Connexin 26 correlates with Bcl-xL and Bax proteins expression in colorectal cancer

Luiza Kanczuga-Koda, Stanislaw Sulkowski, Mariusz Koda, Elzbieta Skrzydlewska, Mariola Sulkowska

 AIM: To evaluate of Cx26 in correlation with Bcl-xL and Bax proteins in colorectal cancer.

 METHODS: Immunohistochemical staining using specific antibodies was performed to evaluate the protein expression of Cx26, Bax and Bcl-xL in 152 colorectal cancer samples and the correlations among studied proteins as well as the relationships between the expression of Cx26, Bax, Bcl-xL and clinicopathological features were analyzed.

 RESULTS: Both normal epithelial cells and carcinoma cells expressed Cx26, Bax and Bcl-xL, but Cx26 in cancer cells showed aberrant, mainly cytoplasmatic staining. Expression of Cx26, Bax and Bcl-xL was observed in 55.9%, 55.5% and 72.4% of evaluated colorectal cancers respectively. We found the positive correlation between Cx26 and Bax expression (r = 0.561, P < 0.0001), Cx26 and Bcl-xL (r = 0.409, P < 0.0001) as well as between Bax and Bcl-xL (r = 0.486, P < 0.0001). Association of Cx26, Bax and Bcl-xL expression with histological G2 grade of tumors was noted (P < 0.005, P < 0.001 and P < 0.002 respectively).

 CONCLUSION: Cytoplasmic presence of Cx26 and its association with apoptotic markers could indicate a distinct role from physiological functions of Cx26 in cancer cells and it could suggest that connexins might be a target point for modulations of apoptosis with therapeutic implications.

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 Key words: Cx26; Bcl-xL; Bax; Apoptosis; Colorectal cancer; Immunohistochemistry

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INTRODUCTION

The most common way of communication between cells is gap junctional intercellular communication (GJIC) mediated by gap junctions (GJs), which are formed from transmembrane proteins called connexins (Cx). A hexameric unit of connexins in one cell (a connexon) couples with a corresponding connexon in a contiguous cell to join the cytoplasm[6]. This allows synchronizing different functions of cells within a tissue. The connexin proteins are encoded by a multigene family, and so far 20 different human Cx genes have been identified[7]. Gap junctions may be heterotypic (each connexon composed of different Cx isotypes) or heteromeric (each connexon composed of more than one Cx isotype)[8]. Gap junction channels allow the exchange of ions, nucleotides, metabolites and other small molecules (<1 ku) including second messengers such as cAMP, IP3 and Ca2+ between adjacent cells[9,10]. GJIC plays an important role in the maintenance of tissue homeostasis probably also by regulating the balance between cell gain and cell loss[11]. Cancers are considered to be the result of a disruption of the homeostatic regulation of a cell’s ability to respond appropriately to extracellular signals of the body, which trigger intracellular signal transduction mechanisms. Cancer cells, among others, have altered ability to programed cell death. On the other hand, the cancer cells of solid tissues appear to have either dysfunctional homologous or heterologous GJIC[6,7]. Altered expression of connexins has been observed in various cancers and forced expression of members of this gene family suppresses tumor growth[6,9,12].

The normal human epithelial cells in the colon express Cx32 and Cx43[10]. In previous studies we also observed for the first time Cx26 expression in the normal colon epithelium as well as in the colorectal cancer[11,12]. However, the involvement of these connexins in apoptosis during colorectal carcinogenesis has not been investigated.

Dysregulation of apoptosis plays an important role in a colorectal carcinogenesis[13,14]. Apoptosis is a complex physiological process that plays a crucial role in tissue homeostasis. Recent data suggest that modulation of molecules involved in the regulation of cell death by apoptosis may be equally important. The main group of genes controlling apoptosis is the Bcl-2 family, which includes both promoters (Bax, Bak, Bad and Bcl-xS) and inhibitors
(Bel-2, Bel-xL and Mel-1)\(^{15}\). Bel-xL is able to form heterodimers with Bak and Bax. The elevated expression of this protein seems to be an early event in colorectal carcinogenesis\(^ {16}\). Bak is a proapoptotic factor\(^ {18}\) and shows a high similarity with some Bel-2 family proteins such as Bel-xL, Bel-w or Bid. As far as now, immunohistochemical studies have shown both lacks of statistically significant differences between expression of Bak in normal epithelial cells of colorectal mucosa and colorectal cancer cells\(^ {16}\) as well as overexpression of Bak in primary colorectal cancer \(\approx\) normal mucosa\(^ {17}\).

Previous studies of apoptosis have focused mainly on the role of intracellular signaling pathways in the regulation of apoptosis. However, some studies have demonstrated correlation between intercellular communication and apoptosis\(^ {18,19}\). On the other hand, interactions between members of Bel-2 family and gap junction proteins, connexins, in colorectal cancer have not been investigated; thus, we examined the expression of Cx26, Bel-xL and Bax by immunohistochemistry and correlations between Cx26 and studied apoptotic markers in colorectal cancer patients.

### MATERIALS AND METHODS

Tissue samples were obtained from 152 patients (78 men and 74 women) who underwent surgical resection because of colon (84 cases) and rectal (68) carcinomas. Our study included 128 colorectal cancers classified histopathologically as adenocarcinoma and 24 as mucinous adenocarcinoma: 108 cases in G2 grade and 44 cases in G3 grade. There were 14 tumors in pT2 stage and 138 in pT3 stage. 82/152 (53.9%) patients had involved lymph nodes at the time of diagnosis. The age of patients ranged from 35 to 92 years (mean 65.4 years). Tumor samples with adjacent normal colon mucosa were collected immediately after tumor removal, fixed in 10% buffered formaldehyde solution for 48 h and then embedded in paraffin blocks at 56 °C according to standard procedures. The resected tumors were histopathologically examined using standard hematoxylin-eosin staining.

### Immunohistochemistry

Paraffin-embedded tissue sections were subjected to immunostaining, using goat polyclonal anti-Cx26, goat polyclonal anti-Bax and goat polyclonal anti-Bel-xL antibodies (Abs) (Santa Cruz Biotechnology, USA) in dilution rate: 1:400, 1:200 and 1:300 respectively. All primary Abs were diluted in PBS with 1.5% normal blocking serum. The studies were performed with avidin-biotin-peroxidase complex (ABC Staining System, SCBi, USA). Slides were counterstained with hematoxylin. In negative controls sections known to stain positively with studied Abs were included in each run with buffer instead of primary antibodies.

The evaluation of immunostaining for Cx26, Bax and Bel-xL was analyzed in 10 different tumor fields and the mean percentage of tumor cells with positive staining was evaluated. The sections were classified as positive when at least 10% of cancer cells expressed the studied antigens.

### Table 1

| Comparative factors | Cx26 | Bax |
|---------------------|------|-----|
| Cx26                | 0.561| <0.0001|
| Bax                 | 0.499| <0.0001|
| Bel-xL              | 0.486| <0.0001|

The expression of Cx26, Bax and Bel-xL did not correlate with age, sex of patient, tumor localization or tumor size. In our study, we noted a tendency toward association between Cx26, Bax expression and adenocarcinoma, but not mucinous adenocarcinoma type \((P=0.07\) and \(P=0.064\) respectively) as well as positive association between Bel-xL expression and adenocarcinoma type of cancer \((P<0.02)\). The subgroup of patients without involved lymph nodes showed unquestionable positive association between Cx26, Bax, Bel-xL expression and adenocarcinoma type of tumor \((P<0.001, P<0.02, P<0.0007\) respectively). On the other hand in the case of mucinous adenocarcinoma we did not find similar relationships. In better differentiated tumors (G2) we observed more Cx26, Bax and Bcl-xL-positive cases \((P<0.005, P<0.001\) and \(P<0.002\) respectively) than in poorly differentiated tumors (G3). A tendency towards negative association between Bax expression and lymph node status \((P=0.063)\), but not between Cx26, Bel-xL and lymph node status was observed.

### Statistical analysis

The significance of the associations was determined using Spearman correlation analysis and the \(\chi^2\) test. Probabilities of \(P<0.05\) were assumed as statistically significant.

### RESULTS

Immunohistochemical analysis of the colorectal cancer sections revealed in 85/152 (55.9%) cases mainly cytoplasmic localization for Cx26. In a few cancer cases (15/152), classified in G2 grade, we focally observed punctate intercellular staining (Figure 1A-D). The adjacent colorectal mucosa also revealed positive immunostaining for this protein, but only punctate immunoreactivity was seen (Figure 1C). Cytoplasmic localization and microgranular staining for Bax (Figure 1E, F) and Bel-xL (Figure 1G, H) proteins was noted in colorectal cancer sections. The adjacent colorectal mucosa also revealed positive immunostaining for these proteins. The studied markers were not detected in control samples, where immunostaining was performed with the omission of the primary antibodies. The positive expression of Bax and Bel-xL was found in 55.5% and 72.4% of the tumors respectively.

Analysis of correlations among assessed proteins revealed the positive correlation between Cx26 and Bax expression \((r=0.561, P<0.0001)\), between Cx26 and Bel-xL \((r=0.409, P<0.0001)\) as well as between Bax and Bel-xL \((r=0.486, P<0.0001)\) (Table 1). Interestingly, correlation between Cx26 and Bax was stronger in better differentiated (G2) than in poorly differentiated (G3) tumors \((P<0.0001\) and \(P<0.006\) respectively).
DISCUSSION

Connexins are typically localized in the cell membrane and normally show a punctate pattern of expression\(^4,20\). Aberrant localization of connexins may contribute to the loss of intercellular communication via gap junctions\(^21\). Our previous observations\(^11\) and present results suggest that impaired communication between neoplastic cells may depend on the subcellular disturbance in the synthesis and localization of Cx26. Consequently, Cx26 protein accumulates in the cytoplasm of cancer cells and it is possible that Cx26 in this localization could play a distinct role from physiological functions.

Cytoplasmic localization of Cx26 might reflect a transcriptional or posttranscriptional defect of this protein during a colorectal carcinogenesis. Previous studies revealed mutations in the extracellular or transmembrane regions of connexins, which contributed to alteration in connexins
localization as well as to loss of GJIC\textsuperscript{22-28}. Immunohistochemical studies of the mutant Cx43 protein revealed nuclear and cytoplasmic localization and no sign of Cx43 in the intercellular area of mutant cells. Furthermore Krutovskikh et al\textsuperscript{22} revealed that subcellular localization of Cx43 in tumor cells could play a role in the regulation of tumor growth. In the other study\textsuperscript{25} mutations in the second extracellular region prevented localization to the plasma membrane but did not decrease the ability of Cx43 to inhibit the growth of tumor cells \textit{in vitro}. Olbina and Eckhart\textsuperscript{24} concluded that regulation of cellular growth by Cx43 does not necessarily require GJIC. It could suggest that cytoplasmic connexins might control tumor progression by the influence on the expression of genes, which are responsible for regulation of cancer cell's functions. Basing on above findings and on our previous\textsuperscript{11,18} as well as present results it could be concluded that Cxs localized in the cytoplasm and in the plasma membrane between cells could play different roles in malignant and normal cells, but additional functional studies of the role of Cxs in signalling pathways are required.

Considerable data demonstrate that connexins could play a tumor suppressor role. Currently it is accepted that the tumor suppressive effect of connexins is associated with inhibition of cell growth and regulation of tissue differentiation. On the other hand, it has been shown that enhancing of apoptosis by a transfer of signalling molecules via gap junctions\textsuperscript{5} can contribute to the tumor suppression, but mechanisms, which regulate these processes are still unclear and it concern connexins localized in the plasma membrane. We suppose that regulation of apoptosis by connexins could be, among others, a result of a control of apoptotic markers such as Bcl-2 family proteins. This theory might explain subcellular localization of connexins in cytoplasmic or nuclear compartments of tumor cells. Krutovskikh et al\textsuperscript{22} proposed that connexins localized in cytoplasm have different signalling activity than those localized in the plasma membrane. Moreover, they supposed that signal transduction functions of connexins require interactions with other intracellular proteins. In fact Huang et al\textsuperscript{18} observed decreased expression of Bcl-2 in Cx43-transfected malignant cells compared to non-transfected cells. They suggested that connexin genes could regulate expression of other genes in tumor cells. Similarly Tanaka and Grossman\textsuperscript{19} found that forced expression of Cx26 (transfected cells with a Cx26 adenovirus vector) in prostate cancer cells suppressed the growth of cancer cells, induced cell cycle arrest at the G2/M phase as well as decreased the expression of Bcl-2 and enhanced apoptosis.

Although the role of connexins in cell growth regulation has been extensively studied, their involvements in apoptosis remain unclear. It has been postulated that GJIC plays a significant role in the regulation of apoptosis in cancerous cells. Krutovskikh et al\textsuperscript{21} have found that due to intercellular communication via gap junctions, cancer cells can spread cell death signals between themselves, and the messenger molecules, which initiate apoptotic process in neighboring cancer cells are probably Ca\textsuperscript{2+} ions. The ability of cells to kill each other through GJ channels has been shown in “bystander death” experiments, where toxin spreads via GJ channels from affected cells into neighboring unaffected cells and eventually kills them\textsuperscript{26,27}. On the other hand, cancer disease is characterized by dysfunction of both, intercellular communication as well as apoptosis\textsuperscript{8,18,27}. Huang et al\textsuperscript{18} found that expression of Cx43 in human glioblastoma cells increased sensitivity of cells to chemotherapeutic agents, which resulted from apoptosis. They reported that Cx43-mediated apoptosis to chemotherapeutic agents is regulated in part through the down-regulation of Bcl-2 expression. It is important to notice that these authors suggested that increased apoptosis after re-expression of Cx43 might not be linked to increased gap junctional communication. In the present study we observed cytoplasmic expression of Cx26, which we consider to be with altered function. Analysis of correlations revealed the positive correlation between Cx26 and proapoptotic Bax as well as between Cx26 and ant apoptotic Bel-xL. It suggests that cytoplasmic Cx26 could perform additional functions in malignant cell, for example, it might be involved in the control of apoptotic process, but functional relationships between cytoplasmic Cx26 and proteins involved in apoptosis require additional studies.

In the present study, we also analyzed correlations between expression of Cx26, Bax, Bel-xL and some clinicopathological features. As described previously\textsuperscript{11,18}, we did not find correlation between Cx26 expression and lymph node status. But we observed a tendency toward negative association between Bax expression and lymph node status. Furthermore, we found that in better differentiated tumors (G2), more Cx26-positive cases were present than in poorly differentiated tumors (G3), but mostly cytoplasmic staining was observed. In some cases of G2 carcinomas punctate immunostaining for Cx26 was seen. These observations indicate that during carcinogenesis in colon and rectum there are possible alterations in Cx26 expression, localization and probably decrease of functional gap junctions. Interestingly, we also observed positive association between Cx26 expression and adenocarcinoma type of tumor. It is well known that mucinous adenocarcinoma is associated with poorer outcome of patients than adenocarcinoma, so the presence of Cx26 in cytoplasm of colorectal tumors could be a good prognostic factor.

Our results showed aberrant expression and localization of connexin 26. Furthermore, cytoplasmic presence of Cx26 and its association with apoptotic markers could indicate a different role of Cx26 in neoplastic cells than participation in gap junctional intercellular communication and it could suggest that connexins might be a target point for modulations of apoptosis with therapeutic implications.

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