THE NUCLEOLUS ORGANIZER REGIONS' STATE AND NUCLEIC ACIDS CONTENTS IN THE COLON TUMOR EPITHELIAL CELLS' NUCLEI

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Summary. The carried out studies have shown, that self-application of the ploidy level, cellular spectrum and the nuclear organizer regions' state cannot fully rely to determine the colon epithelial tumors malignancy grade. DNA content in these tumors nuclei, nuclear organizer regions state and cellular composition may be important secondary indicator in determining the tumor characteristic and predict its further development. Changing's in RNA content and nucleolus organizer regions volume within the tumor cells nuclei can be used as an indirect measure of the cell viability grade and criteria for singling out their morphofunctional types, capable of further development. The correlation level between DNA content and total nucleolus organizer regions volume within the cells nuclei may be additional highly informative criterion for distinguishing between good and malignant colon epithelial tumors. Tumors with high levels of anaplasia (G3) differentiates itself among others, more differentiated ones, by a reduction of cellular heterogenicity level, what gives evidence of clone's dominance with a stable gene and efficient life support systems within them.

Key words: epithelial colon cancer, nucleic acids, nucleolus organizer region.

Introduction. Nucleolus organizer region (NOR) is the subject of active studying in cancer, including colon epithelial tumors. Were shown relationship between the amount of NOR and patients with colorectal cancer survival [16, 19]. Were carried out researches which purpose was to use the number and state of nucleolus organizer regions as a criterion of tumor proliferative activity [15], the malignancy grade [9], invasive and metastatic potential [16], and prognosis of the disease [13]. NOR used as criteria for histological assessment of tumor response to treatment [1, 18]. These data on the possible use of NOR in colon cancer epithelial cells (CCEC) to determine their malignance and further forecast have not received widespread use to date, which indirectly indicates their low information content. Perhaps this is due to the fact that the assessment of NOR state in colorectal cancer cells do not take into account the heterogeneity of both the tumors and their cellular composition.

DNA changes in CCEC nuclei are typical phenomenon [1, 5, 10], which is associated with chromosomal instability, which is realized in polyploidy and aneuploidy [10]. Today received a significant amount of data showing the relationship between changes in CCEC genome and the nature of the disease [11, 14]. A lot of research were done in order to identify the relationship between DNA content (ploidy) in the tumor cells nuclei and histological type of the tumor and, above all, with its malignant potential [1, 11, 17]. Collected results on the amount

of DNA in the cells nuclei and prognosis in colorectal cancer, given the foundation for understanding the importance of this figure, but that in itself is not absolute, due to the variability of the phenomenon.

The above was a pretext to search for more informative objective indicators of malignancy and CCEC prognosis that take account change in the number of DNA and NOR state [5, 6, 8, 14].

The research purpose consist of determining the relationship between nucleic acids contents and the nuclear organizer regions state in the colon cancer epithelial cells.

Object and methods. The research has been carried out with the use of biopsy materials, and material, got from 130 patients with the CCEC: polyps and adenomas (B) - 16; tumors with signs of malignancy (M) - 22 adenocarcinoma G1 (G1) - 6; adenocarcinoma G2 (G2) - 75 adenocarcinoma G3 (G3) - 11. Histological typing of tumors was carried out with use of routine (hematoxylin and eosin) staining.

The resulting material was fixed in 10% formalin buffer pH 7,4 and condensed in wax using histoprocessor Histos-5 (Milestone, Italy). From these blocks were made 5 mm thickness histological sections using microtome Microm NM325 (Thermo Scientific, Germany). Sections were stained with hematoxylin and eosin, and Azur II eosin for overall tumor assessment, Einarson gallocyanin-chrome alum stain (pH 1,62, 37^0C, 24 hours) for the detection of nucleic acids (NK) in cells [2, 3]. Each case of the sections were treated with RNase (MACHEREY-NAGEL GmbH & Co. KG, Germany) for RNA extraction [2]. NOR were detected by silver nitrate impregnation [Pat. №80458]. These samples were researched and photographed using Nikon Eclipse 80i microscope with DS-5SMc/L2 camera using standardized conditions. We analyzed the images of specimens (magnification x400, 1280x960 pixels RGB) with the help of ImageJ 1,46 program. We explored: in 50 cells cross-sectional area of the cell nucleus (Narea), number (nNOR) and the diameter of each NOR and following calculation of their volume (vNOR); in 60 cells: cross-sectional area of the cell nucleus (Narea), integrative optical density of the cell nucleus and nucleus calculated volume (NV) and the content of total nucleic acids (NNK) and DNA (NDNK) number.

As an initial starting point for assessing the content NNK / NDNK in the tumor cells nuclei, we used indicators taken per unit, characterized by lymphocyte nuclei (2c), which were in the stroma of tumors. The cells of each tumor were ranked on the content of DNA in the nucleus. The resulting sequence was divided into the ranks of the step that was equal to the average DNA content in the lymphocytes nuclei: P1 - to 1, P2 - 1-2, P3 - 2-3, and so on. The each tumor cells stained on total NA and NOR were ranked by Narea / NV. These sequences were divided into ranks by average Narea/NV, identified for the rank of DNA content. Within each rank were

determined the number of cells, mean values Narea, NV, NDNK or NNK. The content of RNA in the cell nuclei was determined as the difference between NNK and NDNK for each pair of ranks. These data were processed by standard statistical methods.

Results

The carried out studies have shown that CCEC can be divided into ranks from P1 to P10 by DNA content. However, most of the parameters mean values, starting from P6 with p> 0,05, couldn't used for further statistical analysis. So they were combined into a single rank.

The DNA content mean in the tumor cells nuclei of various grades ranged from 1,86 (G3) to 2.43 (M) (Fig. 1). This marked statistically significant differences between M and B and G1 (p <0,05 according to the *t*- and *f*-tests), and differences between G grades are not significant (p> 0,05).

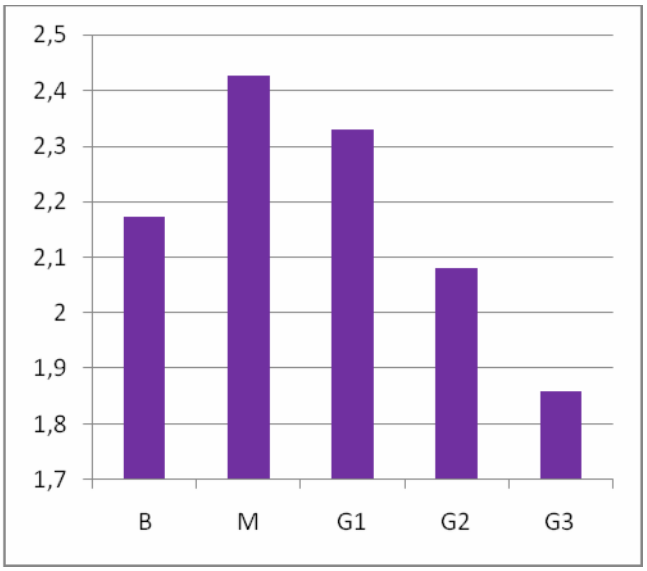


Fig.. 1. The average DNA content (standard unit) in the CCEC with different anaplasia grade (B - adenoma, M - adenomas with signs of malignancy, Gh - carcinoma with the corresponding anaplasia grade).

Tumor ranking by average DNA content in the nuclei has allowed us to share them in three groups: diploid (D - average content of DNA in the nucleus to 1,2), intermediate between di-and tetraploid (D-T - average content of DNA in the nuclei of 1,2 to 2,5), hiperploidy (T + - average DNA content more than 2,5) (Fig. 2). It was found that each group of tumors (anaplasia grade) includes all three identified subgroups (Fig. 2).

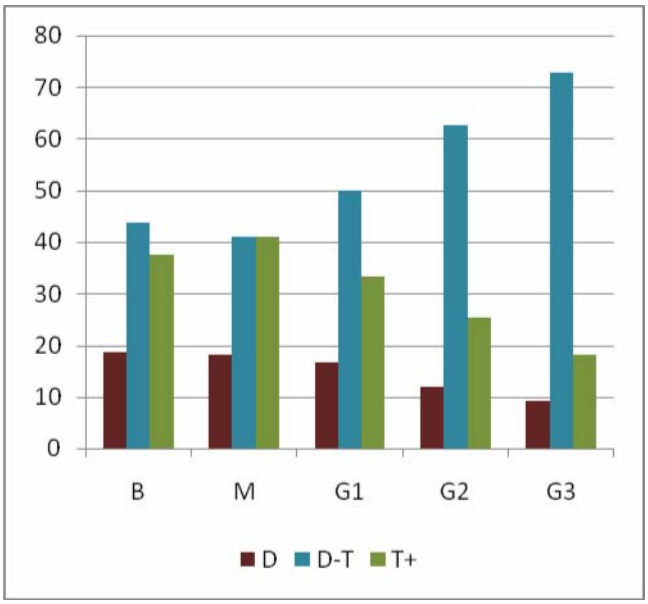
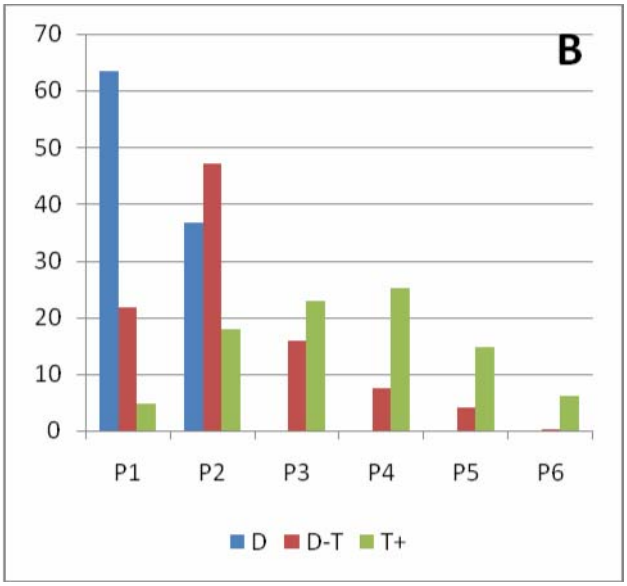
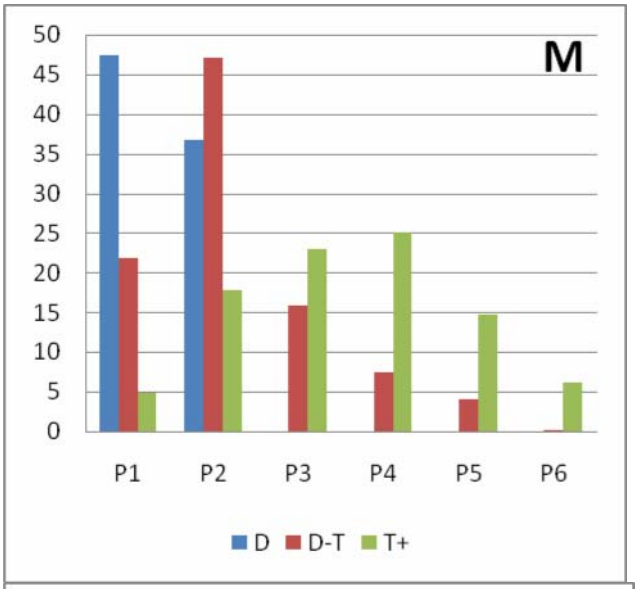


Fig.. 2. CCEC ratio (%) EPTK with different anaplasia grade and average DNA content in cells (B - adenoma, M - adenomas with signs of malignancy, Gh - carcinoma with the corresponding anaplasia grade). D - diploid, D-T - intermediate between di-and tetraploid, T + - hyperploid cells

CCEC structure (spectrum) determined by DNA content in their cells has a moderate degree of dependence on the anaplasia grade (Fig. 3). On the whole, as the increase in mean ploidy shifting the tumor cell spectrum to increase the relative cells number with increased DNA content in the nuclei.




M
50
45
40
35
30
25
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15
10
5
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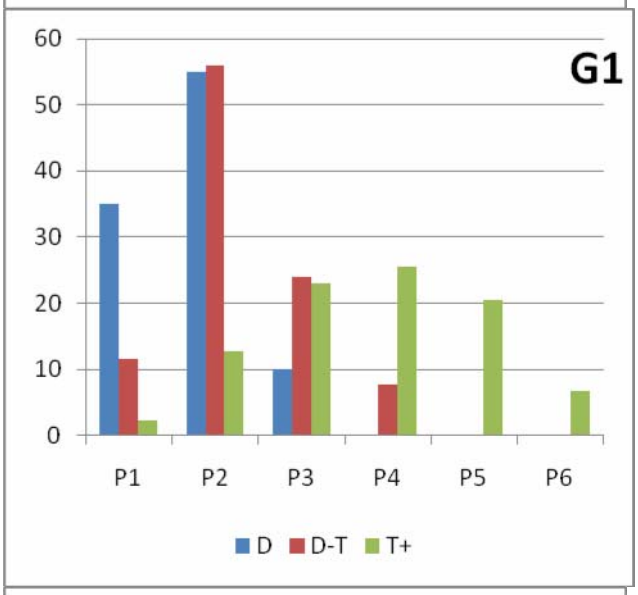
P1
P2
P3
P4
P5
P6
D
D-T
T+


G1
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P3
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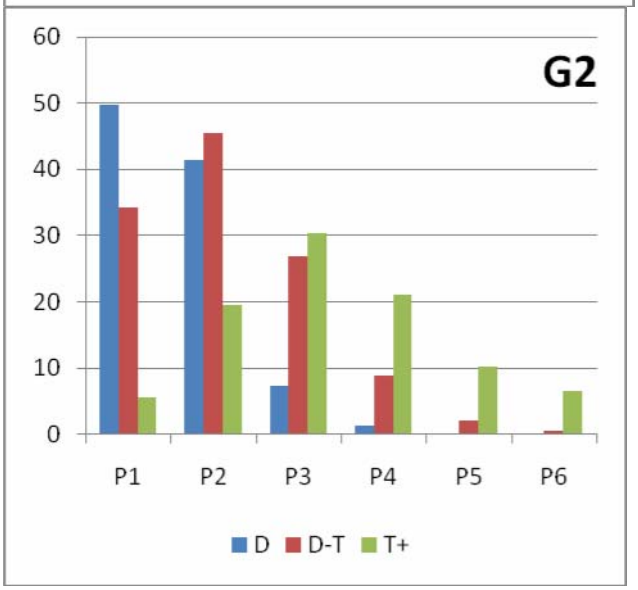
D-T
T+


G2
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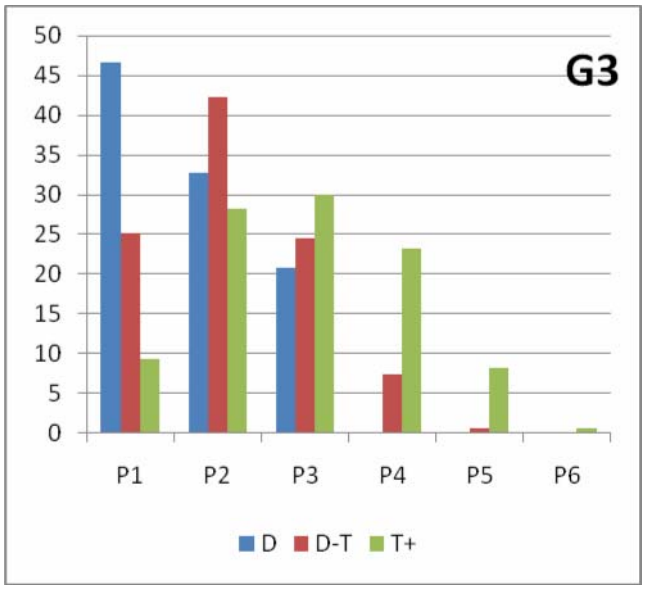


Figure. 3. Relative cells content (%) with different DNA content ranks in different CCEC grade (B, M, G1, G2, G3). LC - Ranks for DNA content; D - diploid, DT - intermediate between di-and tetraploid, T + - hyperploid cells

The RNA content in the tumor cells nuclei showed inverse dependence of DNA - that is, with increasing DNA content decreases its relative content. These changes are not detected depending on the tumor anaplasia (not significantly different) (Fig. 4).

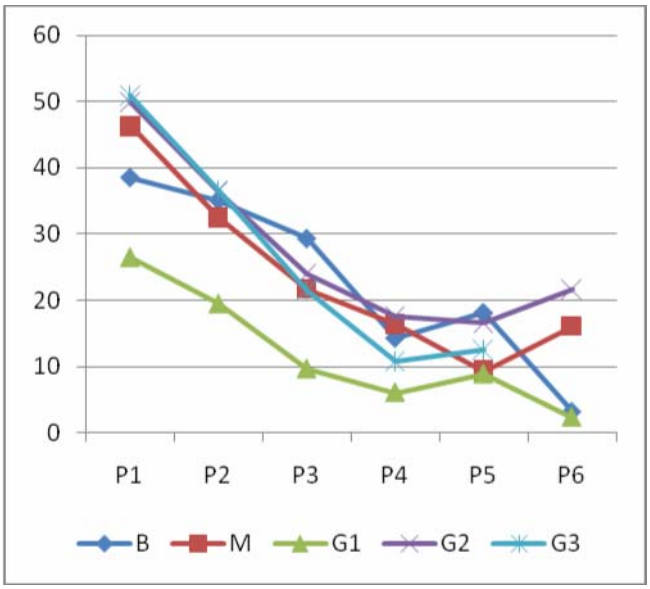


Fig. 4. The relative RNA content (%) in the cells nuclei of different DNA content ranks in different CCEC grade (B, M, G1, G2, G3). PX- ranks for DNA content; D - diploid, DT - intermediate between di-and tetraploid, T + - hyperploid cells

Indicators of DNA, NK and tumor size nuclei (Narea, NV) found a direct dependence and

keeps the ranks P5-P6 (correlation coefficient 0,96-0,98). The cells belonging to the higher ranks these proportions are violated, and the correlation is lower than the values that suggest a direct relationship between these parameters. This is due to a small number of cells within tumors and their large variability. These cells have a nucleus area over 50-52 μm^2 and volume over 300 μm^3. For these cells are characteristic pyknosis phenomena or, conversely, swelling of the nucleus and chromatin lysis. It should also be noted that pyknosis relatively frequently observed in small cells that were referred by us to the P1 rank.

In general, the amount of NOR in the CCEC nuclei with increasing anaplasia grad showed no tendency to decrease, and their total volume increased (Fig. 5), although the difference between tumors' bordering types in general are unreliable. Thus, there is no direct correlation between the number and the total amount of NOR (index 0,34 correlation).

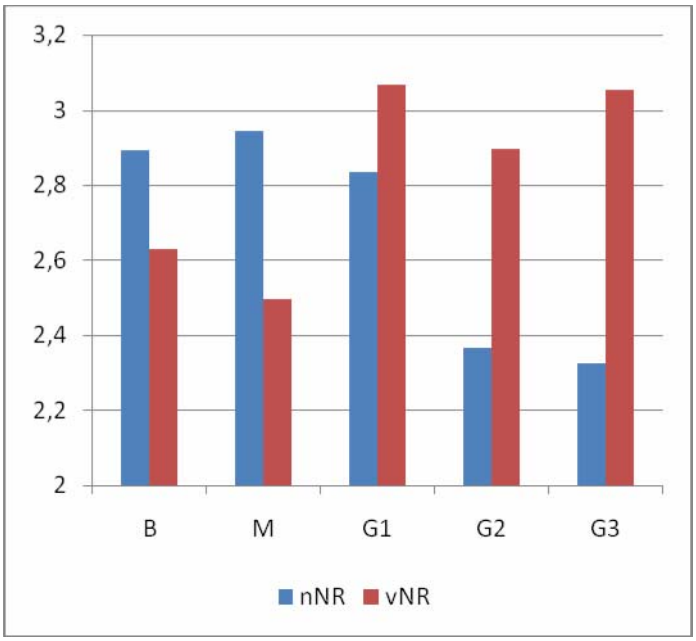
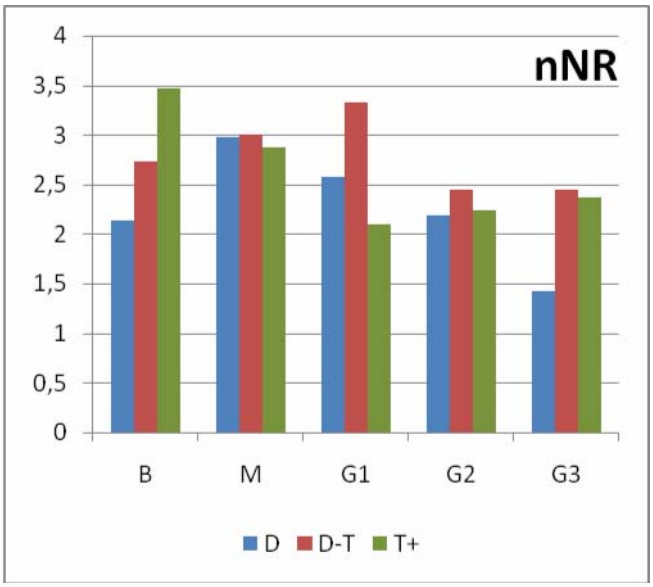


Fig.. 5. Number (nNOR) and total volume (vNOR, μm^2) NOR in the CCEC nuclei with different anaplasia grade (B - adenoma, M - adenomas with signs of malignancy, Gh - carcinoma with the corresponding anaplasia grade).

The number and total amount of NOR (Fig. 6.) In the benign neoplasm cells nuclei (B) is in direct proportion to their ploidy (correlation index 0,98), and marginal (M) and malignant tumors (G1-2) this relationship is broken and the correlation is lower than the value, indicating a direct relationship between these parameters. However, in G3 tumors determined significant direct correlation between vNOR and their cells ploidy (index 0,89 correlation).



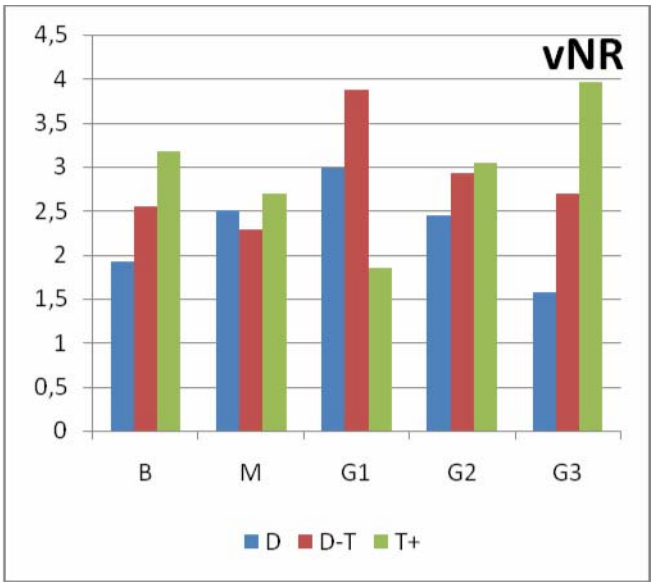


Fig. 6. Number (nNOR) and total volume (vNOR, μm^2) NOR in the CCEC nuclei with different ploidy level (B - adenoma, M - adenomas with signs of malignancy, Gh - carcinoma with the corresponding anaplasia grade). D - diploid, DT - intermediate between di-and tetraploid, T + - hyperploid cells

Assessment of the relationship between the NOR state and the DNA / NA amount in the ranked tumor cell B group showed a high degree of communication between DNA-vNOR in (0,92) and M - (0,96), and between the NA-vNOR (0,87 and 0,97 respectively). In malignant tumors the direct relationship between these parameters is missing.

Proven relationship between the NOR state and RNA content in the tumors nuclei were not found ($p > 0,05$).

So, given that all types of anaplasia grade (B, M, G1, G2, G3) are tumors with different

DNA content corresponding di-, tetra-or-poliploidy then ploidy can not be used as independent parameter to specify the stage of tumor development, as suggested by several authors [1]. Condition (number and volume) of NOR in a whole, does not reflect the grade of tumor transformation and can not be an independent criterion for its assessment.

A variety of cellular structure using DNA content and NOR state, which are links in a single chain of genetic information, are indirect indicators of the tumor cells properties and viability. Accordingly, it is sufficient reason for the inclusion these criteria to the comprehensive assessment of the tumor properties.

Reducing the relative RNA content and lack of proportional increase in the number / amount of NOR in the tumor cells nuclei with increasing DNA content indicates that additional DNA is functionally limited or not active. It can act as decreased viability signs of these cells, leading to aging, apoptosis and elimination of morphological and functional cell types from the tumor [7, 12]. Proof of this is the high level of correlation between DNA content and vNOR in benign tumors, which naturally expected, and after the initiation of this relationship in a series of M-G2, its recovery in G3.

The growth of cellular heterogeneity among the researched CCEC both DNA content and morphological features observed in a number of B to G2. Rightfully suggest that among this cells variety are those that have kept the life-support systems, and those in which they were raised. Other confirmed the prevalence degeneration signs among cells and higher ranks P6, and relatively frequent pyknosis symptoms among P1 cells. This indicates that as the tumor progression from the tumor eliminated viable morphological and functional cell types. However, since cellular diversity are highlighted such as having distorted genotype, but retain a high proliferative activity, effective livelihood and lose specific functions integrate into tissue systems are the basis for the emergence resistant clones capable of infinite existence. This is confirmed by the fact that G3 tumors shrinking range of cellular structure. The predominant cells are D-T, which, given the high proliferative activity, allows us to consider them as a diploid in which DNA synthesis occurs.

Conclusions

The carried out studies have shown, that self-application of the ploidy level, cellular spectrum and the nuclear organizer regions' state cannot fully rely to determine the colon epithelial tumors malignancy grade. DNA content in these tumors nuclei, nuclear organizer regions state and cellular composition may be important secondary indicator in determining the tumor characteristic and predict its further development.

Changing's in RNA content and nucleolus organizer regions volume within the tumor cells nuclei can be used as an indirect measure of the cell viability grade and criteria for singling

out their morphofunctional types, capable of further development.

The correlation level between DNA content and total nucleolus organizer regions volume within the cells nuclei may be additional highly informative criterion for distinguishing between good and malignant colon epithelial tumors.

Tumors with high levels of anaplasia (G3) differentiates itself among others, more differentiated ones, by a reduction of cellular heterogenicity level, what gives evidence of clone's dominance with a stable gene and efficient life support systems within them.

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