RESEARCH ARTICLE

Pathological Implications of Cx43 Down-regulation in Human Colon Cancer

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Abstract

Connexin 43 is an important gap junction protein in vertebrates and is known for its tumor suppressive properties. Cx43 is abundantly expressed in the human intestinal epithelial cells and muscularis mucosae. To explore the role of Cx43 in the genesis of human colon cancer, we performed the expression analysis of Cx43 in 80 cases of histopathologically confirmed and clinically diagnosed human colon cancer samples and adjacent control tissue and assessed correlations with clinicopathological variables. Western blotting using anti-Cx43 antibody indicated that the expression of Cx43 was significantly down regulated (75%) in the cancer samples as compared to the adjacent control samples. Moreover, immunohistochemical analysis of the tissue samples confirmed the down regulation of the Cx43 in the intestinal epithelial cells. Cx43 down regulation showed significant association ($p<0.05$) with the histological type and tumor invasion properties of the cancer. Our data demonstrated that loss of Cx43 may be an important event in colon carcinogenesis and tumor progression, providing significant insights about the tumor suppressive properties of the Cx43 and its potential as a diagnostic marker for colon cancer.

Keywords: Gap-junctions - tumor suppressors - adenocarcinoma - connexin 43

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Introduction

Connexins constitutes a family of structurally related transmembrane proteins that connect two adjacent cells by forming gap junctions (Sohl and Willecke, 2004). Each gap junction is composed of two hemichannels, or connexons and each connexon is constituted of six connexin proteins. Connexins coordinate cell-to-cell communication by allowing the direct transfer of molecules less than 1000 Daltons between the cells, which include ions (electric synapses), amino acids, nucleotides, second messengers (e.g., Ca^{2+}, cAMP, cGMP, IP3) and other metabolites (Cruciani and Mikalsen, 2006). Connexins are multi-functional proteins with diverse role in the cell physiology. Connexins are known to regulate the embryonic development, apoptosis, differentiation, tissue homeostasis and metabolic transport (Dbouk et al., 2009). Connexin 43 (Cx43), encoded by Gja1 gene, is the most widely expressed connexin. Cx43 is abundantly expressed in the human tissues, including the colon cells (Kanczuga-Koda et al., 2004). The carboxy-terminal (CT) region of the Cx43 acts as a regulatory domain that can interact with the cytoplasmic microenvironment and co-ordinate various functions of Cx43. Many functions of Cx43 are mediated by the interaction of its CT domain with other proteins, like Heat Shock Cognate Protein 70. NOV/CCN3, Kir6.1, cytoskeletal proteins, etc (Giepmans, 2006; Gellhaus et al., 2010; Ahmad Waza et al., 2012; Hatakeyama et al., 2013). Besides having junctional properties, Cx43 is also an important player in regulating cell growth and differentiation (Araya et al., 2005), with an established tumor suppressive properties (Kandouz et al., 2013).

The GI-tract cancers are the most prevalent cancers in this cohort of population (Pandith and Siddiqi, 2012). Among the GI-tract cancers, colon cancer has shown an alarming upward trend (Javid et al., 2011). Most of the genetic studies carried in this population are based on the mutational analysis of various genes like Axin 2, TP53, etc (Sameer et al., 2010; Khan et al., 2011). These studies have not been conclusive as far their role in the GI-tract cancers is concerned. In order to understand the role of Cx43 in the GI-tract cancer, we tried to explore its expression status in the human colon cancer in this cohort of population and to correlate its expression status with the tumorigenesis of colon tissue.

Materials and Methods

Patient specimens

Fresh tissue samples of the human colon cancer, together with adjacent non-cancerous normal tissue

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(controls) were collected with consent from the patients who underwent curative surgical resection from March 2010 to December 2013 at the Department of CVTS and the Department of General Surgery, Sher-i-Kashmir Institute of Medical Sciences (SKIMS), Srinagar, J&K, India. For each case, the tissue specimens were reviewed by experienced pathologists, to select a representative tumor block and surrounding normal tissue block. During the period, 80 samples of the colon adenocarcinoma were collected. In all the cases, clinical diagnosis was confirmed by histo-pathological examination. The study protocol was approved by the Sher-i-Kashmir Institute of Medical Sciences, Research Ethics Committee. The adjacent normals taken for the study were obtained by macro-dissection, about 3cm away from the tumorogenic area. Ethical rules, regulations and permission were followed while obtaining the samples. Tissue samples, with pathological evidence of disease were frozen within 10 minutes of surgery, placed in sealed cryo-vials and were subsequently preserved at -80˚C till further use.

Microscopic examination

Each tissue sample was examined by analyzing the medical record and tissue slide of the patient. The clinic-histopathological analysis, like age, gender, histological pattern, depth of invasion of the tumor, presence or absence of metastasis to the lymph node or other organs and the expression of Cx43 was investigated. The tumor stages were determined according to the TNM staging system of the American Joint Committee on Cancer (Greene, 2002). The tissue samples to be analyzed were fixed in 10% neutral-buffered formalin, and the prepared paraffin-embedded tissues were sectioned 4-5 mm in thickness. Hematoxylin and Eosion (H and E) staining was performed, and the slides were examined under a light microscope.

Protein extraction from tissues

Tissue disintegration was performed by cutting the tissue into small pieces with a sterile surgical blade, followed by addition of 0.5% trypsin-EDTA solution (Sigma, St. Louis, MO, USA). The tissue was incubated at 37°C for 5 minutes, followed by centrifugation at 12,000 rpm for 5 min. at 4°C. The pellet was rinsed twice with ice-cold PBS (pH 7.4) (137mM NaCl, 3mM KCl, 4mM Na_2HPo_4, 1mM KH_2Po_4) (sigma Aldrich) and the cell lysis was carried out using the lysis buffer (20 mM Tris Cl (pH 8), 137 mM NaCl, 10% Glycerol, 1% Triton-X-100, and 2 mM EDTA). Protease and phosphatase inhibitors (1mM PMSF, 5-10mM NaF and PIC) (Sigma Aldrich) were added in the lysis buffer freshly and the volume of lysis buffer was adjusted according to the wet mass of the tissue (5ml/gm of tissue). Vortex mixing was done for 1 min and the samples were incubated on ice for 45 minutes, followed by centrifugation at 12,000 rpm for 10 minutes at 4°C. The supernatant was collected and protein concentration was estimated using Bradfords assay.

Western blot analysis

Protein extract, preheated at 100°C for 5 min in reducing SDS sample buffer containing 50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, 100 mM β-mercaptoethanol, was run on 12% SDS-polyacrylamide gel. After gel electrophoresis, separated proteins were transferred to PVDF membrane (Millipore-USA) by semi-dry transfer method in accordance with the manufacturer's instructions (Hoefer-USA). For immune-detection, the PVDF membrane was processed using standardized protocol (Ul-Hussain et al., 2008). Immuno-detection was performed using rabbit anti-Cx43 (1:2000) (Abcam). For loading control, mouse anti-beta actin (1:7000) (Sigma) was included for the immune-detection of beta-actin in the same gel. The secondary detection was performed using fluorescent anti-mouse IRDye 680 (1:20,000) and anti-rabbit IRDye 800 (1:10,000) secondary antibodies (LI-COR). The fluorescence was detected using Odyssey infrared detection system (LI-COR).

Quantification of Western blots

Protein bands of the Western blots were quantified using Licor infrared scanner and in accordance with the protocol described by the LICOR odysee system. Briefly, for the protein quantification, the standard was prepared by adding different concentrations of fluorescently labelled Licor secondary antibodies on the PVDF membrane. The fluorescent spots on the membrane were identified using a shape tool and the fluorescence emitted was measured. Similarly, the fluorescent emitted by the western blot bands (Cx43 and actin) were similarly measured and the amount of protein was estimated using the standard. Each protein band was normalized with the control protein band of beta actin.

Immunohistochemical analysis

Immuno-staining of Cx43 was performed by the HRP-Polymer detection method. Anti-rabbit polyclonal Cx43 antibody (Abcam) (1:200) was used as a primary antibody. The sections of 4μm were cut, dried for 4h at 58ºC and further deparraffinized in xylene. Slides were hydrated in a series of graded alcohol to water. Endogenous peroxidase activity was blocked by incubation with Biocare's peroxidazed 1 solution (Biocare Medical, USA) for 5 minutes. Sections were immersed in the sodium citrate buffer (pH 6.0), incubated at 100°C for 10 minutes and were allowed to cool for 30 minutes. The sections were twice washed with ddH_2O for 5 minutes. The sections were blocked by incubating in the Biocare's background sniper (Biocare medical, USA) for 15-30 minutes at room temperature and finally rinsed in PBS. Thereafter, the sections were incubated with Cx43 primary antibody for 1 hour at room temperature. Post antibody incubation, the sections were washed twice with PBS for 10 minutes, followed by incubation with MACH 1 probe (Biocare Medical, USA) for 15 minutes at room temperature. The sections were then incubated with the HRP-polymer (Biocare Medical, USA) for 30 minutes at room temperature, followed by the incubation in DAB mixture (32μl of chromogen per 1.0 ml of DAB substrate buffer) for 5 minutes. The tissue sections were rinsed with PBS and counterstaining was done using Tacha's automated hematoxylin solution for 30-60 seconds. The

slides were rinsed once with deionized water, followed by the incubation in the Tacha's bluing solution for 1 minute. For the negative control, all the above steps were followed, except the incubation with the anti-Cx43 primary antibody. Immunohistochemical analysis of sections for Cx43 was done by pathologists. The expression of Cx43 in the human colon cancer samples was classified as a weak immunoreactivity (-) or strong immunoreactivity (+). The expression levels of the Cx43 were correlated with the various clinico-pathological parameters, such as age, gender, depth of invasion, the nodal status, the presence or absence of distant metastasis and the tumor stage. For statistical comparisons with selected clinicopathological features, the specimens were divided into groups of connexin-positive and connexin-negative.

Statistical analysis

For the statistical analysis of Cx43 protein expression and various clinico-pathological parameters, the χ^2 test and Fisher's exact test were applied wherever applicable. The level of significance was set at $p<0.05$ for all cases.

Results

Cx43 is down-regulated in human colon cancer

In order to explore the expression dynamics of tumor suppressor Cx43 protein in human colon tissue, we investigated its protein expression in the tumor samples and compared them with the adjacent normal tissue. For this purpose, Western Blot of the protein samples obtained from the normal (N) and cancerous colon (T) tissues was performed using anti-Cx43 antibody. As shown in Figure1A, anti-Cx43 antibody detected a protein band(s) around 40-44 kDa, corresponding to Cx43. As indicated in the Figure 1A, the protein expression of Cx43 in the tumor (T) samples was significantly less as compared to the adjacent normal (N) samples. Out of the total number of samples studied, 75% cancer samples demonstrated down regulation of Cx43 as compared to normals. As a protein loading control, immuno-detection of β-actin was performed using anti β-actin antibody. To quantify the Cx43 expression in the colon cancer samples, densitometry analysis was performed. For this purpose, densitometry of Western blots of Cx43 were normalized with that of the β-actin blots of the same gels. As shown in Figure1B, colon cancer showed ~ 10 fold decrease in the expression of Cx43 in the cancer samples as compared to normal tissue. The decrease of Cx43 expression in the human colon cancer samples was found to be statistically significant ($p<0.05$).

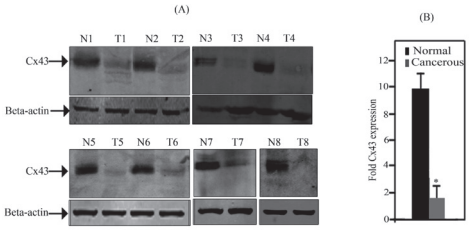

Figure 1. Cx43 Expression in Human Colon cancer: A) Representative Immunoblots of the Cx43 Expression in the Colon Human Samples. Eighty colon cancer samples and their adjacent normal tissue were used in the study. Cx43 expression was found down regulated in 75% of the colon tumor (T) samples as compared to adjacent normal (N) tissue. Expression of the beta actin was used as a loading control. (B) Bar graph represents the densitometeric analysis of Cx43 protein bands in the normal and cancerous colon tissue. Values are expressed as fold expression of Cx43 in carcinoma relative to normal, *statistically significant ($p<0.05$).

Immunohistochemistry analysis confirmed the down regulation of Cx43 in the colon cancer samples

To further ascertain the expression analysis of the Cx43 in the tumor and normal samples, immune-histochemistry analysis was performed. For this purpose, normal and tumor colon tissue slides were prepared as described in material and methods and were examined for the expression of Cx43 using anti-Cx43 antibody. As shown in Figure 2A, strong Cx43 staining was observed between the intestinal cells (red arrows, brown spots) of the normal colon tissue. Moreover, in Figure 2B, weak staining of Cx43 was observed in the cells of cancerous colon tissue. Weak immunoreactivity (-) was observed in 60% cases of colon cancer samples in comparison to their adjacent normals. Therefore, the immune-histochemistry results

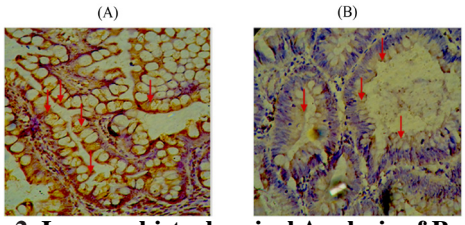

Figure 2. Immunohistochemical Analysis of Paraffin-Embedded Human Colon Carcinoma Using Connexin 43 Antibody. For immuno-histochemistry, the normal and cancerous tissue slides were probed with anti-Cx43 antibody and processed as described in material and methods. A) Normal colon sample showing a strong Cx43 staining (red arrows). B) Cancerous colon sample showing weak Cx43 staining. The samples were counter-stained with hematoxylin. Original magnification, ×200

Table 1. Statistical Correlation of Cx43 Expression with Different Clinico-pathological Features of Colon Cancer Patients*

		Sample	Cx43 -	Cx43 +	p value
Total		80			
Age (±SD) Years		57.8±8.6		55.7±8.60	
Gender	Males	38	31	7	
	Females	42	29	13	0.301
Tumor size (mean±SD), cm		6.14±3.6		5.5±2.2	
Histological type**	Well	47	40	7	
	Mod+poorly	33	20	13	0.018**
T stage**	1 and 2	21	12	9	
	3 and 4	59	48	11	0.04**
N stage	0	38	32	6	
	1 and 2	42	28	14	0.07
M stage	0	74	56	18	
	1	6	4	2	0.637
Clinical stage	I and II	42	33	9	
	III and IV	38	27	11	0.453

*For the statistical analysis, the χ^2 test and Fisher's exact test were applied. The level of significance was set at $p<0.05$ for all cases; **denotes statistically significant values. Cx43 (-) denotes weak expression, while Cx43 (+) denotes strong expression

were consistent with that of Western blotting.

Down regulation of Cx43 is significantly associated with tumor invasion and histological grade of colon cancer

In order to investigate the clinicopathological significance of decreased Cx43 expression, statistical analysis was performed. For this purpose, the correlation between the Cx43 expression and the clinic-pathological features of the patients were compared and analysed. Out of the 80 cases of colon adenocarcinoma, loss of Cx43 expression was observed in 60 cases (75%). Statistical correlation analysis between decreased Cx43 expression with tumor invasion and histological type was found statistically significant (Table 1, $p<0.05$). However, the expression level of Cx43 according to gender, age, lymph node metastasis, metastasis to other organs, and clinical stage did not demonstrate any significant difference (Table 1, $p>0.05$).

Discussion

Cx43, besides forming the gap junction channels, has a well-documented tumor suppressor role (Ableser et al., 2013). In the present study, we explored the expression analysis of Cx43 in the human colon cancer in the Kashmiri population. The role of Cx43 in regulating cell growth has been established from the observation that different types of tumor cells and tumorigenic cell lines show decreased or altered connexin expression and/or localization (Solan et al., 2012). However, most of the previous studies have focused mostly on the expression of Cx43 in various human cancer cell lines (Leithe et al., 2006). Our data present a direct assessment of the expression of Cx43 in human colon cancer samples. Western blot analysis indicated that there exists a strong inverse correlation between the Cx43 protein expression and the colon cancer in this cohort of population.

The importance of functional Cx43 in human colon tissue has been established by mutational studies. The sequence analysis of Cx43 has depicted frame-shift mutations in the carboxy-terminal (CT) domain of Cx43. These frame-shift mutations result in the carboxy-terminal domain truncated Cx43 expression, which is suggestive of the importance of CT-domain of Cx43 in growth regulation. The importance of Cx43 in colon cancer is evident from the studies indicating its frequent mutations and their correlation with the colon sporadic adenocarcinomas (Dubina et al., 2002). In fact, studies have shown that many proteins, involved in growth control interact with Cx43 at its CT domain (Giepmans, 2004). Interestingly, recent report on the role of Cx43 as the colorectal cancer tumor suppressor and the indicator of disease outcome further strengthens our data (Sirnes et al., 2012).

Our data lend more credence to the studies indicating that the Cx43 expression is altered in other cancer tissues, such as in the lung, prostate, breast and ovares (Hu and Liu, 2005; Benko et al., 2011; Plante et al., 2011; Zhao et al., 2013). The statistical analysis of our data demonstrates significant association between the Cx43 down regulation with the tumor invasion and histological type of the colon cancer. The tumor invasion is directly linked with the metastatic potential of cancer cells. Thus, the loss of Cx43 expression may be critical event in determining the metastatic potential of the tumor cells. In fact studies have demonstrated that Cx43 expression prevents mammary tumor metastasis to the lung in a Cx43 mutant mouse model of human diseases (Plante et al., 2011). Our data has a practical significance in terms of the diagnosis of colon cancer is concerned. Tissue investigation for the Cx43 expression will help in understanding the histological type and the invasiveness of the cancer.

The mechanisms by which Cx43 is down regulated in various cancers are complex and not well understood. Studies have shown that various epigenetic factors are responsible for the down regulation of Cx43 during cell transformation (Yi et al., 2007). Our unpublished data indicates that certain oncogenic microRNAs (miRNAs) may be important players in down regulating the tumor suppressive Cx43 protein in colon tissue. Further studies are required to ascertain the molecular mechanisms responsible for the down regulation of Cx43 in colon cancer.

In conclusion, the down regulation of Cx43 expression in the human colon strongly supports the role of this protein in regulating cell growth. The data provide significant insight about the tumor suppressive properties of the Cx43 and its potential as a diagnostic marker for detecting colon cancer. Further studies are required to ascertain the molecular events, which leads to the down regulation of Cx43 in cancers.

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