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THE CORRELATION OF KLF4 EXPRESSION AND CELL ADHESION MOLECULES IN GASTRIC CANCER

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Klf4, transcription factor essential for the regulation of proliferation and differentiation of gastric epithelial cells, and cell adhesion molecules, E-cadherin and β-catenin, have a crucial role in gastric cancer invasion and metastasis. Considering the complex interactions between Klf4 and cell adhesion molecules, the aim of this research was to investigate the immunohistochemical profile and possible association of these proteins with clinico-pathologicalal characteristics of gastric cancer. The tumors with good or moderate histological differentiation were more likely to express retained Klf4 expression ($p < 0.001$). Altered expression of Klf4 was found in 82.6% of the tumors, and significantly correlated with older age and lymph node metastases ($p=0.046$, and $p < 0.001$, respectively). High E-cadherin expression was significantly associated with low histological grade and absence of nodal metastases ($p=0.016$, and $p=0.028$, respectively), while aberrant β-catenin expression was linked to advanced pathological stage, metastatic spread to regional lymph nodes, and younger age ($p=0.027$, $p=0.001$ and $p=0.001$, respectively). In addition, strong correlation was found between Klf4 and E-cadherin expression ($p=0.001$). The translation of the results acquired in molecular studies into the pathological practice is essential for establishing the potential diagnostic, prognostic, and therapeutic application of biomarkers. This study identified significant correlation between Klf4, and immuno-histochemical expression of cell adhesion molecules in gastric cancer tissue. Immuno-histochemical detection of altered expression of Klf4, E-cadherin, and β-catenin may suggest unfavorable prognosis of the disease and contribute to the selection of patients who require closer follow-up after surgery. *Acta Medica Medianae 2017;56(3):143-150.*

Key words: *gastric cancer, Klf4, E-cadherin, β-catenin, immunohistochemistry*

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Introduction

Gastric cancer is one of the most destructive malignant neoplasms, whose molecular pathways of pathogenesis and progression are under intense investigation for the purposes of prevention and more effective therapeutic strategies. Despite the global trend of significant decline in morbidity and mortality, gastric cancer remains one of the leading causes of death among malignant diseases (1, 2). The aggressiveness of gastric cancer is one of its main features, but the basic mechanisms of this aggressive behavior are not fully understood. Numerous studies in the past few decades have strongly associated epithelial - mesenchymal transition (EMT) regulated by a set of transcription factors and signaling pathways of cancer cells proliferation, by invasion and metastasis (3). EMT is the result of a complex interplay of many signaling pathways. Recently published studies have highlighted the key role of Kruppel-like factor 4 (Klf4), zinc finger-like transcription factor, in the negative regulation of EMT. By transcriptional regulation of its target genes, Klf4 plays an important role in carcinogenesis, cell proliferation and differentiation (4). It was found that its expression is reduced or absent in the majority of gastric carcinomas (5, 6).

Klf4 suppresses the expression of mesenchymal phenotype associated genes during EMT, which equips the cancer cell for metastatic spread (4, 6). Therefore, a lack of Klf4 results in the loss of EMT suppression, leading to cancer progression (7). A recent study has suggested an inverse correlation of Klf4 and β-catenin expression in gastric cancer (8). In a study of moderately differentiated human gastric cancers, the altered expression of both markers was significantly associated with advanced tumor stage. β-catenin is an oncoprotein encoded by CTNNB1 gene and is

reported to play an important role in gastric carcinogenesis (9). β-catenin is involved in cadherin-mediated cell adhesion to the plasma membrane. E-cadherin/β-catenin cell adhesion complex has a crucial role in the maintenance of cell integrity and orientation, and disruption of its function contributes to increased cell motility and gain of mesenchymal properties. A loss of E-cadherin expression releases intracellular β-catenin, causing its translocation into the cell nucleus, where β-catenin acts as a transcriptional regulator of downstream target genes involved in cell prolife-ration, differentiation, migration, and angiogenesis, including CyclinD1, c-Myc, CD44, and vascular endothelial growth factor (4).

Considering the complex interplay of Klf4 and cell adhesion molecules, we aimed to investigate the immunohistochemical profile and possible association of these proteins' expression in a spectrum of gastric cancer specimens of various histological grades and pathological stages. The purpose of this research was to investigate the correlation of Klf4 expression and cell-adhesion molecules in gastric carcinoma, and to examine the association of Klf4, E-cadherin, and β-catenin expression to clinicopathological characteristics of gastric cancer.

Material and methods

Tissue samples

The study comprised archival specimens of tumor tissue obtained from 69 patients who underwent total or partial resection of the stomach at the Department of General Surgery, Clinical Center Niš. Following a fixation in 10% buffered formaldehyde, tumor specimens were processed using the conventional histopathological methods in the Center for Pathology, Clinical Center Nis, and then embedded in paraffin. Four μm thick histological sections from the paraffin blocks were deparaffinized and rehydrated and then stained with Hematoxylin Eosin routine method (HE), and used to determine the histological type of the tumor, degree of differentiation (histological grade) and pathological stage. Pathohistological analysis was performed by two independent pathologists using a light microscope (Olympus BX43, Olympus Corporation, Tokyo, Japan). The examined tumors were divided into two groups, with respect to the presence of glandular formation in the tumor tissue, in accordance with the Lauren classification (10, 11).

Immunohistochemical analysis

The selected representative samples of the tumor tissue were first dewaxed through a series of xylene (4 times; 5 minutes each), and rehydrated in a series of alcohol washes (3 times; 5 minutes each). Pretreatment with antigen retrieval in order to increase the binding affinity of primary anti-bodies for immunohistochemical staining was carried out in a citrate buffer (pH 6), by heating for 20 minutes in a microwave at 800W. After washing in phosphate buffered saline (PBS, pH 7.4), the slides were exposed to endogenous peroxidase blocking for 20 minutes in 3% hydrogen peroxide solution (H_2O_2) in methanol. This was followed by an abundant rinsing in PBS, and later incubation in a water bath with the primary antibody at room temperature for 1 hour. In this study, for immunohistochemical analysis we used the following mouse monoclonal primary antibodies: Anti-E-Cadherin (clone 36, BD Transduction Laboratories, BD Biosciences, San Jose, California, SAD, at dilution 1:100), Anti-β-Catenin (clone 14, BD Transduction Laboratories, BD Biosciences, San Jose, California, SAD, at dilution 1:250), and for detection of Klf4 polyclonal antibody to human KLF4 (clone H180, Santa Cruz Biotechnology, Santa Cruz, CA, USA, at dilution 1:200). The labeled antigens were, after thorough rinsing in PBS, detected using DAKO EnVision kit (EnVision® + Dual Link System-HRP (DAB +), DakoCytomation) which was used as a universal immunoperoxidase polymer, with which the samples were incubated at room temperature for 1 hour. Visualization of the reaction was performed with diamino-benzidine-tetrahydrochloride (DAB), which marked the site of an antigen-antibody reaction with brown colored precipitation. The sections were then rinsed with water and counterstained with Mayer's hematoxylin. Negative controls were carried out by omitting the primary antibody.

Scoring of immunohistochemical staining

Positive immunohistochemical reaction, i.e. the presence of the investigated proteins in the tissue sections, was verified in the form of brown staining of cell membranes, cytoplasm and cell nuclei. The expression of E-cadherin and β-catenin was evaluated in relation to the distribution of positive reaction, i.e. in relation to the proportion of positive tumor cells. Tumors were divided into: 1) tumors with diffuse expression of the marker, i.e. uniformly positive, if more than 90% of tumor cells were stained, 2) heterogeneously positive - between 10 and 90% cancer cells stained, and 3) negative tumors, where the immunoprecipitation was present in 0 to 10% of the cells. Only tumors with uniform staining were regarded as high expression, while the tumors in Groups 2 and 3 were considered tumors with low/decreased expression.

Klf4 expression was observed as nuclear and cytoplasmic staining. In relation to the percentage of stained cells, all the analyzed tumors were divided in two groups: tumors with ≥50% stained cells, with moderate to strong expression Klf4, which was considered preserved Klf4 immunoreactivity (high expression group), and tumors with reduced or absent expression of Klf4, which was considered a pathological, aberrant finding (low expression group).

Statistical analysis

All data were analyzed using statistical software for data processing SPSS version 20.0. Continuous variables were presented as the mean ± standard deviation. The frequencies of categorical variables were tested by using χ^2 test with Yates's correction. Univariate and multi-variate analysis of clinicopathological variables was performed using a Cox regression analysis. P ≤0.05 values were considered statistically significant.

Results

The average patient age at the time of diagnosis was 62.6±8.1 years, with the youngest patient 46 old, and the oldest 79 years old. For the purposes of statistical analysis, all patients were dichotomized in two age groups: those over 60 years (N=45, 65.2%), and those below 60 years of age (N=24, 34.8%). There were 48 male (69.6 %) and 21 female patients (30.4%). The tumors exhibiting predominantly tubular or papillary architectural pattern were classified as intestinal type tumors (42, 60.9%), while poorly cohesive tumors were designated as diffuse type (27, 39.1%). Thirty tumors (43.5%) were well or moderately differentiated, while the remaining were classified as poorly differented, high grade cancers. The majority of analyzed tumors were in advanced pathological stage (Table 1).

Table 1. Overview of pathologic characteristics of the analyzed tumors

Criterion	Distribution	Number of patients N (%)
Lauren's classification	Intestinal type	42 (60.9)
	Diffuse type	27 (39.1)
Tumor differentiation (Tumor grade)	well/moderate	30 (43.5)
	poor	39 (56.5)
Pathologic tumor stage (TNM)	Stage I	3 (4.3)
	Stage II	12 (17.4)
	Stage III	18 (26.1)
	Stage IV	36 (52.2)

High Klf4 immunohistochemical staining of paraffin-embedded gastric cancer tissue sections was observed in 12 cases (17.4%), while low expression was prevalent, comprising 57 (82.6%) tumors (Table2). Staining for Klf4 was nuclear, strong or intermediate, and cytoplasmic, with intermediate intensity (Figure 1, A, B). In the adjacent-non-neoplastic tissue, preserved glandular epithelium showed strong cytoplasmic and nuclear staining of Klf4. High Klf4 expression was more frequently found in older patients (>60), and in patients without metastasis to regional lymph nodes (p=0.046, and p <0.001, respectively). Moreover, tumors with good or moderate histological differentiation were more likely to express retained Klf4 expression (p <0.001). Normal expression of Klf4 was observed in only one tumor with poor differentiation. However, there was no statistically significant difference between Klf4 expression in intestinal type and diffuse type gastric cancer, although 80% of the tumors with high Klf4 expression were with intestinal architecture.

Table 2. Correlation of clinicopathologicalal features of gastric cancer and immunohistochemical expression of Klf4

Characteristic		Klf4 expression		
Value	Total (N=69)	Low (N=57)	High (N=12)	*p*
Patient gender	Male	39	9	*0.744*
	Female	18	3	
Age at diagnosis	≤60	23	1	*0.046**
	>60	34	11	
Lauren's classification	Intestinal type	32	10	*0.108*
	Diffuse type	25	2	
Differentiation (Histologic grade)	Well/Moderate	19	11	*0.000**
	Poor	38	1	
Pathologic stage (TNM)	I / II	11	4	*0.278*
	III / IV	46	8	
Localization	Upper portion	29	4	*0.348*
	Lower portion	28	8	
Lymphangioinvasion	Absent	32	10	*0.108*
	Present	25	2	
Nodal metastases	Absent	7	11	*0.000**
	Present	50	1	

χ^2 test with Yates' correction was performed; value of $p \leq 0.05$ was considered statistically significant(*)

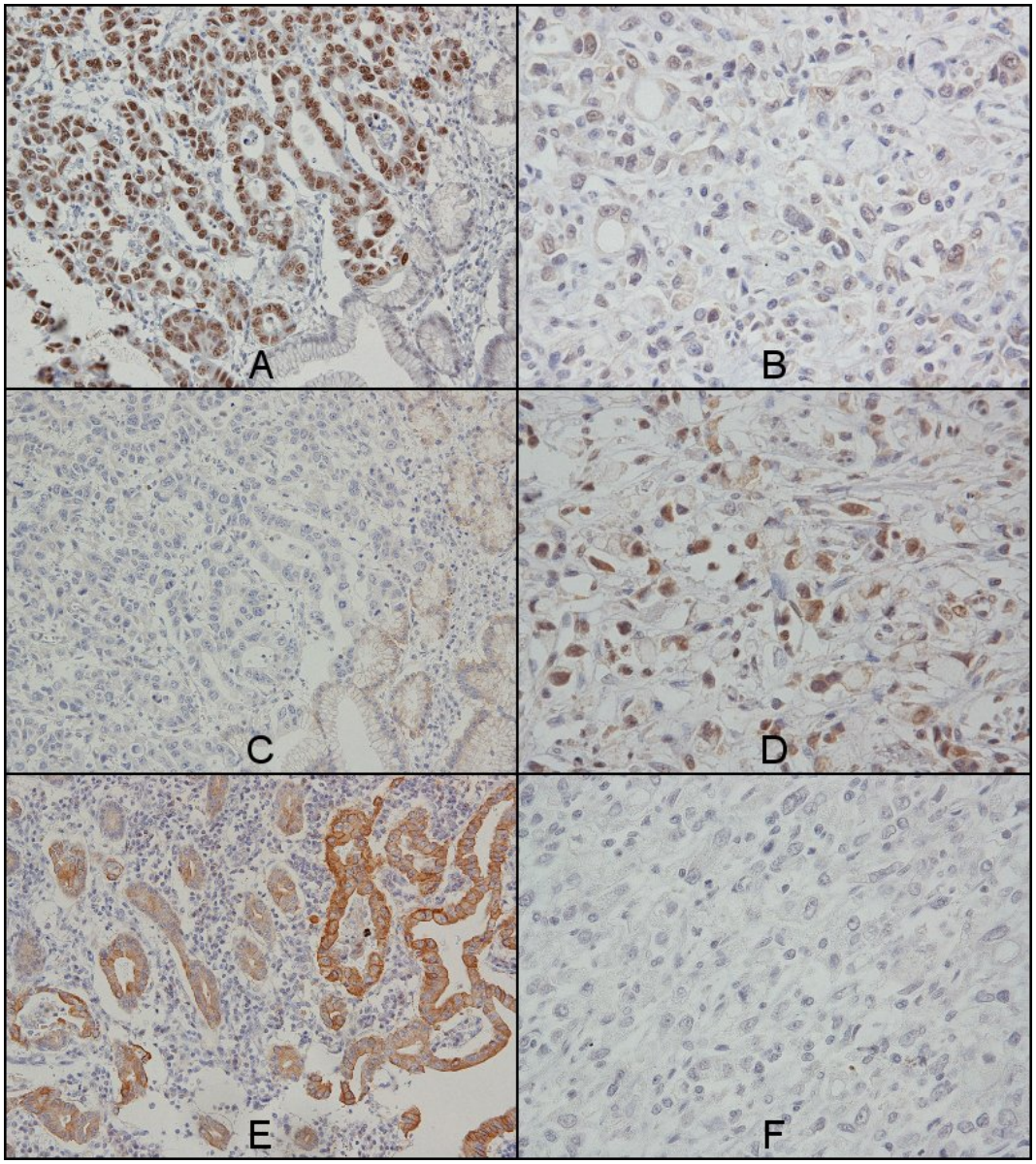

Figure 1. Immunohistochemical expression of Klf4 and cell adhesion proteins in intestinal (A, C, E), and diffuse type gastric cancer (B, D, F): Diffuse nuclear and cytoplasmic expression of Klf4 in intestinal type (A), and reduced expression in diffuse type of gastric cancer (B); β-catenin expression absent in intestinal type (C), and displaying nuclear staining in poorly cohesive gastric cancer (D); Reduced, heterogeneous expression of E-cadherin in intestinal (E), and complete loss of expression in diffuse carcinoma (F). Original magnification x400.

Regarding the expression of investigated cell adhesion proteins, high E-cadherin expression was found in 39 (56.5%) of the tumors, while β-catenin was positive in 21 (30.4%) of analyzed gastric cancer tissue samples. E-cadherin stained cell membranes, while β-catenin expression was perimembranous, cytoplasmic, and also nuclear, which was a more frequent finding in diffuse type gastric cancer than in tumors with glandular histological pattern (Figure 1). The correlation of E-cadherin and β-catenin expression and clinicopathological parameters is shown in Table 3. There was no significant difference in the distribution of their positivity according to Lauren's histological classification. However, E-cadherin was significantly associated to low tumor grade and absence of nodal metastases (p=0.016, and p=0.028, respectively). In contrast, high β-catenin expression was associated with advanced pathological stage and metastatic spread of the cancer to regional lymph nodes (p=0.027, and p=0.001, respectively). In addition, the expression of β-catenin was strongly linked to younger patient age (p=0.001).

The analysis of the correlation between Klf4, and the expression of E-cadherin and β-catenin (Table 4) showed that retained Klf4 expression is more frequently associated with low β-catenin staining, and normal E-cadherin expression. However, only the correlation between Klf4 and E-cadherin was statistically significant (p=0.001).

Table 3. Correlation of clinicopathologicalal features of gastric cancer and immunohistochemical expression of E-cadherin and β-catenin

Characteristic		E-Cadherin expression			β-Catenin expression		
Value	Total (N=69)	Low (N=30)	High (N=39)	*p*	Low (N=48)	High (N=21)	*p*
Patient gender	Male	19	29	*0.430*	32	16	*0.572*
	Female	11	10		16	5	
Age at diagnosis	≤60	14	10	*0.080*	23	1	***0.001****
	>60	16	29		26	19	
Lauren's classification	Intestinal type	17	25	*0.621*	30	12	*0.790*
	Diffuse type	13	14		18	9	
Differentiation (Histologic grade)	Well/Moderate	8	22	***0.016****	20	10	*0.793*
	Poor	22	17		28	11	
Pathologic stage (TNM)	I / II	8	7	*0.397*	14	1	***0.027****
	III / IV	22	32		34	20	
Localization	Upper portion	11	22	*0.145*	24	9	*0.612*
	Lower portion	19	17		24	12	
Lymphangioinvasion	Absent	19	23	*0.806*	28	14	*0.598*
	Present	11	16		20	7	
Nodal metastases	Absent	12	6	***0.028****	18	0	***0.001****
	Present	18	33		30	21	

χ^2 test with Yates' correction was performed; value of $p \leq 0.05$ was considered statistically significant(*)

In multivariate Cox regression analysis that tested the significance of clinicopathological parameters on the expression of Klf4 and cell adhesion molecules E-cadherin and β-catenin, several associations were statistically confirmed as relevant ($p < 0.05$). The reduction and loss of Klf4 expression was independetnly predicted by the presence of lymph node metastases, and older patient age. Patient age and regional tumor spread to lymph nodes were also significant factors for β-catenin expres-sion, while regression analysis in a relevant model established only low tumor grade as a statistically significant predictor in E-cadherin membranous staining.

Table 4. Correlation between Klf4 expression and immunostaining of cell adhesion proteins, E-cadherin and β-catenin, in gastric cancer

		Klf4 expression		*p-value*
		Low (%)	High (%)	
β-Catenin	Low	39 (56.5)	9 (13.0)	0.744
	High	18 (26.1)	3 (4.4)	
E-Cadherin	Reduced	29 (42.0)	1 (1.5)	**0.001***
	Normal	28(40.6)	11(15.9)	
	Total	57(82.6)	12(17.4)	

χ^2 test with Yates' correction was performed; value of $p \leq 0.05$ was considered statistically significant(*)

Discussion

Klf4 is a transcription factor that has been found to act as an important regulator of proliferation and differentiation of epithelial cells in the gastrointestinal system (12, 13). Altered expression of Klf4 inevitably contributes to the process of epithelial to mesenchymal transition, which represents a crucial step during carcinogenesis, and is characterized by the loss of epithelial cell markers, including cell adhesion molecules, and gain of mesenchymal cell markers in invasive tumors (14). Reduced Klf4 expression is associated with unfavourable biological behavior and shorter overall survival. Research data suggested that restoration of normal Klf4 expression inhibits the growth of gastric cancer tumor cells in vitro, and supresses their tumorigenicity in animal models (15). In the present study, the majority of the investigated samples of gastric cancer showed aberrant, decreased or complete loss of Klf4 immunohis-tochemical expression. This is in accordance with the previously published results (5, 7, 8).

The results did not show significant correlation of Klf4 expression, neither the expression of E-cadherin and β-catenin with histological pattern of gastric cancer. Intestinal type tumors are associated with environmental factors, including H.pylori infection, nutritional factors, smoking and obesity, while diffuse type carcinoma is more frequently seen in younger patients, with a recognizable familial heritable component (10). Diffuse growth pattern of gastric cancer has been linked to decreased or aberrant expression of E-cadherin, which was recognized as a marker of agressive cancer behavior, with progression of the disease and dissemination to distant sites (16, 17). Dysregulation of E-cadherin induces dysfuncion of cellular pathways that influence cell polarity, invasiveness and cell migration in gastric carcinogenesis (18, 19). In this study, low E-cad-

herin staining did not correlate with diffuse type of gastric cancer, but it was associated with poor differentiation of the tumors.

Based on the presence of CDH1 gene mutation, which encodes for E-cadherin, even the specific entity of diffuse gastric cancer has been described, where inheritable mutation is associated with the occurence of signet ring cell type gastric cancer in younger patients (17, 18). However, in our knowledge all the tumors included in the present study were sporadic, and there was no hereditary burden in any case. Although aberrant expression of E-cadherin was more frequently observed in diffuse type than intestinal type gastric cancer, this association did not gain significance in statistical analysis. Interestingly, reduced E-cadherin expression was indeed more frequently observed in female patients, at younger patient age, and higher histological grade, which are all properties significantly associated with familial gastric cancer. The results suggest that genetic and epigenetic changes that alter E-cadherin expression are important events in sporadic gastric cancer, but may not represent the key dominant mechanism responsible for biological agressiveness of gastric cancer.

Nevertheless, the loss of E-cadherin interaction in epithelial cell leads to deterioration of cell-cell contacts, which is an essential step in EMT (4, 9). This loss leads to dissociation of E-cadherin/β-catenin complex, and underlies the transcriptional activation of β-catenin. Activating mutations of the Wnt/β-catenin pathway are well recognized genetic alterations involved in the development of premalignant and malignant lesions of gastrointestinal tract (20, 21). The alterations in this signaling pathway initiate the process of cell transformation. The data from the previous studies suggested that β-catenin expression was upre-gulated in gastric cancer tissues compared to adjacent normal gastric mucosa (8, 19). The altered β-catenin expression was linked to advanced tumor stage. Our results indicated significant association between high pathologic tumor stage, however this accociation was not confirmed in regression analysis model. Overexpression of β-catenin strongly correlated with the occurence of lymph node metastases, indicating the influence of β-catenin transactivation of complex downstream molecular pathways included in EMT, crucial for metastatic progression.

The interaction of Klf4 and cell adhesion molecules has been implicated in gastric carcinogeneses, and the alteration of their immunohistochemical expression was associated with clinicopathological parameters that indicate poor prognosis, including advanced tumor stage and metastatic spread. It has been suggested that Klf4 acts as a antagonist of β-catenin on nuclear level, thus its loss drives the gastric cancer cell to acquire mesenchymal phenotype (4, 7). Although the inverse correlation of Klf4 and β-catenin have been reported previously (8), statistically significant correlation between these markers could not have been established in this research. This may be due to limitation caused by number of gastric cancer samples that were used, and, their significant heterogeneity, since the study included well, moderate, and poorly differtiated tumors. However, the tendency of tumors with retained E-cadherin to express Klf4 was noted. In addition, even the tumors with high grade characteristics which expressed E-cadherin were also positive for Klf4 staining. This contributes to the notion of tight relationship between Klf4 and regulation of cell adhesion moleculs, which expression is essential for preservation of gastric tissue architecture.

Although the molecular crosstalk and interactions of Klf4 and cell adhesion molecules are under intense investigation, translation of the acquired results in pathological practice is essential for establishing these biomarkers as potential diagnostic, prognostic, and therapeutic targets. In conclusion, this study identified significant correlation between Klf4 immunohistochemical expression and cell adhesion molecules in gastric cancer. Immunohistochemical detection of altered expression of the investigated proteins may suggest an unfavourable prognosis of the disease and contribute to more precise stratification of the patients that require closer attention after surgery.

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KORELACIJA EKSPRESIJE KLF4 I ĆELIJSKIH ADHEZIONIH MOLEKULA U KARCINOMU ŽELUCA

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Klf4, transkripcioni faktor neophodan za regulaciju proliferacije i diferencijacije ćelija želudačnog epitela i ćelijski adhezioni molekuli, E-kaderin i β-katenin, imaju ključne uloge u invaziji i metastaziranju karcinoma želuca. Uzimajući u obzir složenost interakcija između Klf4 i ćelijskih adhezionih molekula, cilj ovog rada bio je da ispita profil imunohistohemijske ekspresije i moguću povezanost ovih proteina sa kliničko-patološkim karakteristikama karcinoma želuca. Tumori sa dobrom i umereno dobrom histološkom diferencijacijom su učestalije pokazivali očuvanu ekspresiju Klf4 ($p < 0,001$). Izmenjena ekspresija Klf4 nađena je kod 82,6% tumora i značajno je korelirala sa starijim životnim dobom bolesnika i metastazama u limfnim nodusima ($p=0,046$ i $p < 0,001$). Jaka ekspresija E-kaderina bila je značajno povezana sa niskim histološkim gradusom i odsustvom nodalnih metastaza ($p=0,016$ i $p=0,028$), dok je izmenjena ekspresija β-katenina bila udružena sa uznapredovalim patološkim stadijumom, metastatskim širenjem u regionalne limfne noduse i mlađim životnim dobom bolesnika ($p=0,027$, $p=0,001$ i $p=0,001$). Translacija rezultata dobijenih u molekularnim istraživanjima u patološku praksu je ključna u cilju ostvarivanja potencijalne primene biomarkera u dijagnostičke, prognostičke i terapijske svrhe. U ovoj studiji utvrđena je značajna korelacija između imunohistohemijske ekspresije Klf4 i ćelijskih adhezionih molekula, E-kaderina i β-katenina, u tkivu karcinoma želuca. Imunohistohemijska detekcija izmenjene ekspresije Klf4, E-kaderina i β-katenina mogla bi ukazati na nepovoljnu prognozu bolesti i doprineti selekciji bolesnika koji zahtevaju pažljivije praćenje nakon operacije. *Acta Medica Medianae 2017;56(3):143-150.*

Ključne reči: *karcinom želuca, Klf4, E-kaderin, β-katenin, imunohistohemija*