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Original article

Prognostic importance of Claudin-1 and Claudin-4 expression in colon carcinomas

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ABSTRACT

In this study, we analyzed Claudin-1 and Claudin-4 expressions in colon carcinomas. We investigated the relationship between the expression of these tight junction proteins and clinicopathologic parameters. Claudin-1 and 4 expressions were determined by immunohistochemical methods, and the rate of cells expressing these tight junction proteins were calculated with stereologic methods. Fifty-nine colon cancer cases were enrolled in the study group. Claudin-1 and 4 expressions were found to be significantly lower in cases with lymph node metastasis. Mean staining rates of Claudin-1 and 4 in lymph node (+) cases were 36.1 ± 20.1 and 58 ± 28.9, while in lymph node (−) cases, these were 63.8 ± 25.9 and 72.3 ± 25.6, respectively ($p = 0.0005$ for Claudin-1, $p = 0.049$ for Claudin-4). The mean staining rate for Claudin-1 in adenomatous polyps was significantly higher than incarcinomas (77.13 ± 23.4 and 50.6 ± 26.93, respectively) ($p = 0.003$), while it was quite similar for Claudin-4 (65.4 ± 26.9 and 65.3 ± 27.9, respectively).

In this study, we demonstrated Claudin-1 and 4 expressions in colon cancer cases. Claudin-1 expression seems to be more prominent in adenomatous polyps as compared with cancer cases. Expression of Claudin-1 decreases significantly in the presence of lymph node metastasis and diminishes in mucinous carcinoma cases, indicating a negative correlation between Claudin-1 expression and neoplastic progression.

Introduction

Claudins are members of a protein family containing at least 24 integral membrane tight junction proteins. These protect cell polarity in epithelial and endothelial cells, and have paracellular barrier functions [1]. Loss of cellular organization has an important role in cancer pathophysiology. One of the key components is the tight junction proteins. The tight junction is part of the apical junction complex, and is closely associated with both paracellular permeability and cell polarity [2]. Tight junction proteins are considered to play a critical role in neoplastic processes. They connect the extracellular medium with intracellular signal pathways and cytoskeleton [3]. Neoplastic cells often show structural and functional deficiencies in tight junctions. Cancer progression can be explained partly by loss of tight junctions. Claudin-1 is localized in the tight junctions, and its expression has shown to be decreased in breast and colon carcinomas [4–6]. However, there are other studies which have demonstrated upregulation of Claudin-1 in colorectal, pancreatic, and thyroid carcinomas [7–10]. Upregulation of Claudin-3 and 4 has also been reported in colorectal, ovarian, gastric, breast, prostate, and pancreatic cancers [7,11–15]. Claudin over-expression seems to be an early step in carcinogenesis for at least some cancer types. The reason for this discordance in Claudin expression among different tissue types has not yet been clarified, but this situation might be due to differences regarding tissue or micro environmental properties [16]. There are only few studies that have analyzed the role of tight junction proteins in the development of colorectal neoplasia. In the current study, we analyzed Claudin-1 and Claudin-4 expressions in colon carcinomas using immunohistochemical methods. We tried to demonstrate the association of the expression of these tight junction proteins with clinicopathologic parameters such as tumor grade, invasion depth, lymph node metastasis, tumor localization, and gender.

Materials and methods

Fifty-nine colon cancer cases, registered at Karadeniz Technical University Medical Faculty between 2003 and 2006, were enrolled in the study group. The study was approved by the local ethics committee. Pathological reports and paraffin blocks were obtained from the pathology archive. Histological diagnoses of tumors were performed according to WHO criteria [17]. Rectal cancers were not included in the study. Pathologic staging was performed according to American Joint Committee on Cancer criteria [18]. Every

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patient underwent an operation. None of these patients received neo-adjuvant chemoradiation. All tissue samples were formalin-fixed and paraffin-embedded. The corresponding HE slides were reviewed for confirmation of diagnosis by two pathologists. Adenomatous polyps located close to the tumors were detected in 11 of 59 cases. Each case was classified according to grade (well, moderately, poorly, and undifferentiated), mucinous differentiation, invasion depth, and lymph node metastasis. Areas at the deep invasive front of the tumor and rich in non-necrotic tumoral glands were identified for immunohistochemical study.

Immunohistochemistry

For immunohistochemical study, 5-μm sections were prepared for each antigen (Claudin-1 and Claudin-4) from paraffin blocks of previously selected colon tumors. Following deparaffinization, antigen retrieval was performed in 10 mM citrate buffer using a microwave oven. After a 60-min incubation with primary antibody (dilution 1:50 for anti-Claudin-1 and 4), a biotinylated secondary antibody and Histostain-SP kit were used. DAB kit was used as a chromogen for immunohistochemical study. Slides were counterstained with hematoxylin, dehydrated, cleared, and mounted. Negative control stainings were carried out by substituting non-immune rabbit or mouse serum and PBS for the primary antibodies. Positive controls consisted of normal colonic mucosa. The percentage of immunostaining in tumor cells was assessed by counting positive and negative cells using stereological methods, and expressed as staining rate (%).

Stereology

Stereology is a method that deals with the three dimensional interpretation of planar sections of tissues. It provides practical techniques for extracting quantitative information about three dimensional material from measurements made on two dimensional planar sections of the material [19]. In our study, cell counting was performed using stereologic analysis methods. In this study, the images obtained from stained sections were transformed to the computer screen by a video-camera mounted on a light microscope and analyzed by a stereological workstation (Stereo Investigator 6.0-microbrightfield; USA). The above mentioned workstation had some features such as a personal computer and a computer-controlled motorized specimen stage (BioPrecision MAC 5000 controller system), and a light microscope (Leica DM4000 B). Immunoreactive tumor cells stained with primary antibody Claudin-1 and Claudin-4 were counted separately using a 20× Leica Plan Apo objective (NA = 1.40), which allowed accurate recognition. Immunoreactive tumor cells were counted according to the unbiased counting rules of optical fractionator [19]. The optical fractionator's approach is a combination of performing counting with the optical dissector and with fractionator sampling for the estimation of population size [20]. In our study, a pilot study was done at the beginning of the stereological analysis. It was found that one of 2.250.000 μm^2 (in *X*, 1.50 mm; in *Y*, 1.50 mm) step size for microscopic sampling would be suitable for stereological analysis in our study. In all steps, an unbiased counting frame sizing of 4900.00 μm^2 (70.0 mm × 70.0 mm) was used. According to the optic dissector counting rules, each dissector probe, that means three-dimensional counting box, has a lower height than section thickness. The height of the dissector probe was 70-μm. Thickness sampling fraction was dissector height (16 μm)/mean section thickness. All immunoreactive tumor cells were counted in each dissector probe sampled on the sections during stereological analysis [21]. While the focus plane approaches to section, images taken from the top of the section to the bottom are changed, the first optical section seen in optical section plane (OPS). The upper surface of the section determined if the type of tissue could be clearly distinguished. After reaching the upper surface of the section, the focal plane of lens was adjusted to scan the section thoroughly from the upper to the bottom surface. After determination of the upper surface of section, 5-μm-thickness of a guard zone from the surface of section was left. The immunoreactive tumor cell nuclei were counted after OPS. In this study, we also estimated the coefficient of error value for the optical dissector application. In general, the highest CE rate is 5%, as in our study [22].

Statistical analysis

Statistical analyses were performed using SPSS 12.0.1 for Windows (SPSS Inc.; Chicago, IL). Data are expressed as percentage, mean, and standard deviation (SD) in the case of normally distributed data. Normality of distribution was assessed with the Kolmogorov–Smirnov test. Student's *t*-test was used to determine differences in expression between tissue groups. Pearson correlation was used to determine correlations between Claudin-1 and Claudin-4 expression. For *t*-tests and Pearson's correlation, statistical significance was taken as $p \leq 0.05$.

Results

Clinicopathological features

The mean age of patients on the date when surgery was performed was 60.5 ± 13.8 (minimum 19, maximum 97). Fifty-nine cases were included in the study, 37 of them being male (62.7%) and 22 women (37.3%). In 19 cases (32.2%), tumors were located in the right colon, while 38 (64.4%) tumors were found in the left colon, and 2 (3.4%) tumors at the transverse colon. The reference point was set as splenic flexura. Rectum tumors were not included in the study. Tumor size changed between 2 and 13 cm (5.04 ± 2.04 cm). Forty-seven of 59 cases invaded the subserosa, while another 6 invaded the muscle layer, and 6 the serosa and surrounding organs. Forty cases had well differentiated, 13 cases had moderately differentiated, and 6 cases had poorly differentiated tumors, while none of our study cases had undifferentiated tumors. All of the six cases with poorly differentiated tumors had mucinous tumors. Lymph node metastases were present in 29 cases (49.2%). The clinicopathological features are summarized in Table 1.

Table 1
Clinicopathological characteristics of 59 colonic cancer patients.

Variable	*n*
Age at surgery (years)	
Mean	60.5 ± 13.8
Range	19–97
Gender	
Female	22
Male	37
Tumor localization	
Right colon	19
Left colon	38
Transvers colon	2
Lymph node involvement	
Negative	30
Positive	29
Differentiation	
Poorly differentiated	6
Moderately differentiated	13
Well differentiated	40
Depth of tumor invasion	
Muscularis propria (T2)	6
Subserosa (T3)	47
Serosa and surrounding organ invasion (T4)	6