Morphological and quantitative analysis of *BCL6* expression in human colorectal carcinogenesis

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Abstract. The aim of the present study was to determine whether *BCL6* is expressed during malignant transformation of the large bowel and to assess whether, and to what extent, immunoreactivity is related to the different stages of neoplastic progression. Samples of normal colorectal mucosa (n=22), microadenomas (n=22) and colorectal cancer (n=22), were analyzed by immunohistochemistry, immunofluorescence coupled with confocal microscopy and western blotting. Our results clearly outlined the marked increase occurring in both intensity and density of *BCL6* protein expression in the normal mucosa-microadenoma-carcinoma sequence. Immunohistochemistry and immunofluorescence analyses showed that *BCL6* is expressed at low levels in normal mucosa and increases in microadenoma and in cancer with statistical significance. These results were confirmed by western blotting data. The increasing expression of *BCL6* in human colorectal cancer development suggests the involvement of *BCL6* in tumor progression, from the earliest stages of carcinogenesis with significant increase in cancer. The enhanced understanding of the biological role of *BCL6*, previously shown to exert a key role in lymphomagenesis, may lead to a re-evaluation of this protein and may highlight the importance of performing further studies in order to identify novel therapeutic targets for colorectal cancer.

Introduction

BCL6 is a transcriptional repressor which has emerged as a critical regulator of germinal centers (GCs) of lymph nodes. *BCL6* is also a frequently activated oncogene in the pathogenesis of human B cell lymphomas, most of which derive from the GC B cells (1). Especially in the last 10 years, knowledge concerning this gene and its expression has improved considerably. Identification of factors acting together with the *BCL6* protein as well as delineation of several target genes have allowed a determination of its biological function. This set of *BCL6* targets points to a number of cellular functions which are likely to be directly controlled by *BCL6* during GC development, including activation, survival, DNA-damage response, cell cycle arrest, cytokine-, toll-like receptor-, TGFβ-, WNT-signaling and differentiation (2).

BCL6 protein is a 92- to 98-kDa nuclear phosphoprotein that is produced at low levels in several tissues and is expressed at high levels exclusively in GC B cells (3,4). High levels of *BCL6* gene expression in non-Hodgkin's lymphomas have a favorable prognostic value (5,6). *BCL6* contains an N-terminal BTB/POZ domain and 6 Kruppel-type (C2H2) zinc-finger (ZnF) motifs in the C terminus (7). The BTB/POZ domain displays a conserved protein-protein interaction motif that is required for the repressive activity of the protein (8). The BTB domain interacts with several other proteins which are members of the BTB/POZ-zinc finger family (BAZF, LRF, PLZF) (9-11). Although *BCL6* contributes to lymphomagenesis by allowing conditions favorable to lymphoma pathogenesis, its precise role in the malignant transformation remains to be fully elucidated.

Logarajah *et al* (12) and Bos *et al* (13) reported that *BCL6* has an important function in breast cancer. Walters *et al* (14) evaluated the *BCL6* protein expression in a subset of mesenchymal tumors. However, to our knowledge, there are no data on *BCL6* expression/role in the adenoma-carcinoma sequence of colorectal carcinogenesis.

Colorectal cancer remains the second leading cause of cancer-related mortality in Western countries, and it develops mainly from adenomatous polyps and shows a morphological and genetic progression through an adenoma-carcinoma sequence, both in hereditary and in sporadic colorectal cancer (15). Microadenomas (MAs) have been generally accepted as precancerous lesions on the basis of histopathological characteristics, biochemical and immunohistochemical features, as well as

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genetic and epigenetic alterations (16-19). The purpose of the present study was two-fold: (i) to determine whether *BCL6* is expressed during malignant transformation of the large bowel and (ii) to assess whether, and to what extent, *BCL6* immunoreactivity is related to the different stages of neoplastic progression.

Therefore, samples of human normal mucosa, MA and cancer were evaluated with different methods, i.e., immunohistochemistry, immunofluorescence and western blot analysis.

The results obtained showed a significant expression of *BCL6* protein in preneoplastic and neoplastic lesions and suggest a possible involvement of *BCL6* during the malignant transformation of colorectal mucosa.

Materials and methods

Study population. Twenty-two samples of normal colorectal mucosa (NM) were collected from 10 patients during colonoscopy, at least 2 samples from each patient. One sample from each patient was fixed in formalin and embedded in paraffin for immunohistochemistry; the others were frozen at -80˚C. All patients had normal colonoscopy. Twenty-two MAs were also identified in 6 patients, and removed after operation for colorectal cancer on surgical specimens; the precise localization of the lesion was made staining the resected mucosa with a 0.1% methylene-blue solution in saline, and observing it under a dissecting microscope in order to take and process only the fields of the lesion, as previously described (16,19). All MAs, defined as dysplastic aberrant crypt foci at histology were referred to as MAs. Ten MAs were fixed in formalin and embedded in paraffin, the others were frozen at -80˚C. Finally, 22 samples of colorectal cancer (K) were collected from 11 patients operated on for cancer, fixed in formalin or frozen at -80˚C. All cases of cancer were Dukes' C adenocarcinomas.

The patients enrolled in the present study, who underwent colonoscopy or surgical resection for colorectal cancer at the University Hospital of Modena, provided written informed consent to the study protocol, which was approved by the local Ethics Committee.

Immunohistochemistry. Ten samples of NM, 10 of MA, and 10 of K were fixed in a 10% formalin solution for 1 h. They were then dehydrated, embedded in paraffin wax, and sectioned (3-μm thick); the sections were placed on Super Frost Plus microscope slides (Menzel). Prior to immunohistochemistry, routine histology of all tissue samples was carried out after hematoxylin and eosin (H&E) staining of the sections. Slides were dried overnight at 37˚C, dewaxed in 2 changes of fresh xylene, and rehydrated in a descending alcohol series. Antigen retrieval involved treatment with a protease (Pronase 1:20; DakoCytomation) for 7 min at 37˚C. Prior to immunohistochemical staining, the sections were washed with PBS and blocked with 20% Swine Serum (Normal; DakoCytomation) in PBS for 30 min to reduce nonspecific antibody binding. Primary antibody was then applied at appropriate dilutions for 30 min at room temperature. Polyclonal rabbit anti-mouse BCL6 antibody (DakoCytomation) was used as primary antibody at a dilution of 1:200 in PBS. Endogenous peroxidase activity was blocked by treating the slides with 0.3% hydrogen peroxide in methanol for 12 min. After washing with PBS, biotinylated anti-mouse secondary antibody (1:20; DakoCytomation) was incubated for 30 min, and the slides were incubated with pre-diluted streptavidin-HRP conjugates for 30 min according to the manufacturer's instructions. In order to reveal the color of the antibody staining, DAB substrate solution (freshly made immediately prior to use: 0.05% DAB-0.015% H_2O_2 in PBS) was used. Slices were counterstained with Mayer's hematoxylin, dehydrated in ascending alcohol series and covered with a coverslip (Histovitrex mounting medium; Carlo Erba). For all these procedures, the negative control was obtained by staining samples without the primary antibody. Human B cell lymphomas were used as positive control.

Scoring system. One or 2 slices for each sample were scored as described below for immunofluorescence analysis. For each group of colorectal tissue and lesions, about 25 x100 microscopic fields were scored. Moreover slices for each group of normal mucosa and lesions were scored by 2 independent observers and there was <5% variance between the results of the 2 counts. To quantify the staining area, the slices were processed with an ImageJ software. The images were converted into 16-bit images and then thresholded (the same value was used in all analyzed images). The resulting thresholded images were binary and they showed only the staining area. The total staining area was calculated by the software.

Immunofluorescence confocal microscopy. Immunofluorescence analysis was carried out to evaluate the expression of *BCL6* protein. Six samples of NM, 6 of MA and 6 of K, were fixed in 4% paraformaldehyde in PBS, cryoprotected in 15% sucrose in PBS, and frozen in isopentane cooled in liquid nitrogen. Horizontal cryosections of the samples were cut (10-μm thick), and H&E staining was performed on sections to control tissue integrity. After a treatment with 3% BSA in PBS for 30 min at room temperature, the cryostatic sections were incubated with the primary antibodies (mouse anti-*BCL6*; DakoCytomation) diluted 1:25 in PBS containing 3% BSA for 1 h at room temperature. After washing in PBS, the samples were incubated for 1 h at room temperature with the secondary antibodies diluted 1:20 in PBS containing 3% BSA (sheep anti-mouse FITC-conjugated; Sigma). After washing in PBS and in H_2O, the samples were counterstained with 1 μg/ml DAPI in H_2O and then mounted with anti-fading medium (0.21 M DABCO and 90% glycerol in 0.02 M Tris, pH 8.0). Negative control samples were not incubated with the primary antibody. The confocal imaging was performed on a Leica TCS SP2 AOBS confocal laser scanning microscope. For DAPI and FITC double detection, samples were sequentially excited with the 405-nm/25-mW lines of a blue diode laser and the 488-nm/20-mW lines of the Argon laser. The emission signals from DAPI and FITC were detected by 2 photomultiplier tubes. Excitation and detection of the samples were carried out in sequential mode to avoid overlapping of signals. Sections were scanned with laser intensity, confocal aperture, gain and black-level setting kept constant for all samples. Optical sections were obtained at increments of 0.3-μm in the z-axis and digitized with a scanning mode format of 512x512 or 1,024x1,024 pixels and 256 grey levels. The confocal serial sections were processed with Leica LCS

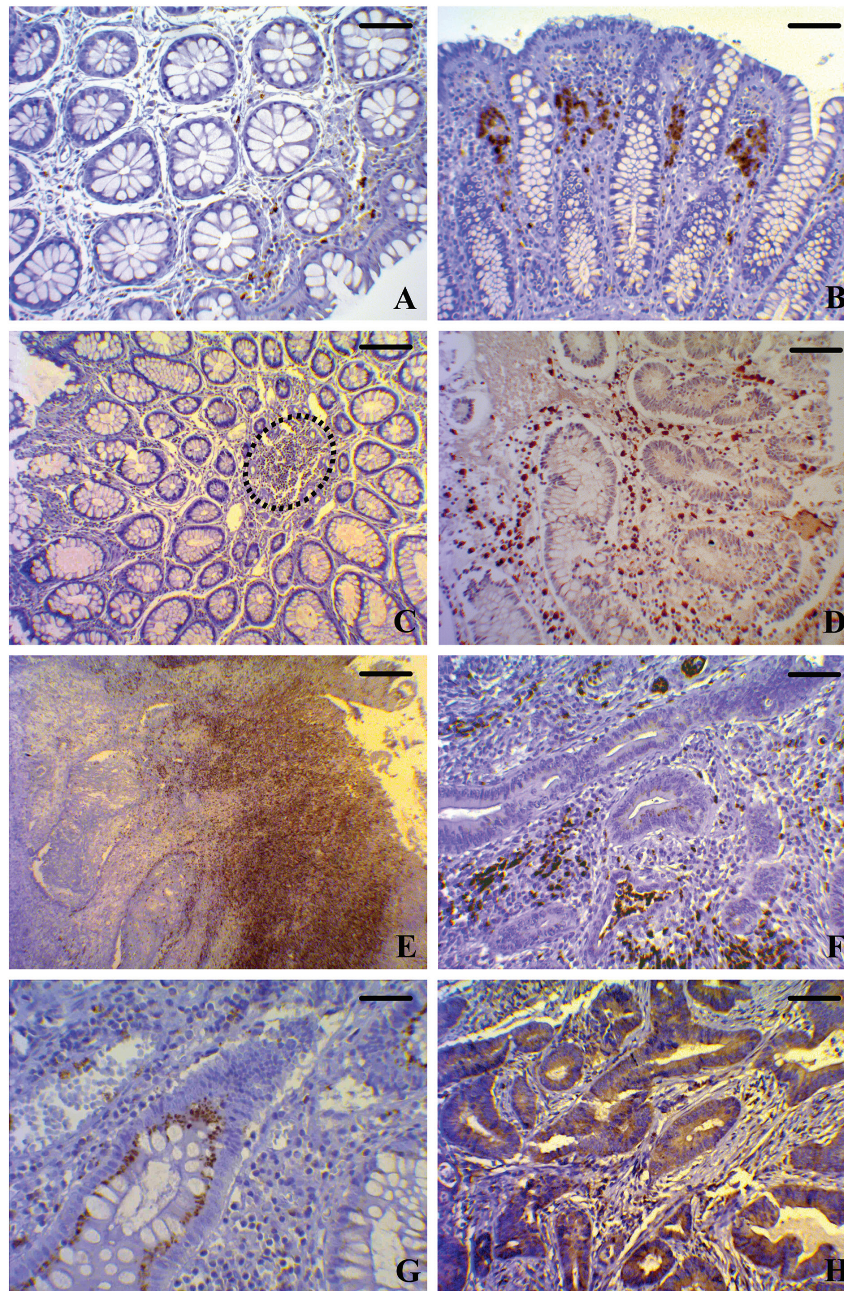

Figure 1. Immunohistochemical staining of *BCL6* in different human colonic tissues. (A and B) Normal mucosa at different magnifications. (C and D) MA at different magnifications. (E and F) Colorectal cancer at different magnifications. (G and H) High magnifications of MAs and colorectal cancer, respectively. The expression of *BCL6* in samples of normal mucosa is low and restricted to the stromal compartment, as is well evident in both (A) transverse and in (B) longitudinal sections. The *BCL6*-positive cells show nuclear and cytoplasmic localization of the protein. MA samples are characterized by high expression at both the cytoplasmic and the nuclear level of fibroblast-like cells and leukocytes that fill the stromal compartment (dotted line). (C) Transverse section; (D) longitudinal section. In MA, the epithelial cells of the crypt show well evident *BCL6* immunostaining, particularly in the (G) cytoplasm. In the *lamina propria* of colorectal cancer, the number of *BCL6*-positive fibroblast-like cells and leukocytes is significantly higher than in MA. (E and F) The protein is localized at both the cytoplasmic and the nuclear level. In colorectal cancer samples, diffuse high cytoplasmic and nuclear *BCL6* expression is present in tumor cells (H). (A and D: bar, 50 μm; B: bar, 70 μm; C: bar, 100 μm; E: bar, 200 μm; F and H: bar, 50 μm; G: bar, 25 μm). MAs, microadenomas.

software to obtain 3-dimensional projections. Image rendering was performed by adobe Photoshop software.

Evaluation of BCL6 immunofluorescence. The original green fluorescent confocal images were converted to grey-scale and median filtering was performed. An intensity value ranging from 0 (black) to 255 (white) was assigned to each pixel. Background fluorescence was subtracted and the immunofluorescence intensity was calculated as the average for each selected area. To quantify *BCL6* expression, all blocks were completely sectioned and 3-4 slices were examined at a magnification of x40 for each patient. Starting randomly, 3 microscopic fields for each section were used for sampling all sections within the unbiased sampling frame. The fluorescence intensity at the selected areas, linearly correlated with the number of pixels, was quantitatively analyzed using the standard imaging analysis software of the NIS-Elements system. To each sample was assigned a code number and

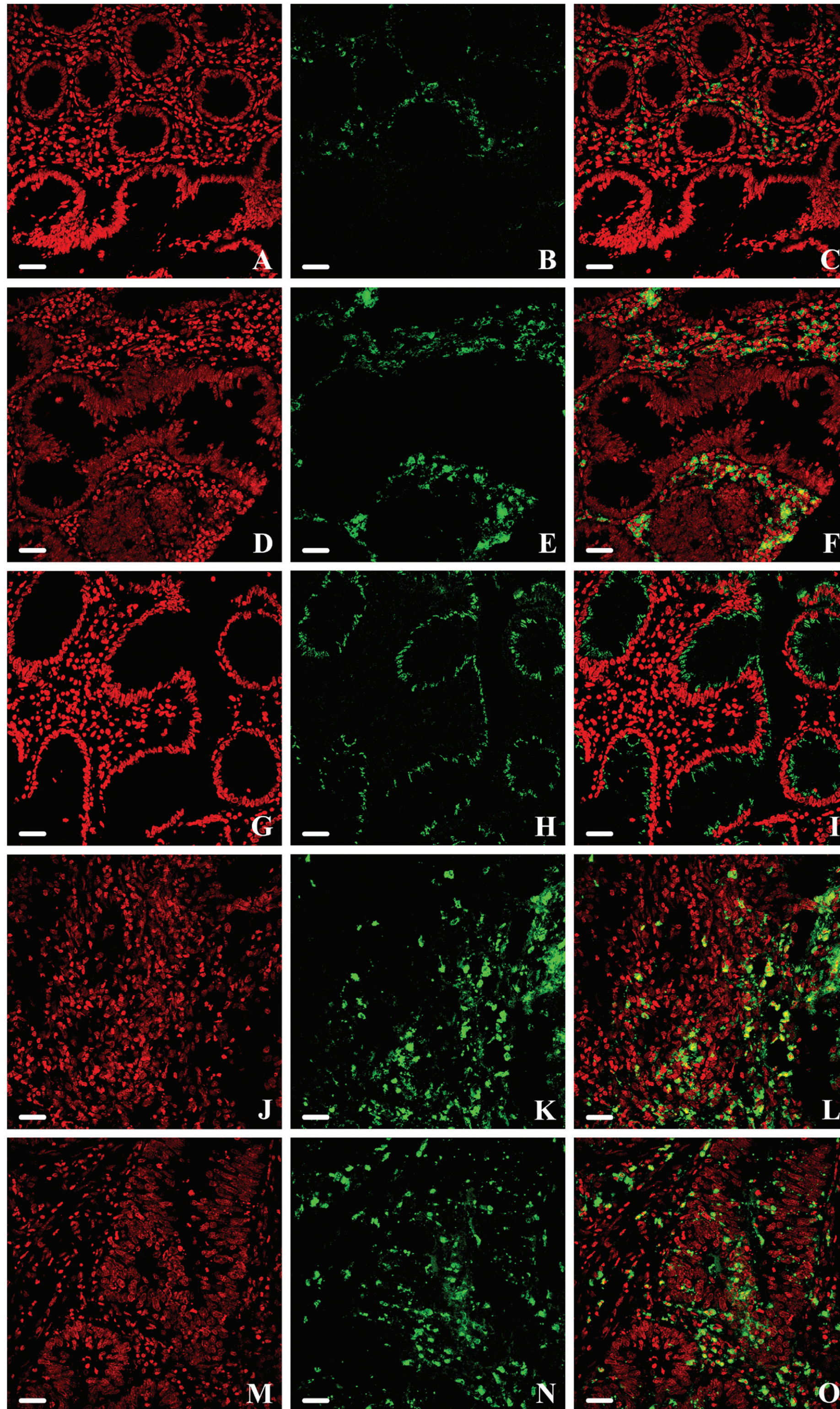

Figure 2. Confocal analysis of colonic mucosa cryosections labelled by DAPI (red) and anti-*BCL6* (green). (A-C) Normal colorectal mucosa (C, merge): the *BCL6* protein is localized mainly in stromal cells at the nuclear and cytoplasmic levels; the amount of immunostaining is low. (D-I) MA (F and I, merge): the marked increase of *BCL6* staining with respect to normal mucosa is evident; (D-F) the protein is present in the nucleus as well as in the cytoplasm of stromal cells. (G-I) Crypt epithelial cells show marked staining mainly at the lateral borders of goblet cells. (J-O) Colorectal cancer. (J-L) In the stromal compartment, the presence of many *BCL6* labelled cells at the nuclear and cytoplasmic levels is evident. The immunostaining is significantly enhanced with respect to MAs. (M-O) *BCL6* is also strongly expressed in the nucleus and in the cytoplasm of cancer cells. (A-C: bar, 50 μm; D-I: bar, 30 μm; J-O: bar, 25 μm). MAs, microadenomas.

the score, referred to as immunofluorescence intensity score, determined by an observer who was blind to tissue groups during analysis (20).

Western blot analysis. Whole cell lysates were obtained from 6 samples of NM, 6 of MA and 6 of K, extracted with hypotonic buffer (50 mM Tris-Cl, pH 7.8, containing 1%

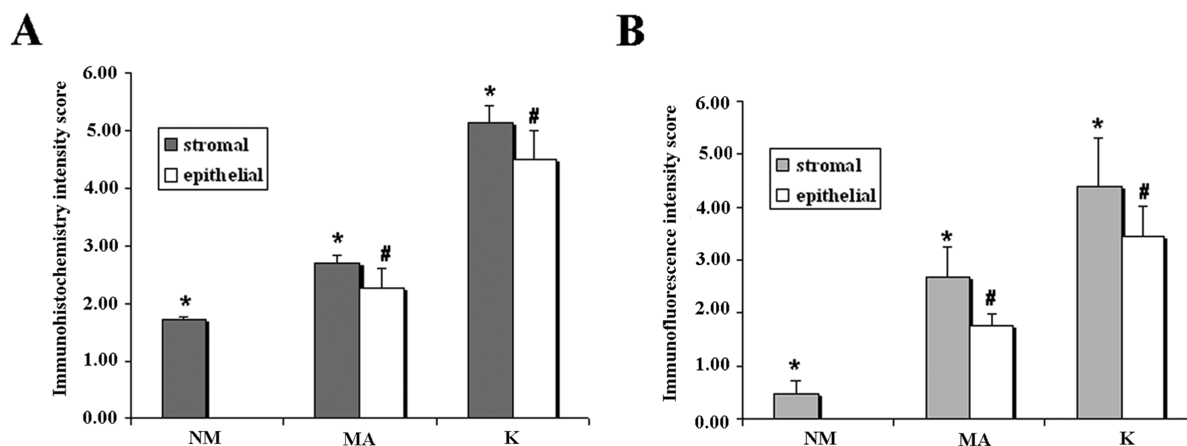

Figure 3. (A) Immunohistochemistry intensity score of *BCL6* in human normal colorectal mucosa (NM), microadenoma (MA) and colorectal cancer (K) in stromal and epithelial compartments. *#P<0.05 between all group pairs of samples. Bar, SD. (B) Immunofluorescence intensity score of *BCL6* in NM, MA and K in stromal and epithelial compartments. *#P<0.05 between all group pairs of samples. Bar, SD. No positivity was recorded in the epithelial compartment of NM in either (A) or (B).

Nonidet P-40, 140 mM NaCl, 0.1% SDS, 0.1% Na deoxycholate, 1 mM Na_3VO_4 and freshly added protease inhibitor cocktail). Lysates were then cleared by centrifugation for 15 min in a refrigerated centrifuge (1,500 rpm) and immediately boiled in SDS sample buffer. Protein extract (40 µg) from each sample (NM, MA and K) was electrophoresed on SDS-PAGE and transferred to nitrocellulose membranes. The protocols of the western blotting were performed as previously described (20). The membranes were blocked with 3% dry milk and 2% BSA in PBS-T and were then incubated with mouse anti-*BCL6* (DakoCytomation) antibody, diluted 1:1,000 overnight at 4˚C under agitation. After washing, the membranes were incubated with secondary HPR-conjugated goat anti-mouse IgG antibody (1:10,000) for 30 min at room temperature. Immunoreactive proteins were detected with ECL (Amersham). Furthermore, the membranes were stripped and incubated with anti-mouse β-tubulin (Sigma) to control and correct for loading error. Densitometry analysis was performed using a Kodak Image Station 440CF system (Rochester, NY, USA).

Statistical analysis. All quantitative data for NM, MA and K are reported as means ± SD. The difference in *BCL6* average expression in the different groups of colorectal lesions was tested for statistical significance using ANOVA one-way analysis, followed by Student-Newman-Keuls tests. P<0.05 was considered to indicate a statistically significant difference between groups.

Results

Morphological evaluation. The quality of staining obtained by the immunohistochemical method was generally very good; *BCL6* staining was evident as brown spots both in the cytoplasm and in the nucleus of stromal cells, easily allowing their identification even at low magnification. Examples of *BCL6* staining are shown in Fig. 1, in which panels A and B show low levels of *BCL6*-positive cells (fibroblast-like cells and leukocytes) in NM, especially in the stroma adjacent to the crypts. In all samples of normal mucosa, appreciable *BCL6* staining of epithelial cells was not found. In MAs, *BCL6* expression was higher compared to normal mucosa and it was very evident in the stromal compartment (Fig. 1C and D). Moreover, a consistent epithelial positivity was observed in MA and K: the protein aggregated in small clumps appears distributed in the cytoplasm at the lateral and basal portions of the epithelial cells and also partially in their nucleus (Fig. 1G). Concerning the stromal compartment, the samples of K were characterized by a higher number of *BCL6*-positive cells with respect to MA (Fig. 1E and F); in carcinoma, positive cells were not evenly distributed in the tissue, but tended to gather in some areas, usually at the edge of the neoplastic tissue (Fig. 1E), not only in the stroma inside the tumor (Fig. 1F), but also among the neoplastic cells at the margins of the lesions (Fig. 1H). Inside the epithelial-positive cells, *BCL6* protein accumulated predominantly at the apical region of cells towards the lumen of colonic crypts, as well as at the nuclear level (Fig. 1H). The semi-quantitative evaluation of immunostaining intensity reported as immunohistochemistry intensity score in Fig 3A, showed that the level of *BCL6* protein in both the epithelial and the stromal compartment had an increasing trend from NM to K, with statistical significance of the different expression among the groups. In order to obtain a better resolution of subcellular structures in very thick samples, the confocal analysis of *BCL6* was performed in a subset of NM, MA and K samples (Fig. 2). Moreover, this technique allowed to define the precise distribution pattern and the quantification of *BCL6* protein. The staining patterns of *BCL6* varied from a diffuse mode to a granular one, both in the cytoplasm and in the nucleus of stromal cells, appearing consistent and unchanged in the same samples, without appreciable variations in intensity and localization among the cells. In all NM samples, a few scattered areas in the stroma compartment exhibited a moderate *BCL6*-reactivity, although the epithelial cells were not marked (Fig. 2A-C). The results of immunofluorescence analysis were in line with those of immunohistochemistry: intense staining was evident in MA, in stromal cells surrounding colonic crypts (Fig. 2D-F); moreover, a moderate expression appeared in epithelial cells,

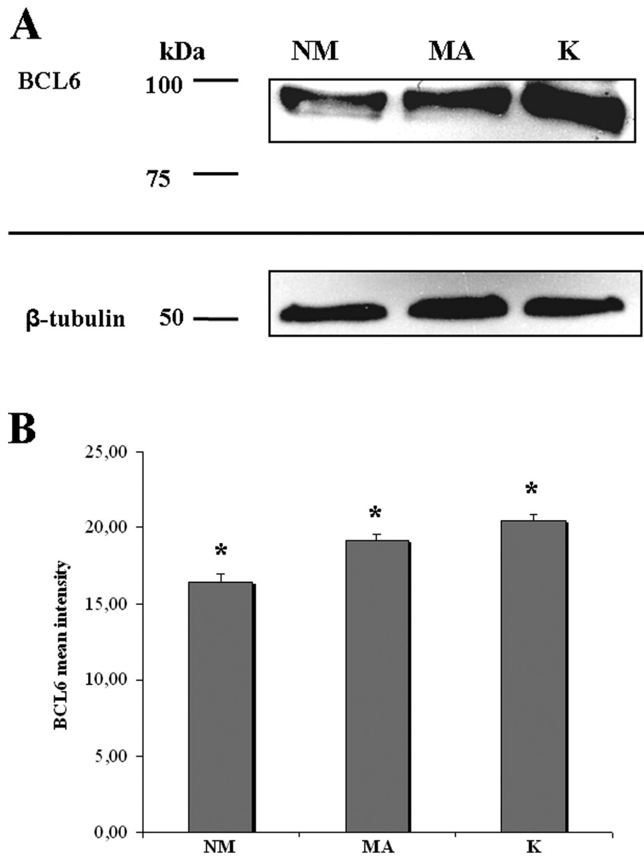

Figure 4. (A) Western blotting of *BCL6* in human normal colorectal mucosa (NM), microadenoma (MA) and colorectal cancer (K). (B) Mean densitometric values of *BCL6* protein expression level. *P<0.05 between all group pairs of samples. Bar, SD.

but only at the cytoplasmic level (Fig. 2G-I). *BCL6*-positive cells were observed throughout the resected tissue in all colon cancer lesions and they were particularly numerous within the central tumor stroma (Fig. 2J-L). Some cells (both the epithelial and stromal ones) showed a strong *BCL6* immunoreactivity in the cytoplasm as well as in the nucleus inside some tumor areas (Fig. 2M-O); in particular, they appeared located deeply, inside the tumor lesion. These features were confirmed by the measurement of the *BCL6* expression levels, both in the epithelial and in the stromal compartment, by means of immunohistochemistry and immunofluorescence, that showed statistically significant differences in the 3 different types of samples (Fig. 3A and B). These results were in agreement with those obtained by immunohistochemical analysis.

Western blot quantification. To confirm the findings of the morphological evaluation previously described, cell lysates of NM, MA and K were analyzed by western blotting. The protein profile of *BCL6* clearly showed 1 single weak band at the expected molecular weight (98-kDa) in NM, an intense band in MA and a very intense band in K (Fig. 4A). Densitometric analysis and normalization (with equal amounts of protein loading) of the immunoreactivity signal from protein extracts of NM, MA and K showed that the level of *BCL6* protein has an increasing trend from NM to K, with statistical significance of the different expressions (Fig. 4B).

Discussion

The aim of the present study was to evaluate *BCL6* expression during the neoplastic progression of human intestinal mucosa. *BCL6* is a transcriptional repressor required in mature B cells during the germinal center (GC) reaction (3). The critical functions exerted by *BCL6* during normal B-cell development can be hijacked by the malignant transformation process, thus indicating its critical involvement in colorectal cancer. Indeed, *BCL6* is targeted by genetic aberrations and acts as an oncogene in GC-derived lymphomas (2); despite this, its precise role in the malignant transformation process remains to be fully elucidated. In non-hematolymphoid neoplasms, *BCL6* expression has been demonstrated in subsets of high grade ductal human carcinomas of the breast, both at the gene and the protein level (12,13).

To our knowledge, this is the first comprehensive report from morphological and quantitative analyses of *BCL6* in human colorectal carcinogenesis, both in the epithelial and the stromal compartment. The results clearly outline that there is a marked increase in the intensity and density of staining for *BCL6* protein in the normal mucosa-microadenoma-carcinoma sequence. Immunohistochemistry and immunofluorescence analyses showed that *BCL6* is expressed in normal mucosa, although at low levels, whereas it significantly increases in microadenoma (MA) and in cancer with statistical significance. These results were confirmed by western blotting data, with no difference according to the various techniques used. In *BCL6*-positive cells, both the nucleus and the cytoplasm appeared stained and this finding is in accordance with previous data on cellular localization of *BCL6* in gastric cancer (21). Moreover, our observations consistently showing that the *BCL6* protein is expressed only in the stromal compartment of the normal mucosa support some observations of other authors; *BCL6* was considered to be produced at low levels in various tissues, as reported in EST libraries obtained from several epithelial tissues, although Hirata *et al* (21) reported that *BCL6* is expressed in normal gastric epithelial cells and that it is not expressed in normal mucosa of colon and oesophagus. However, in their study, the authors did not examine the stromal compartment of the colon and did not show quantitative data.

In addition, our data showed that the *BCL6* protein is highly expressed in epithelial cells, in premalignant lesions as well as in carcinomatous tissues. For this reason, we hypothesized that the increased expression of the *BCL6* protein is due to the transformation of the epithelium, that represents an important source of this protein.

Another notable consideration is that the *BCL6* expression/production by the epithelium appears to be linked to the early stages of carcinogenesis, since the positivity is strongly detectable in MAs. Although MAs are not the only microscopic lesions in the large bowel, they represent the most common precursors of cancer in this organ (22,23). In these neoplasms, the transformation process begins in the crypts and seems to result from qualitative, quantitative and spatial reorganization of some pathways that represent the physiologic regulator of epithelial homeostasis (24-26). Although the key role played by MAs in colorectal tumorigenesis is widely acknowledged, the modifications of gene expression that trigger or accompany their development have never been comprehensively studied.

This investigation identifies, for the first time, *BCL6* as a putative marker of colorectal tumorigenesis and may also provide new insight for future molecular characterization of colorectal precancerous lesions.

Recently, Walters *et al* (14) examined a series of mesenchymal tumors with immunohistochemical and fluorescence *in situ* hybridization techniques. They reported that *BCL6* expression is relatively common in solitary fibrous tumor (SFT) and that, markedly, *BCL6* protein seems to be significantly more expressed in malignant SFT, by the histological point of view, as compared to benign SFT. This finding is significant, inasmuch as our results suggest that the *BCL6* protein may play some roles in the malignant transformation occurring in the large bowel. Our data should be validated with further investigations on premalignant and malignant lesions and/or different subtypes of colorectal cancer.

As is well known, microarray data serve as a springboard and reference point for further studies on the molecular basis of colorectal transformation along the adenocarcinoma pathway; in this regard, Saadi *et al* (27) recently carried out a gene set enrichment analysis (GSEA) to demonstrate that genes of the stromal cellular compartment discriminate pre-invasive from invasive disease and suggest outcome prediction in digestive tumors, including colorectal cancer. The authors identified several genes, including *BCL6,* and underlined that the upregulation of this protein shows a trend for significantly poorer outcome in esophageal adenocarcinoma. Although the genes identified are relevant to Barrett's carcinogenesis, it is likely that these genes are involved in a dysregulated process common to other gastrointestinal tumors rather than being isolated critical oncogenes.

Furthermore, we consider immunohistochemistry critical in performing the identification of proteins which are *current* as well as *new putative* therapeutic targets. On the basis of our excellent results obtained by immunohistochemical analysis, fully confirmed by immunofluorescence and western blotting methods, it would be useful to determine whether the *BCL6* protein can be considered a histological marker for colorectal cancer.

Considering the well documented accuracy of the prognostic relevance of *BCL6* in large B-cell lymphomas (28-30), it is possible that quantification of *BCL6* expression may have a potential role in risk evaluation of large bowel tumors.

In conclusion, the findings of the present study clearly show the qualitative and quantitative expression of *BCL6* in human colorectal cancer development (in particular, the localization of *BCL6* expression in precancerous as well as tumor epithelia), and demonstrate that *BCL6* appears to be involved in tumor progression, from the earliest stage of carcinogenesis. The improved understanding of the biological function of *BCL6*, the key role of which was already demonstrated in lymphomagenesis, should lead to a re-evaluation of the importance of this protein in other tissues and may highlight the relevance of performing further studies in order to identify novel therapeutic targets for colorectal cancer.

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