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Gradual Loss of Functional Gap Junction within Progression of Colorectal Cancer – A Shift from Membranous CX32 and CX43 Expression to Cytoplasmic Pattern During Colorectal Carcinogenesis

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Abstract. *The aim of this study was the assessment of expression and location of CX32 and CX43 in colorectal adenomas and carcinomas as well as analysis of expression of these proteins in association with clinical and pathological features of tumors and evaluation of mutual relationships between CX32 and CX43. Patients and Methods: The study included 151 primary colorectal carcinoma and 71 colorectal adenomas. The control group comprised 30 colon samples. Connexins were detected with immunohistochemistry. Results: There was a lack of membranous distribution of connexins or a shift from moderately membranous immunoreactivity to predominantly cytoplasmic accumulation of CX32 and CX43 in studied colon tumors. Mentioned alterations were found in adenomas and augmented in cancer. Expression of Cx32 was significantly associated with grading of colorectal cancer, implicating a role of intracellular CX32 in regulation of tumor growth and differentiation. A strong correlation was present between CX32 and CX43 in node-positive cases and absent from node-negative ones. Conclusion: To our knowledge, our study is the first illustration for a gradual loss of functional gap junctions within progression of colorectal neoplasia. An intracellular location of connexins, the site of their common and the most frequent detection within cancer cells in our study may be of significance. Independenty of its role in functional gap-junctions, cytoplasmic CX32 could be involved in cancer differentiation, resulting in a higher rate of CX32 positive moderately differentiated tumors (G3) than poorly differentiated CX32-positive ones (G3).*

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Intercellular junctions of the gap type are the channels that are composed of connexins (CX) and are located among different cells of various layers of the gastrointestinal wall. Although, studies have mainly focused on human muscularis propria, expression of CX43 and CX32 was also detected in enterocytes of human colon (1); our previous research was aimed at evaluation of CX43, CX32 and CX26 at this site (2). One of most important features of neoplastic cells is uncontrolled growth and altered differentiation. In vitro studies that were conducted for the first time in 1966 showed that intercellular communication is very restricted among neoplastic cells (abolished homologous gap junctions) (3). Lack of gap junctions was then detected at the junction of malignant with benign cells (spoilt heterologous gap junctions) in hepatic carcinogenesis (4). Furthermore, oval cells, which are believed to be liver stem cells can differentiate into hepatocytes or biliary cells via restriction of certain connexin expression. Namely, CX32 was discovered as being expressed in hepatocytes, while biliary cells expressed Cx43. Besides this blockage of CX32 or CX43 can result in an onset of hepatocellular or cholangiocellular carcinomas from differentiating oval cells with impaired gap junction intercellular communication (GJIC) (5). Discohesion and isolation of cells allow malignant cells to escape the stream of signaling which normally keeps cell proliferation under control. This escape can partially result from a decrease of connexin expression and functional GJIC (5). Carcinogensis is also accompanied by the changes in the cellular membrane and subsequent

aberrant re-distribution of connexins in the cell (2). On the other hand, restoration of GJIC and upregulation of connexin expression can be induced by agents such as cyclic AMP agonists which increased CX43 protein in lung epithelial cells (6).

Such alterations prevent cell to cell adhesion which is indispensible for the formation of gap junctions. In consequence, a clone of cells selectively acquires the ability for fast and unrestricted growth along with disruption of tissue integrity. As yet, most solid cancers have been shown to have qualitative and quantitative aberration of gap junctions, which are consistent with alteration of connexin expression (7-9). CX26 expression and cellular distribution were found to be altered in colorectal adenomas and carcinomas in comparison with unaffected intestinal mucosa in our previous studies (2, 10-12) and another subsequent publication (13). However, the mechanisms involved connexin expression and its significance have been addressed in few publications, mostly in animal models of connexin signaling, or experiments on cell lines to explain pathogenesis of colorectal neoplasia. Therefore, the aim of this study was to assess expression and location of CX32 and CX43 in colorectal adenomas and carcinomas in relation to that in unaffected colon mucosa.

Patients and Methods

Patients and tissues. Specimens used in this study were obtained from 151 colorectal carcinomas and 71 colorectal adenomas surgically removed. Villous and serrated adenomas were excluded and only tubular and villotubular adenomas with low-grade intraepithelial neoplasia (LGIN) were included in the study to construct a relatively homogenous group. The control group constituted 30 samples that were collected from the proximal surgical colon not less than 10 cm distant from the malignant tumor margin. For immunohistochemical studies, these selected tissues did not reveal significant pathological changes under microscopic examination. Tissue fragments were sampled immediately after tumor removal, fixed in 4% buffered formaldehyde solution and embedded in paraffin blocks at 56˚C according to standard procedures. Histopathological examination was performed using standard hematoxylin-eosin staining.

Immunohistochemistry. CX32 was detected with a goat polyclonal antibody sc-7258 (Santa Cruz Biotechnology, USA) at a dilution of 1:200, which recognizes amino acids in the carboxyl terminus of human CX32. CX43 was detected with a goat polyclonal antibody sc-6560 (Santa Cruz Biotechnology, USA) at a dilution of 1:300 which recognizes epitope mapping at the *C*-terminus and is recommended for the detection of human CX43. Immunohistochemical studies, including negative controls with omission of the primary antibodies, were performed as described elsewhere (2, 14).

Evaluation of immunohistochemical staining. Expression of CXs in the studied samples of the normal human colon and adenomas was classified using a three-point scale: – no immunoreactivity; +/– weak immunoreactivity observed partially in the texture of a certain benign lesion; (+) strong immunoreactivity observed in most of a texture of a given sample. The expression of CXs in colorectal cancer was analyzed in 10 different tumor fields and was assessed according to a 3-point scale: 0, <10% positive cells; 1+, 10-50% positive cells; 2+, >50% positive cells. For statistical comparisons with selected clinicopathological features, the specimens were divided into groups of connexin-positive (connexin expressed at level 1+ or 2+) and connexin-negative (connexin expressed at level 0) tumors.

Statistical analysis. The association of CX32 with selected clinicopathological features was evaluated using the chi-square test. Differences in CX32 expression between primary tumors and lymph node metastases were assessed using Mann-Whitney *U*-Wilcoxon rank sum W-test. Probabilities of $p<0.05$ were assumed as being statistically significant.

These human studies were performed in agreement with the ethical standards laid down in the 1975 Declaration of Helsinki and its latest revision in 2004 and approved by the Ethics Committee of Medical University of Bialystok). All the participants gave their informed consent before they were included in the study.

Results

Assessment of CX32 expression and distribution in colonic mucosa and colonic adenomas. CX32 was detected in the form of quite large granules between epithelial cells in colon mucosa (Figure 1a). Expression and distribution of CX32 was described in benign colonic epithelium in our earlier report in detail (2). In most of the adenomas (82%) there was positive, but predominantly weak, staining. The distribution of CX32 was exclusively membranous only in 19% of positive cases. Mixed membranous-cytoplasmic immunoreactivity was observed in 64% of CX32 positive adenomas and exclusive cytoplasmic staining was present in 17% of positive cases (Figure 2a).

Cellular location of CX32 and evaluation of its expression in reference to selected clinical features of colorectal cancer. Positive immunoreactivity for CX32 was found only in 44 examined tumors (29% of all cases): 23 of them exhibited with weak staining, and 21 of them with strong staining. There was exclusively finely granular cytoplasmic staining in all CX32-positive malignant tumors (Figure 3a).

There was no statistically significant relationship of Cx32 expression with patients' age, gender, tumor site, histopathological type of colorectal cancer or lymph node involvement. CX32 expression was statistically higher expressed in moderately differentiated tumors in comparison with poorly differentiated ones (Table I).

Assessment of expression and location of Cx43 in intestinal mucosa and adenomas. Similarly to CX32, membranous immunoreactivity to CX43 was membranous in colonic mucosa, particularly in the basal part of colon crypts (Figure 1b). Detailed descriptions of expression and location of CX43 were shown in neoplasia-spared colon in previous report (2).

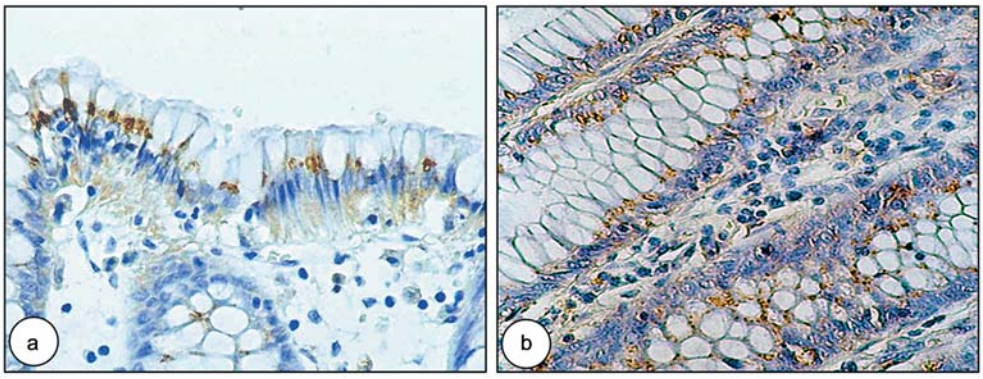

Figure 1. *Immunoreactivity to CX32 (a) and Cx43 (b) in the mucosa of normal human large intestine. Immunopositive deposits of CXs are mainly distributed in between goblet cells in colon mucosa. Original magnification ×200.*

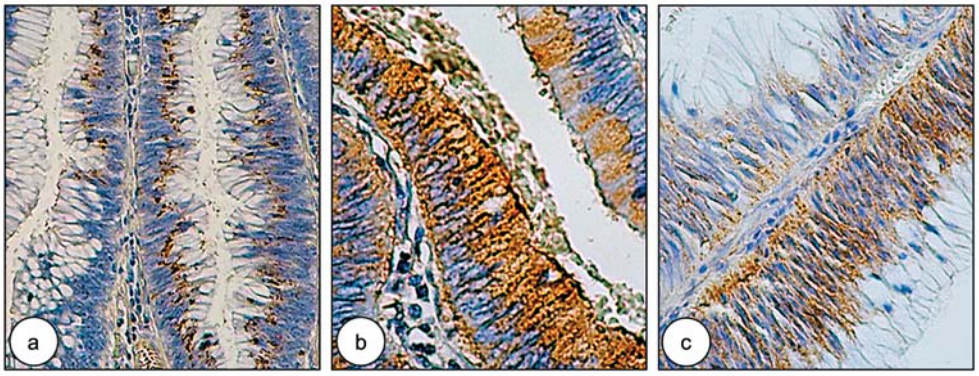

Figure 2. *Anti-Cx32 and anti-Cx43 staining (a) and CX43 (bc) in colorectal adenoma. Coarsely granular intercellular immunoreactivity to Cx32 between cells presenting low grade interaepithelial neoplasia (LGIN) (a). Strong granular cytoplasmic staining against CX43 with focal membranous immunoexpresion of CX43 in LGIN (b) and coarsely granular immunostaining for CX43 with a mostly intercellular location in glandular tubes of an LGIN (c).*

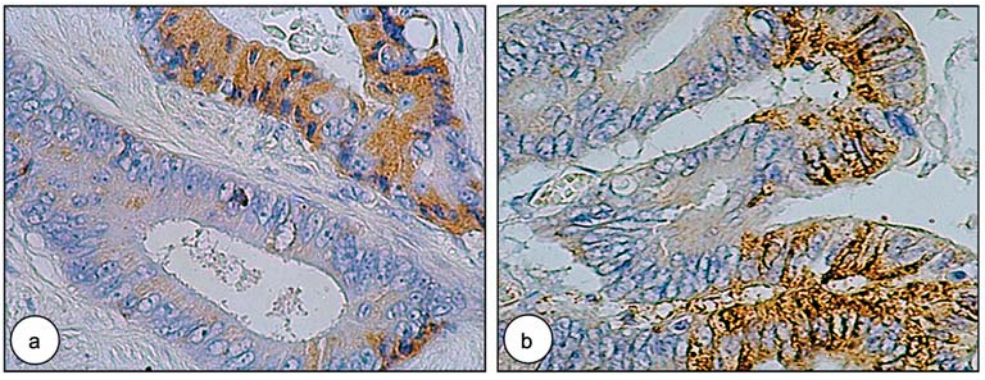

Figure 3. *Expression of CX32 (a) and Cx43 (b) in the colorectal carcinoma. Heterogeneous, cytoplasmic, finely granular distribution of CX32 in part of a colorectal cancer cell population (a). Mixed, cytoplasmic and membranous anti-CX43 labeling of some cancer cells is apparent (b).*

Table I. *Comparison of CX32 expressions in colorectal cancer in groups with different clinical and pathological features of colorectal cancer patients.*

Patients'groups	CX32 (–), n	CX32 (+), n	*p*-value
Age (years)			
≤60	31	17	0.246
>60	76	27	
Gender			
male	58	21	0.468
female	49	23	
Location			
Rectum	52	21	0.922
Colon	55	23	
Hp-type			
adc	87	41	0.065
adc muc	20	3	
Grade			
2	69	37	0.016
3	38	7	
pT			
pT1+pT2	12	2	0.199
pT3+pT4	95	42	
pN			
(–)	48	23	0.406
(+)	59	21	

Hp-type, histopathological type; adc, adenocarcinoma; Adc muc, mucinous adenocarcinoma; grade, grading of cell differentiation; pT, tumor ingrowth depth; pN, lymph node involvement.

Table II. *Comparison of CX43 expressions in colorectal cancer between subgroups of different clinical or pathological features of colorectal cancer patients.*

Patients'groups	CX43 (–), n	CX43 (+), n	*p*-value
Age (years)			
≤60	21	27	0.994
>60	45	58	
Gender			
men	32	47	0.405
women	34	38	
Location			
rectum	26	47	0.052
colon	40	38	
Hp-type			
adc	57	71	0.630
adc muc	9	14	
Grade			
2	42	64	0.120
3	24	21	
pT			
pT1+pT2	5	9	0.526
pT3+pT4	61	76	
pN			
–	28	43	0.318
+	38	42	

Hp-type, histopathological type; adc, adenocarcinoma; Adc muc, mucinous adenocarcinoma; grade, grading of cell differentiation; pT, tumor ingrowth depth; pN, lymph node involvement.

Positive CX43 labeling was revealed in 63 cases (89%) of adenomas. Weak immunoreactivity was found in 49% and strong staining was detected in 51% of positive adenomas. Finely granular membranous immunoreactivity was found in 31 cases and 32 adenomas exhibited a mixed, membranous and cytoplasmic distribution of CX43 (Figure 2bc).

Cellular distribution of Cx43 and evaluation of its expression in association with clinical and pathological features of colorectal cancer. There were CX43-positive 85 colorectal tumors (56%) in the studied group. Expression of CX43 was weak in 28 and strong in 57 neoplasms among CX43-positive tumors. In most of studied slides, there was finely granular pattern of staining. A total of 59 tumors showed cytoplasmic immunoreactivity for CX43; only 6 tumors were characterized with membranous labeling of malignant cells, while in 20 cases there was mixed immunoreactivity (membranous and cytoplasmic) (Figure 3b).

There was no statistically significant relationship between the expression of CX43 and either age or gender of patients, or location and histological type of colorectal cancer, depth of mural malignant ingrowth or lymph node involvement (Tabele II).

Comparison between CX32 and CX43 in colorectal cancer. Mutual relations were drawn between CX32 and CX43 in colorectal cancer. There was positive statistically significant correlation between CX32 a CX43 in patient whole group ($p<0.0001$, r=0.286), in the subgroup of patients with lymph node-positive cancer ($p<0.0001$, r=0.429), in moderately differentiated (G2) colorectal adenocarcinomas ($p=0.022$, r=0.222) and poorly differentiated ones (G3) ($p=0.037$, r=0.313), in histopathological type of conventional adenocarcinoma ($p=0.002$, r=0.275). No statistically sound relationship was revealed in the comparison between CX32 and CX43 expressions for cancer without nodal involvement ($p=0.336$, r=0.116) nor in the subgroup of mucinous adenocarcinomas ($p=0.107$, r=0.345).

Discussion

The detection of reduced and aberrant, cytoplasmic expression of CX32 and CX43 in most of human colorectal adenomas in comparison with normal colonic mucosa in our current studies is novel – there is a complete lack of such an observation of CX32 and CX43 alteration in any previous reports on humans; it is worth mentioning that CX26 was

found to undergo similar changes in our past studies (2, 10). Thus, it is difficult to refer to publications of other authors. However, one work was focused on immunohistochemical detection of CX26, CX40 and CX43 but it employed an experimental rodent model of familial adenomatous polyposis (*multiple intestinal neoplasia mice*) (15). The presence of CX26 was discovered in adenomatous epithelium and – what is more interesting – there was a selective increase of CX43 in adjacent stromal cells (15).

In another report, King *et al*. (16) and Aasen *et al*. (17) observed a decrease of CX43, CX26 and CX30 in a comparison of cervical intraepithelial neoplasia with normal paraepidermal cervical epithelium. On the basis of these findings, loss of connexin expression and resultant impairment of gap junction communication was recognized as a possible early event of carcinogenesis in precancerous lesions. The results of our present studies confirm this hypothesis and suggest that an analogous process occurs in colonic adenomas which due to a grade of dysplasia are currently viewed as a type of intraepithelial neoplasia of the colon. On the other hand, Habermann *et al*. (18) reported an increase of CX32 and CX43 expression in frozen sections of hyperplastic prostates, while expressions were dramatically reduced in prostate adenocarcinoma. Similarly, Sawey *et al*. (19) noticed an increase of CX26 and CX43 in rodent skin rodent papillomas and abolishment of expression in malignant skin lesions. Habermann *et al*. (18) thought that increased expression of CX32 and CX43 was able to reflect acceleration of metabolism in benign prostate hyperplasia. Nevertheless, a reduction of connexin expression can mark the loss of gap communication and be involved in numerous genetic and epigenetic alterations that drive unrestricted proliferation and uncontrolled tumor cell growth. Such relationships are revealed and explained in the light of our present detection of CX26 in colorectal adenomas and carcinomas. Alterations of connexin expression may also be dependent on the tissue type.

The results of our studies indicate that colorectal cancer exhibits aberrant expression and subsequent abnormal cellular distribution of CX32 and CX43. There was a decrease or lack of connexin expression in comparison to normal colon mucosa in a number of cases for evaluation of each connexin type. Moreover, in malignant lesions there was positive staining for connexin of surprisingly aberrant location, namely predominantly cytoplasmic in opposition to membranous in normal mucosa. Consequently, a minority of cancers tumors exhibited mixed membranous and cytoplasmic distribution of connexins, particularly of CX43. Dysplastic, adenomatous lesions tended to lose membranous immunoreactivity with a number of adenomas exhibiting connexin accumulation in the cytoplasm. On the grounds of our findings, an aberrant distribution of connexins appears to result in a loss of intracellular gap-type communication (20) and contributes to growing disintegration, autonomy and discohesion of firstly benign neoplastic and later gradually dedifferentiating malignant cells, which would favor cancer dissemination (20). The decrease and/or aberrance of connexin expression found here and by the others indicates that intercellular communication can be very restricted among malignant cells and in interactions between a cancer cell and a benign one. Such isolation of cancer cells would help malignant growth escape from signaling that normally maintains control of proliferation (21).

Connexins which accumulate in the cytoplasm of neoplastic cells could perform a novel function, which is different from the physiological role of membranous connexin. Several mutations result in alterations of extracellular and membranous fractions of connexin protein (20, 22), leading to an aberrant cellular location of connexins. As a result, intercellular communication gap junctions was abolished (20, 22). Moreover, Krutovskikh *et al*. (20) suggested, that intercellularly located CX43 may regulate tumor growth and fulfill the function of cytoplasmic signaling protein in neoplastic cells. *In vitro* structural destruction of extracellular CX43 caused inhibition of membranous translocation of CX43, but the growth inhibitory impact of CX43 remained unchanged. A hypothesis was proposed that cell growth and differentiation can be maintained by connexins independently from the presence of functional gap junctions (23). Cytoplasmic connexins probably control growth and progression of tumors by means of their impact on gene expression crucial for regulation of apoptosis, cell cycle and cell differentiation. Huang *et al*. (24) showed a decrease of antiapoptotic Bcl-2 as a result of transfection of CX43 gene into tumor cells. Furthermore, Chen *et al*. (25) demonstrated a decrease of cyclin-dependent kinase expression in CX43-transfected neoplastic cells. Qin *et al*. (26) observed that growth suppressive properties of connexins might be independent from gap junction communication in breast cancer lines and were likely to be mediated *via* a decrease of gene expression that regulated tumor growth *e.g*. fibroblast growth factor receptor-3 (FGFR-3). Moreover, Zhang *et al*. (27) discovered CX43 inhibited expression of S-phase kinase-associated protein 2 (Skp2), which was involved in degradation of p27. Thus, CX43 indirectly participated in inhibition of tumor cell proliferation that was dependent on elevation of cellular levels of protein p27 (27).

There was a number of reports of immunohistochemical detection of aberrant connexin expression in various tumors in which a decrease of connexin expression was described which referring mainly to membranous expression of connexins (28-30). Hardly any research was concerned with other, nuclear or cytoplasmic locations of connexins distributed in such an aberrant fashion in dysplastic and cancer cells. In the present study aberrant expression and

distribution of CX32 and CX43 were depicted in adenomas, hence impairment of gap junction communications appears to be an relatively early event of colorectal carcinogenesis. No relation of connexin expression was revealed with nodal involvement but a strong and exclusive, statistically significant association between these connexins in node-positive cancer was found in this work. However, it is too early to delineate a possible engagement of CX32 and CX43 in dissemination of colorectal cancer on the ground of this finding alone. Detailed studies are clearly required to elucidate the role of aberrant connexin expression in the biology of colorectal cancer.

Our study is the first illustration of a gradual loss of functional gap junctions within progression of colorectal neoplasia.

The intracellular location of connexins – the place of their common and the most frequent detection within cancer cells in our study- may play a significant role in neoplastic progression.

Independently from functional gap-junctions, cytoplasmic CX32 could be involved in cancer differentiation, resulting in a higher rate of CX32-positive moderately differentiated tumors (G3) than poorly differentiated CX32-positive ones (G3).

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