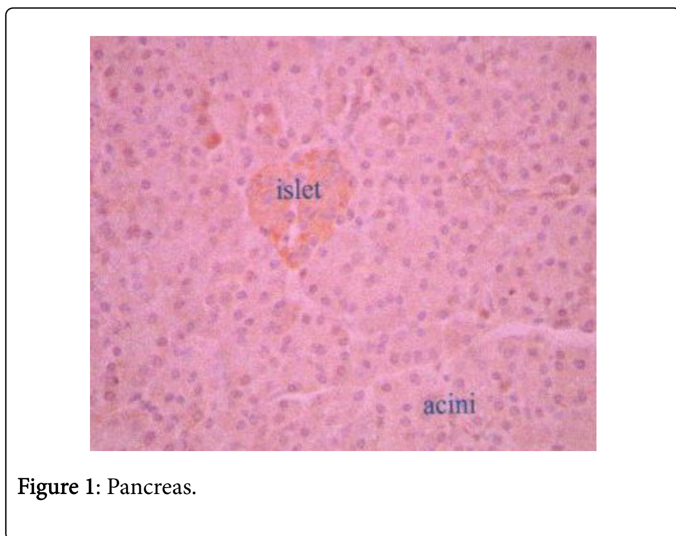

Figure 1: Pancreas.

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

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

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 still positive (Table 2).

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) was 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. This staining was continuous along this horizontal zone (Figure 2). The lamina propria, smooth muscle and lymphoid aggregates all stained negative. Inflammatory or plasma cells were positive, as were the cancerous areas.

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

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

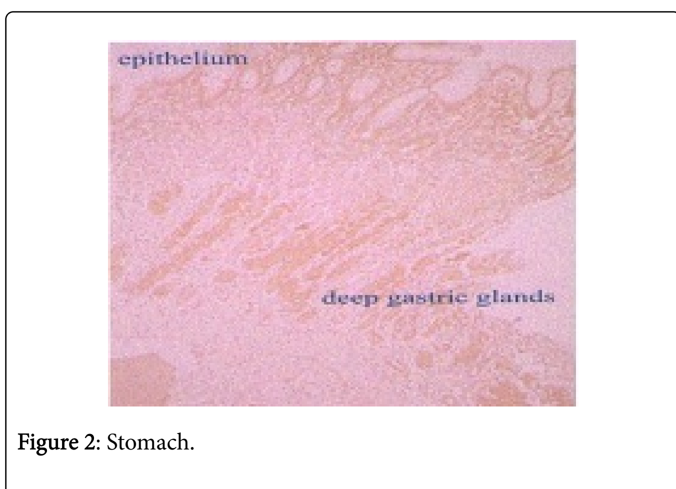

Figure 2: Stomach.

Small intestines: In duodenal biopsies (Table 3), the Brunners glands tended to be 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 showed scattered positive cells (which might possibly have been plasma or mast cells). The parasympathetic ganglion of the mesenteric plexus (Auerbach's plexus) demonstrated positive 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. Associated with this staining pattern was a positive granular staining of the paneth (enteroendocrine) cells. The staining in cancerous tissue only showed weak staining (Figure 3).

Large bowel: Most of the sections (obtained from the lamina propria, muscularis, MALT and smooth muscle) were found to be negative. Similar to what we found in the small bowel, the parasympathetic ganglia of the mesenteric plexus (Auerbach's plexus) demonstrated granular staining in all sections we studied. The epithelium showed moderate positive staining in the granules (Para nuclear locations 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 showed strong diffuse positive cytoplasm staining.

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

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

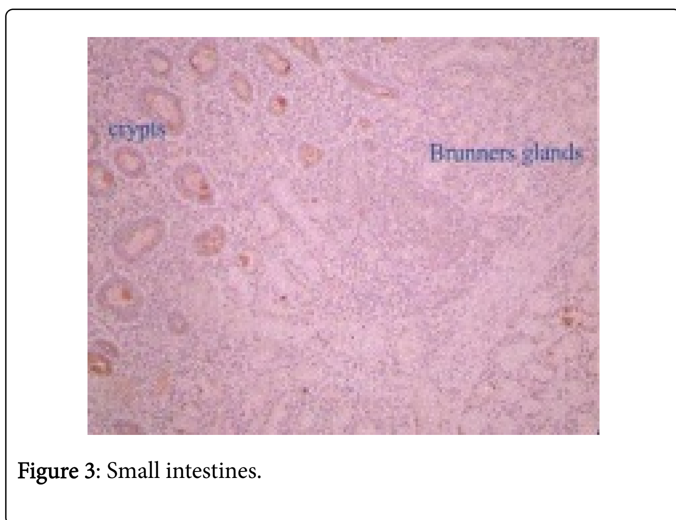

Figure 3: Small intestines.

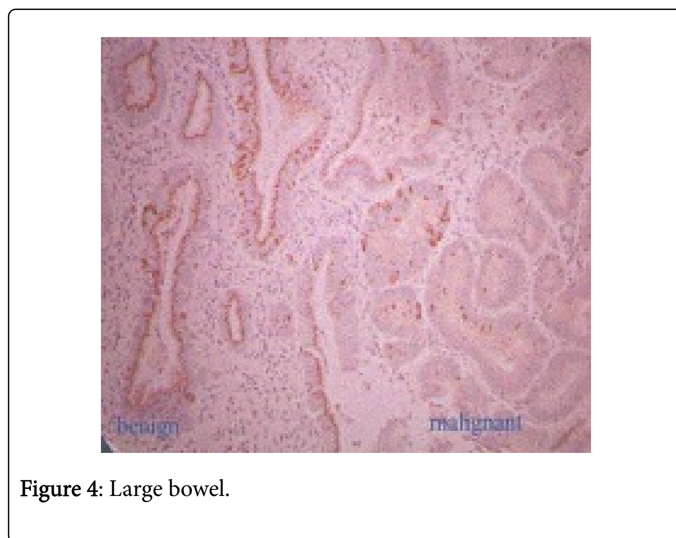

Figure 4: Large bowel.

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

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

Expression of BCL-G in Leukemic patients

Experiments were performed first to demonstrate that BCL-G was present in CD34$^+$ and in day 14 CFU-GM cells. Approximately 90 percent of the tested cells expressed BCL-G (Figure 5). To exclude any interference, normal FITC-IgG were set as a control (data not shown).

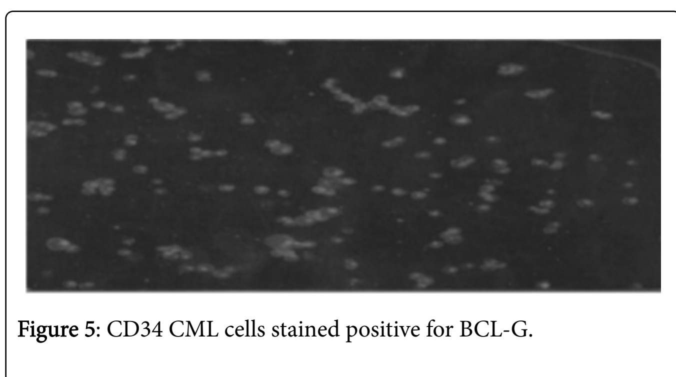

Figure 5: CD34 CML cells stained positive for BCL-G.

Discussion

This study represents the first time that the distribution and staining pattern of BCL-G has been ever described in such a large range of different types of normal and malignant tissues as well as from leukemia patients.

We found that the majority of kidney sections showed strong positive Para nuclear staining in the cytoplasm of the distal tubules. In the wide range of tissues we examined there was a consistent pattern of staining variability seen for BCL-G. Epithelia from all sources we studied expressed a 'weakly positive' pattern of cytoplasmic staining. We confirmed this as a reproducible reaction of the tissue since it displayed a similar pattern when we again re-ran the sections on different days. In addition, most epithelia, taken from any source, showed positive apical Para nuclear granules. These granules are very specific and consistent in their position within the cytoplasm in relationship to the nucleus. To us, this suggests that the protein may be involved in shuttling to and/or from the nucleus (or localized in lysosomes if being broken down), or that the protein is located within organelles associated with the nucleus (such as endoplasmic reticulum, the Golgi apparatus), or even contained within the mitochondria.

To study the nature of any possible relationship between cellular differentiation and expression levels of BCL-G, three malignancies with graded differentiation patterns were examined. The anatomical variation of the staining localization within the epithelium suggested that the expression levels of BCL-G intensified with increasing differentiation of cancer.

One staining pattern of note showed increasing staining in the cytoplasm whereby granules which increased in intensity with differentiation, with the basal couple of layers being completely spared of any staining. In the other staining pattern of note, we demonstrated positive granules in open nuclei that were predominantly in the middle half of the epithelial layer.

Of the difference between benign and malignant tissue, we observed that cancerous areas tended to show a reduction in staining intensity, being limited to a diffuse 'weak positive' staining of the cytoplasm but unfortunately there were often also a focus of positive granules.

Rickards and colleagues showed that BCL-G is downregulated in prostate cancers and this was enough to reduce its sensitivity to UN-induced cytotoxicity (REF9). Similarly, the same group demonstrated a reduction in BCL-G expression in breast cancer tissues with no link to patient survival rate (REF10).

There is not enough data on the relationship of CML to BCL-G (REF11). However, we believe enhanced expression of the BCL-G as anti-apoptotic gene may be correlated with BC transformation itself, or the poor response to treatment, or both. Therefore, it could be inferred that the presence of fully functional genes regulating both cell cycle and apoptosis will maintain the balance between the rate of cell division and apoptosis of any population in vivo. Thus, any malfunction or loss of BCL-G or any one of its family, may lead to an increase in their self-replication.

This BCL-G protein could have an as yet unrecognized function which may eventually account for this variability. We feel that the range of staining patterns of BCL-G expression in different cells with different distributions may have functional implications beyond apoptosis, or that this distribution is closely linked to the interaction of BCL-G to the apoptosis specific proteins. Consequently, we wish to compare the staining pattern of BCL-G with other apoptotic markers which play an important role in tumor suppression.

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