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EPITHELIAL-MESENCHYMAL TRANSITION AND STEM CELLS IN COLORECTAL CANCER PROGRESSION

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Abstract

Introduction. Colorectal cancer (CRC) is the third leading cause of cancer-related death worldwide. Studying invasion and metastasizing mechanisms in colorectal carcinogenesis is gaining momentum. The **purpose** of the study was to analyze immunohistochemical (IHC) expression levels of epithelial and mesenchymal phenotypic markers, as well as cancer stem cells markers on I-IV (pTNM) stages of CRC. **Materials and methods.** Histopathological and IHC studies of 53 CRC were conducted. IHC study was carried out using antibodies against E-cadherin, CK-20, α -SMA, vimentin, CD44, and ALDH1. **Results.** CRC is characterized by low E-cadherin expression level that decreases during the I-IV stages sequence and by medium CK-20 expression level that decreases during the studied sequence. Furthermore, CRC is characterized by medium α -SMA and vimentin expression levels that increase during the studied sequence. These findings indicate parallel epithelial phenotype losing and mesenchymal phenotype acquiring by the cancer cells that

happen during colorectal adenocarcinoma progression from the I to the IV stages. CD44 expression by the tumor stromal cells was described in this study. The median of CD44+ cells area equal to 61,26 (42,58; 79,15) % of CRC stromal cells. This index significantly increases from the I to the III stages of the tumor. ALDH1 expression by both cancer cells and stromal cells was described as well. The median of ALDH1+ stromal cells area equal to 40,22 (22,54; 47,77) %, the median of ALDH1+ cancer cells area equal to 42,15 (32,06; 50,42) %. The index of ALDH1+ stromal cells area significantly increases from the I to the III stages of the tumor whereas the index of ALDH1+ cancer cells area significantly increases from the III to the IV stages of the tumor. Moreover, correlations between the studied markers expression were revealed that proves the connection between epithelial-mesenchymal transition and stemness acquisition in CRC progression.

Key words: Colorectal Cancer; Epithelial-Mesenchymal Transition; CD44 Antigen; Aldehyde Dehydrogenase 1.

Introduction. Colorectal cancer (CRC) is the third leading cause of cancer-related death worldwide. It is the fourth most common type of cancer in both men and women as well. Unfavorable prognosis in CRC conditioned by late diagnosis – almost 80% of all diagnosed cases are revealed on metastatic stage [1].

Considering the relevance of the problem, studying invasion and metastasizing mechanisms in colorectal carcinogenesis is gaining momentum. Of particular interest to the issue of CRC progression mechanisms, is the theory of cancer stem cells. Cancer stem cells are subpopulations of tumor cells that localize in the stroma of cancer (mesenchymal stem cells) and among malignant epitheliocytes (namely cancer stem cells). These cells have properties of adult stem cells, including self-renewal, symmetric and asymmetric cell division, differentiation potential. Moreover, cancer stem cells are able to survive, maintain self-renewal and differentiation beyond a primary tumor [2, 3].

The theory of cancer stem cells closely related to epithelial-mesenchymal transition (EMT) that forms invasion and metastasizing basis in different types of cancer. EMT is a process by which epitheliocytes lose their normal properties like polarity, cell-cell adhesions, and gain mesenchymal cells properties, like mobility, ability to synthesize and destruct extracellular matrix components, etc [4]. According to current literature data, losing epithelial phenotypic properties that happens during EMT associated with acquiring of stemness by transformed cells [5].

The **purpose** of our study was to analyze immunohistochemical expression levels of epithelial and mesenchymal phenotypic markers, as well as cancer stem cells markers on I-IV (pTNM) stages of colorectal cancer.

Materials and methods. Samples of 120 colorectal adenocarcinomas that were removed during surgical treatment at Surgical Unit of Zaporizhzhia Regional Clinical Oncology Center were analyzed retrospectively from January 2018 to January 2020. Tissue samples of 53 colorectal adenocarcinomas were selected for the current histopathological and immunohistochemical (IHC) study.

The features of histological structure of the studied samples were examined in sections stained by hematoxylin and eosin, as well as PAS-staining. Based on the pTNM classification for CRC studied samples were divided into the following groups: I stage (10 cases), II stage (15 cases), III stage (16 cases), IV stage (12 cases).

IHC study was conducted according to protocols provided by used antibodies manufacturers. Antibodies against E-cadherin (Clone NCH-38, DAKO, USA), keratin 20 (Clone Ab-1, ThermoScientific, USA), vimentin (Clone Ab-2, ThermoScientific, USA), and smooth muscle actin (Actin, Smooth Muscle, Clone Ab-1, ThermoScientific, USA) were used for cells phenotype studying. The expression levels were estimated by the method of photo digital morphometry: using Color Deconvolution plugin intensity of immunostaining was calculated and expressed in conventional units of the optical density (CUOD). The obtained results were graduated on the four levels: lack of expression – 0-20 CUOD, low expression level – 21-50 CUOD, moderate expression level – 51-100 CUOD, and high expression level – more than 100 CUOD.

Antibodies against CD44 (CD44 Std. / HCAM Ab-4, Thermo Scientific, USA) and ALDH1 (ALDH1A1, Clone 5A11, Thermo Scientific, USA) were used for cancer stem cells studying. The areas of CD44+ and ALDH1+ cells were estimated by the method of photo digital morphometry: calculation of immunopositive pixels number in a digital image with further comparing to total pixels number in the image was carried out. Number of immunopositive pixels was expressed in % and indicated relative area of immunostained cells.

Statistical processing of the results was performed on a personal computer using program “Statistica® for Windows 13.0” (StatSoft Inc., License № JPZ804I382130ARCN10-J). The median (Me), the lower and the upper quartiles (Q1; Q3) were calculated. Comparison was performed using the Mann-Whitney U-test. The correlation analysis was performed using

Spearman's rank correlation coefficient (r). The results were considered as statistically significant when $p < 0,05$.

Results. According to the obtained results, colorectal adenocarcinoma (CRA) is characterized by low E-cadherin expression level with the median equal to 45,94 (32,04; 58,26) CUOD. E-cadherin expression is revealed in cancer cells that form gland-like structures and clusters, that was observed in 100% studied samples. Additionally, CRA is characterized by medium CK-20 expression level with the median equal to 54,28 (41,55; 70,27) CUOD. CK-20 expression is revealed in cancer cells that form gland-like structures and clusters in 76,6% of studied cases, and in isolated cells located in tumor stroma in 40% of studied cases. 23,4% of studied cases were CK-20-negative cases. Comparative analysis revealed statistically significant increase of the epithelial phenotype markers expression in the studied sequence (fig. 1, fig. 2).

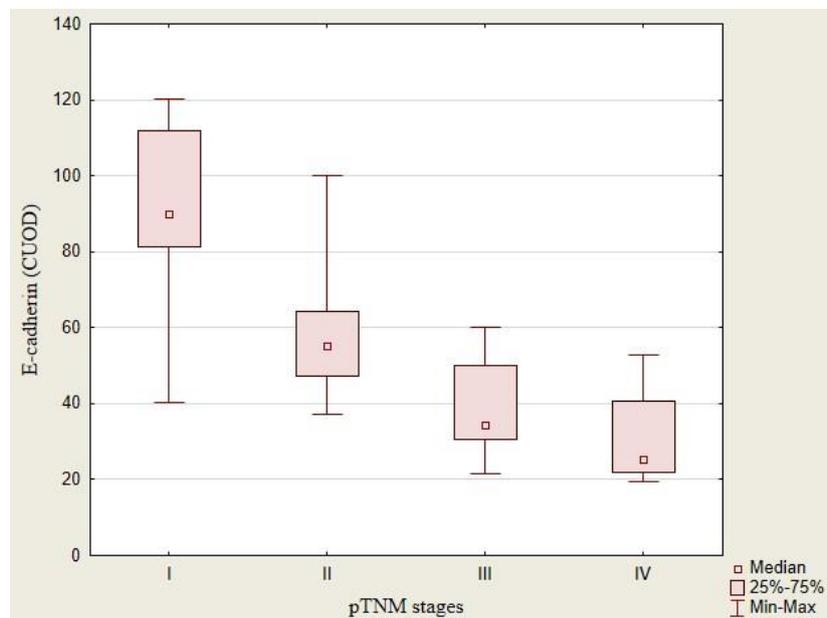


Figure 1. The medians of E-cadherin expression in colorectal adenocarcinoma on I, II, III, and IV pTNM stages

It was established that CRA is characterized by medium α -SMA expression level with the median equal to 75,71 (60,22; 90,34) CUOD and by medium vimentin expression level with the median equal to 95,23 (80,22; 110,21) CUOD. Expression of both markers is revealed in spindle-shaped cells of tumor stroma with the prominent tendency to accumulation of these cells around cancer cells clusters in 100% studied cases. Comparative analysis revealed statistically significant increase of the mesenchymal phenotype markers expression in the studied sequence (fig. 3, fig. 4).

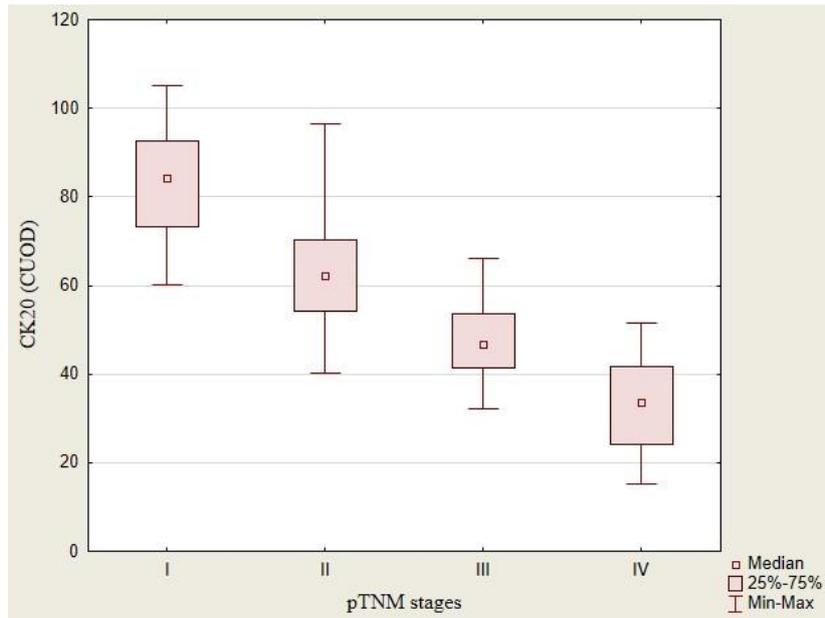


Figure 2. The medians of CK-20 expression in colorectal adenocarcinoma on I, II, III, and IV pTNM stages

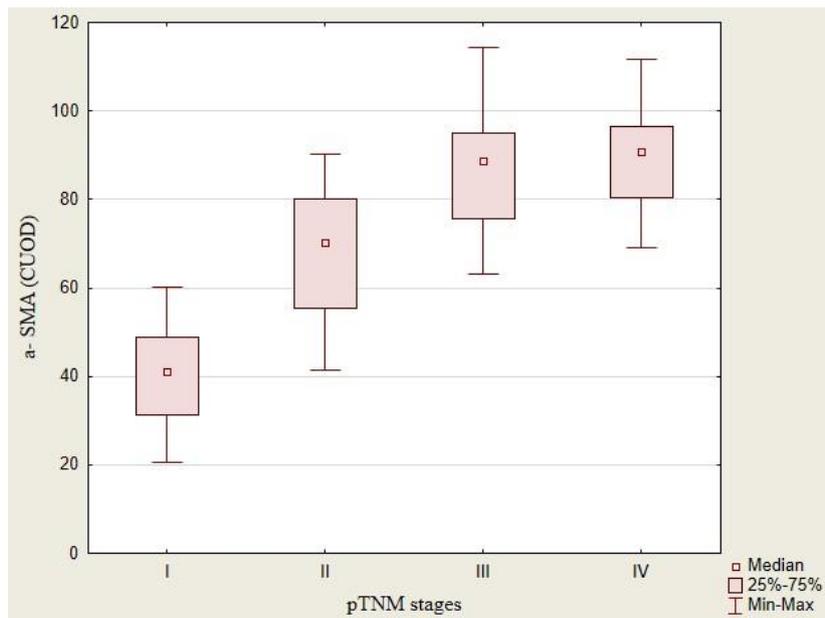


Figure 3. The medians of α -SMA expression in colorectal adenocarcinoma on I, II, III, and IV pTNM stages

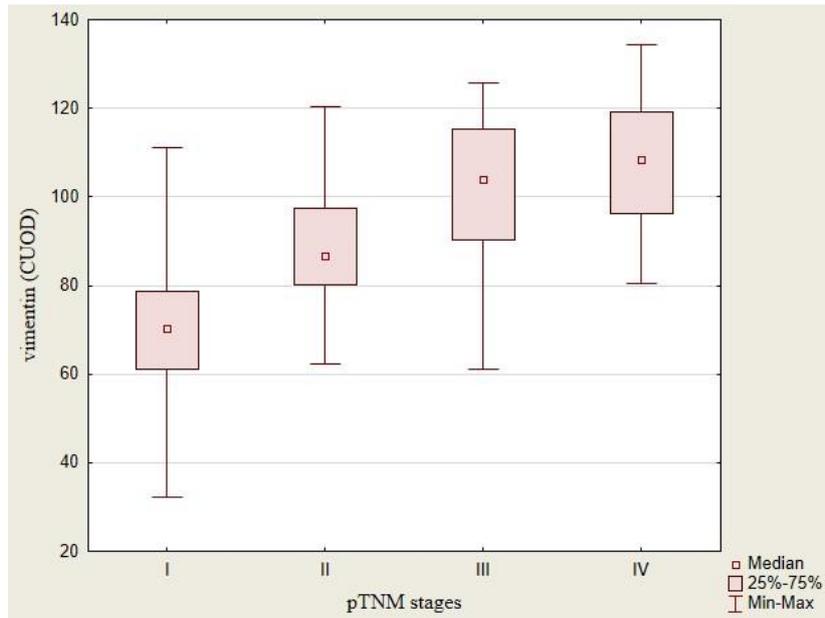


Figure 4. The medians of vimentin expression in colorectal adenocarcinoma on I, II, III, and IV pTNM stages

Moreover, it was established that CRA is characterized by the median of CD44+ cells area equal to 61,26 (42,58; 79,15) %. CD44+ cells are revealed in 100% studied cases, these cells located exclusively in tumor stroma. Comparative analysis revealed statistically significant increase of CD44+ cells area during the CRA progression from the I to the III stages (fig. 5).

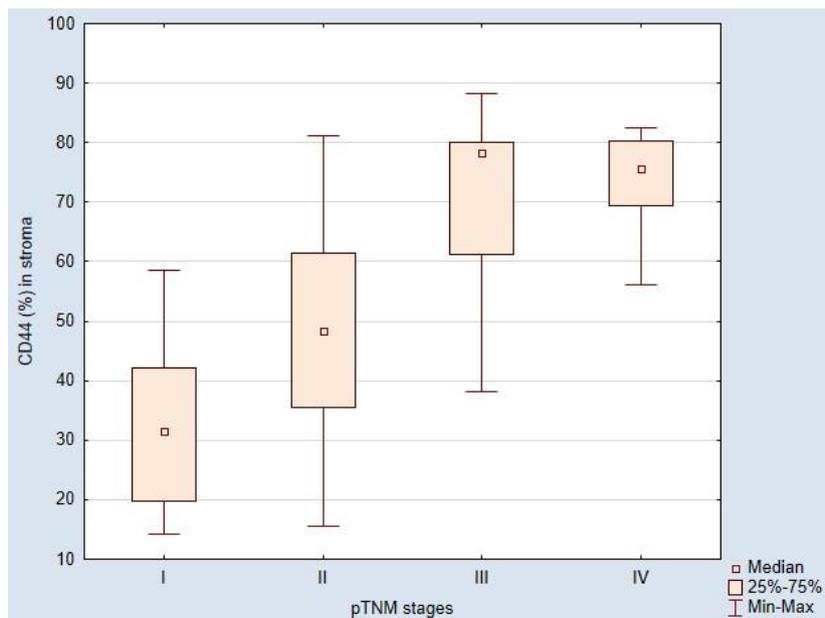


Figure 5. The medians of CD44+ cells area in colorectal adenocarcinoma on I, II, III, and IV pTNM stages

Regarding ALDH1 expression it was established that the expression characterizes both stromal cells and cancer cells. ALDH1+ stromal cells are revealed in 100% studied cases with the median of the area equal to 40,22 (22,54; 47,77) %. Comparative analysis determined statistically significant increase of ALDH1+ stromal cells area during the CRA progression from the I to the III stages (fig. 6). ALDH1+ cancer cells are revealed in 50% of studied cases of CRA II-IV stages with the median of the area equal to 42,15 (32,06; 50,42) %. Comparative analysis determined statistically significant increase of ALDH1+ cancer cells area during the CRA progression from the III to the IV stages (fig. 7).

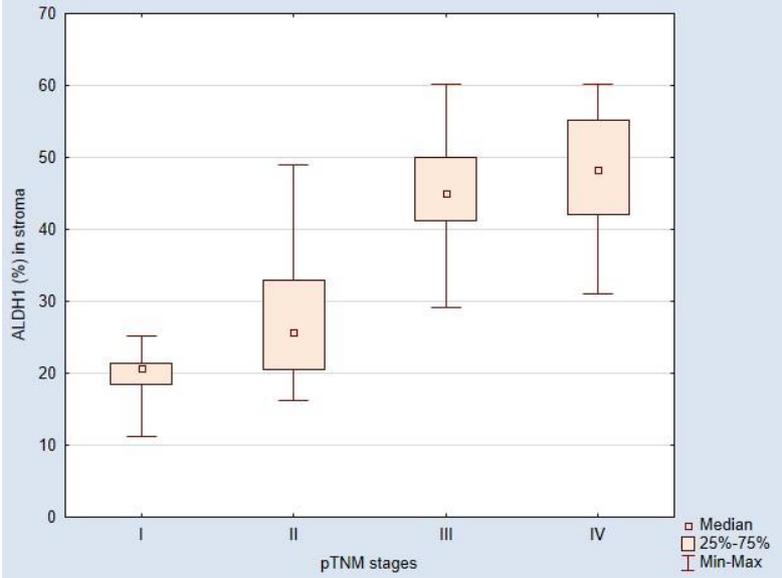


Figure 6. The medians of ALDH1+ stromal cells area in colorectal adenocarcinoma on I, II, III, and IV pTNM stages

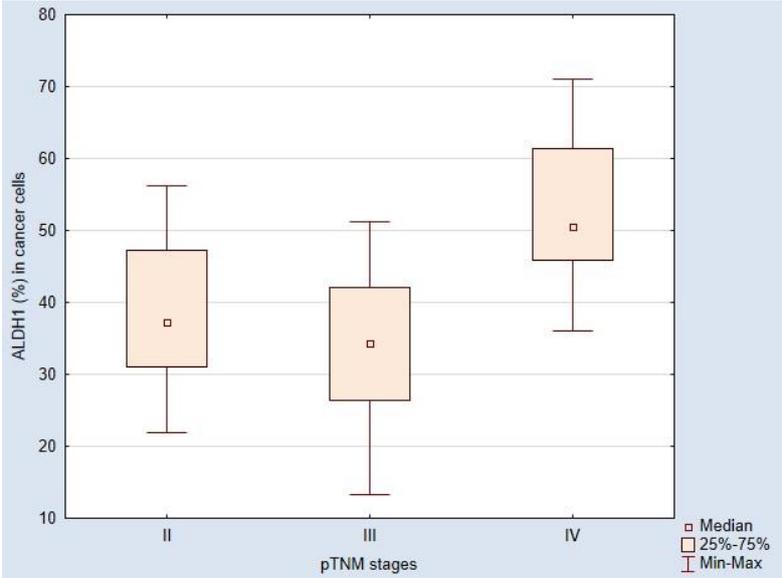


Figure 7. The medians of ALDH1+ cancer cells area in colorectal adenocarcinoma on I, II, III, and IV pTNM stages

Correlative analysis revealed reverse strong connection between ALDH1+ cancer cells area and E-cadherin expression ($r=-0,71$) as well as direct medium strength connection between ALDH1+ cancer cells area and vimentin expression ($r=0,65$) in colorectal adenocarcinoma.

Discussion. Based on the obtained results regarding epithelial phenotypic markers, CRA is characterized by low E-cadherin expression level that decreases during the I-IV stages sequence. Based on literature data, decreasing E-cadherin expression in colorectal carcinogenesis may be mediated by CDH1 gene mutations or by activation of SNAI1 and SNAI2 genes. The SNAI genes participate EMT activation and at the same time, these genes suppress CDH1 expression [4, 6]. Additionally, CRA is characterized by medium CK-20 expression level that decreases during the studied sequence. It is well known that CK-20 is widely used for differential diagnosis between CRA, breast cancer, liver cancer, lung cancer, etc [7]. However, according to the current literature 10-20 % of CRA cases are CK-20-negative [7, 8] that is consistent with the obtained results (23,4 % of studied cases are CK-20-negative). It was reported that CK-20-negative colorectal carcinomas are tumors with high level of microsatellite instability (Jaudah Al-Maghrabi et al., 2018) [7].

Based on the obtained results regarding mesenchymal phenotypic markers, CRA is characterized by medium vimentin expression level that increases during the I-IV stages sequence. Tendency to accumulation of vimentin-positive cells around structures formed by cancer cells was revealed as well. It is known that vimentin-positive cells produce cytokines that participate regulation of cancer cells survival, phenotypic changes, and angiogenesis [9]. The revealed tendency reflexes involvement of paracrine mechanisms of regulation. The information about direct correlation between vimentin expression and Slug expression, that is one of the EMT activators, was reported (Y. Toiyama et al., 2013) [10]. Even though, the exact mechanism of vimentin and Slug interaction is still unclear. Additionally, CRA is characterized by medium α -SMA expression level that increases during the studies sequence. Some studies demonstrated new aspects of α -SMA-positive cells significance: expansion of α -SMA-positive cells area leads to increasing density of tumor stroma that leads to increasing pressure on cancer cells clusters. As a result, cancer cells separation and invasion happen. Furthermore, α -SMA-positive cells form so-called “tracks” which are directions for cancer cells movement [5, 11, 12].

Our findings regarding phenotypic markers expression reflex EMT that happens during the CRA progression. Based on the literature data EMT is closely connected with acquiring stemness by malignant cells [5]. In the current study, significant increase of CD44+

cells area during the CRA progression from the I to the III stages was established. CD44 expression by cancer cells reflects their acquisition of stem properties, since the molecule is involved in the activation of signaling cascades described earlier, which ensure high cell survival [13]. Furthermore, CD44⁺ cancer cells are cells that undergo EMT [14]. The mechanism of CD44 involvement in EMT is the next. One of the driver transcription factors for EMT is ZEB1. EMT-induced ESRP1-suppression controls CD44 alternative splicing that is the reason for switching from CD44^v (variant forms) expression to CD44^s (standard form) expression. CD44^s controls ZEB1 expression independently that works as the main mechanism of CD44^s expression supporting as well as the EMT activation mechanism [15].

Moreover, in the current study significant increase of ALDH1⁺ stromal cells area during the CRA progression from the I to the III stages and significant increase of ALDH1⁺ cancer cells area during the CRA progression from the III to the IV stages were established. Based on the literature data, high ALDH1 expression level is associated with increased number of DR4- and DR5-receptors. These receptors activate MEK/ERK-pathway which participate in EMT-activation [16]. In our study, it was also established that ALDH1⁺ cancer cells area correlates with E-cadherin expression level as well as ALDH1⁺ cancer cells area correlates with vimentin expression. These findings prove the connection between EMT activation and stemness acquisition that occurs during the CRA progression.

Conclusions:

1. Parallel epithelial phenotype losing and mesenchymal phenotype acquiring by the cancer cells that happen during colorectal adenocarcinoma progression from the I to the IV pTNM stages.
2. Colorectal adenocarcinoma is characterized by significant increasing of CD44⁺ and ALDH1⁺ stromal cells areas during colorectal adenocarcinoma progression from the I to the III pTNM stages.
3. II-IV stages of colorectal adenocarcinoma differ by presence of ALDH1⁺ cancer cells that reflexes epithelial-mesenchymal transition.

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