MITOTIC ACTIVITY AND NUCLEIC ACIDS CONTENT IN THE CELLS' NUCLEI OF NEUROBLASTOMAS

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Summary. The anaplasia grades' growth in neuroblastoma is attended by a decrease in the average DNA content and increased proliferative activity. However, a wide variation range in the ganglioneuroma and neuroblastoma don't allow (except in some cases) to use it as an independent criterion for determining the malignant potential of a particular individual tumor. Ganglioneuroblastoma and neuroblastoma can be distinguished with their cellular spectrum, due to a decrease or, conversely, increase the mitotic activity by increasing the DNA content in tumor cells. On the other hand, neuroblastoma can be divided into those that increase in the average DNA content in the cell nuclei, due to high proliferation activity (large proportion of cells in which DNA synthesis occurs) and those in which this component increased, due to an increase the cells proportion in the cellular spectrum with high DNA content (polyploid).

Key words: neuroblastoma, Ki-67, nucleic acids.

Nowadays it is well-known that Ki-67 protein is high-informative cellular proliferation marker. As a result, unlimited (uncontrolled) mitotic activity is the typical tumor quality; the determination of this protein is widely used in oncology, and the part Ki-67-positive tumor cells (Ki-67 the index of marking) often correlates with clinical cancer trend.

Neuroblastoma is also the object of examination for identifying their proliferative activity by determination of protein Ki-67 expression [10, 11, 12]. In addition, the dependence of Ki-67 high level with unfavorable and interim MKI were shown (Shimada index [14, 15]). In addition, the high level of Ki-67 were seen in aneuploid tumors and in tumors of III and IV grades that immediately points on the direct link among high proliferation level and unfavorable disease trend, and the bad tumor reaction on the treatment.

However, NB proliferative activity is not as meaningful prognostic factor as

in tumors of some other histogenetic types (for example, neuroendocrine [1]). First, this connects with NB biological peculiarities, which are child's embryonic tumors; it needs to extrapolate proliferative activity to such background one, which is naturally high enough for the tissues of child's organism. That was the reason to search the links between the Ki-67 expression and other markers, which characterize these or other NB cellular features.

On the other hand, the limitation of informational content of proliferative activity in NB like prognosis factor is connected to heterogeneity of these tumors including their cellular structure by contents of nucleic acids [2]. Presence of NB with different cellular ploidy indicates on different pathogenetic mechanisms [8, 9], which lead to NB appearance. It is logical that it will reflect on proliferative activity of their cells. Accordingly, mitotic activity of tumor cells should not be the independent factor; it should be used as the derivative prognosis factor.

The object of the work was to establish the mitotic activity peculiarities within neuroblastoma cells with different nucleic acids content.

The object and methods of investigation

The research has been carried out with the use of biopsy materials, and material, got from 102 patients with neuroblastoma: ganglioneuromas (GN) – 3; ganglioneuroblastomas (GNB) – 14; neuroblastomas (NB – the neuroblastoma) – 26. Histological typing of neoplasms were made with usage of routine (hematoxylin and eosin staining) and immunohistochemical research.

The received material was fixed in 10% formalin buffer pH 7.4 and condensed in wax using tissue processor 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, Einarsons' gallocyanin-chrome alum stain (pH 1,62, 370C, 24 hours) for the detection of nucleic acids (NK) in cells [3, 4]. Each case of the sections were treated with RNase (MACHEREY-NAGEL GmbH & Co. KG, Germany) for RNA extraction [3].

Immunohistochemical reactions were made with monoclonal mouse anti-

human Ki-67 antigen, clone MIB- 1 (Dako, Denmark) and detection system EnVision TM FLEX, (Dako, Denmark) according to the required protocol, and necessary controls. Gills' hematoxylin was used as a nuclear counterstain. The tissues samples with definite positive reactivity were used like positive control, and for the negative control the procedure without usage of primary antibody were made.

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 stained by gallocyanin-chrome alum: cross-sectional area of the cell nucleus, integrative optical density (NIntDen) of the cell nucleus and the total nucleic acids (NNA) and DNA (NDNA) number. RNA number was figure out as the difference between the nucleic acids (NA) and DNA number. To appraise the NA content within tumor cells nuclei were used their content in the lymphocytes nuclei as the equivalent unit. The cells in each tumor were ranked by the DNA content in the nucleus. The resulting sequence was divided into the ranks by 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 [2]. Each tumor stained on total NA and Ki -67 expression were ranked by the nucleus size according to the average DNA values. Within each rank the relative cells number, the average value of the Narea, NintDen, NDNA, NNA and and MI Ki- 67 were determined. These figures were processed by standard statistical methods.

Results and discussion

In this study, we have shown that the average DNA content in tumor's cells nuclei decrease by the extend of neoplastic transformation degree increases: GN (4.68 ±0.21) – HNB (3.07 ± 0.02) – NB (2.16 ± 0.03), and the mitotic activity raises (4.4 ± 1.5, 15.8 ± 0.5 and 31.8 ± 0.5) (Fig.1). Correlation indexes between mitotic activity and anaplasia grade is 0.99 and DNA quantity is -0.94.

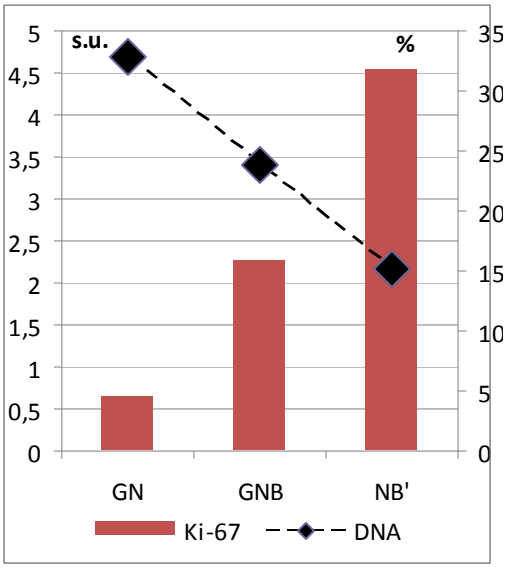


Fig.. 1. DNA content (standard unit) within NB cell nuclei and the percentage expressing Ki-67 (GN - ganglioneuromas, GNB - ganglioneuroblastomas, NB '- actually neuroblastoma).

According to average DNA content in tumor's cells nuclei were divided into three groups: diploid (D - average DNA content in the nucleus is to 1.2), intermediate between di- and tetraploid (D+ - average DNA content in the nuclei is from 1.2 to 2.5), tetra- and hyperploid (T+ - average DNA content in the nuclei is more then 2.5). Among examined HN and HNB the tumors T+ are predominate, and D were absent in our list. Among NB', in comparison to more differential tumors, the essential predominance of D+ tumors is seen. However, in difference of that the big part of all tumors were the D subgroup tumors [2]. Accordingly, statistically reliable comparison of proliferative activity among tumors with different anaplasia grade can be done only in T+ tumors (Fig.2), which differed much from common meanings (Fig. 1). Among NB the biggest mitotic activity were seen in D+ tumors (Fig. 2).

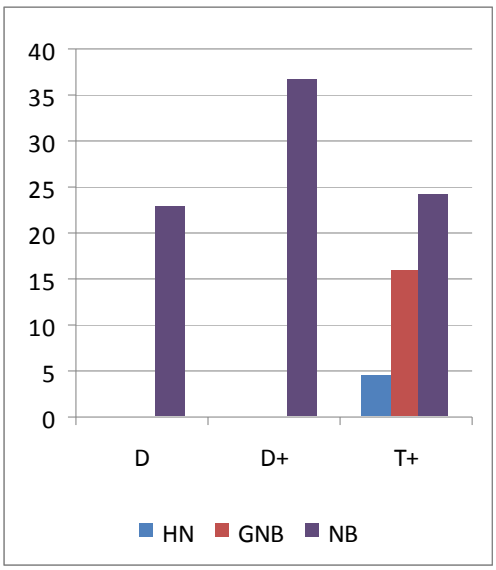


Fig. 2. Mitotic activity (percentage of Ki-67 expressing tumor cells nuclei) in NB with different average DNA content in cells (GN- ganglioneuromas, GNB - ganglioneuroblastomas, NB '- actually neuroblastomas; D - diploid, D + - intermediate between di-and tetraploid, T + - tetra-and hyperploid tumors).

By DNA content, NB cells can be divided into ranks from P1 to P12. However, most of the parameters' mean values, starting from P5 is statistically unreliable ($p > 0.05$) and couldn't be used for further statistical analysis, were merged into a single rank, named P5 +. Cellular complement of NB, which was identified by the DNA contents in their cells, shows temperate stage of dependence from the tumors differentiation grade [2]. In general, with the differential grade diminution, the tumors' cell spectrum shows the tendency to decrease the quantity of cells with big a DNA mount in their nucleus, it happens when the relative tumor cells quantity increases in this way. In HN' this index has diphasic curve with a pick in P4 range (Fig. 3).

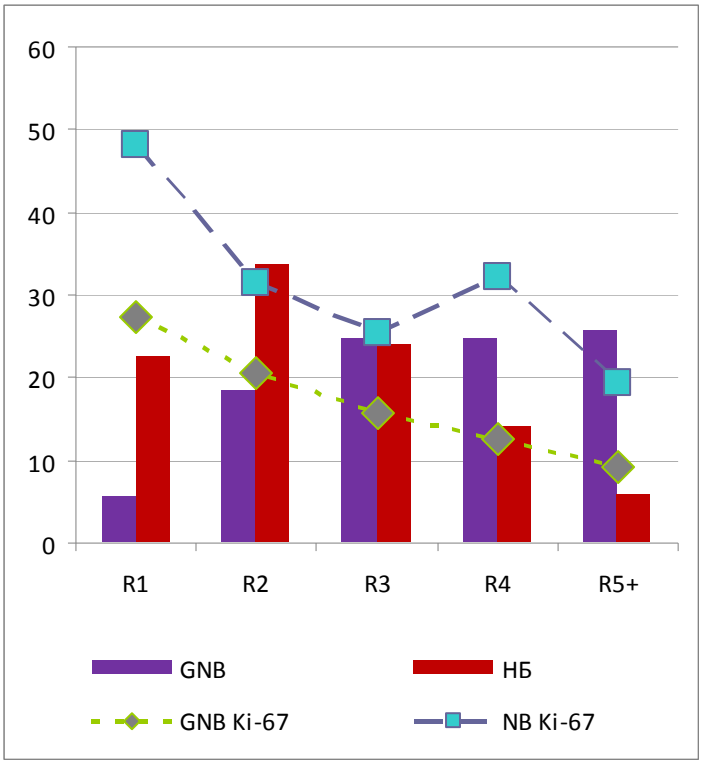
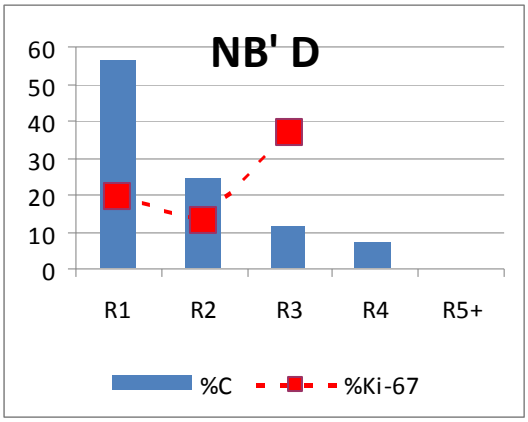
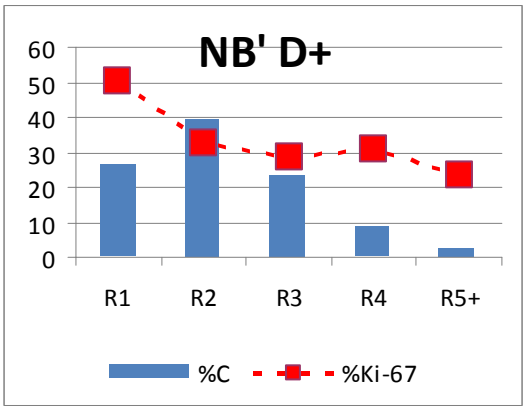


Fig.. 3. Relative content (percentage) different ranks cells by DNA content and mitotic activity (percentage of tumor cells, which nuclei expressing Ki-67) in NB (GNB - ganglioneuroblastomas, NB '- actually neuroblastomas, Ki-67 -percentage labeled nuclei. RX - Ranks the content of DNA).

Among NB of different ploidy the biggest mitotic activity is seen in the D+ group (36.9 ± 0 .7) and fundamentally less in D (22.9 ± 0.5) and T (24.1 ± 0.9) groups. In general, the mitotic activity distribution among cells ranges in these groups shows on its increase in the way of rise the DNA contents in the tumors cell's nuclei (Fig. 4).





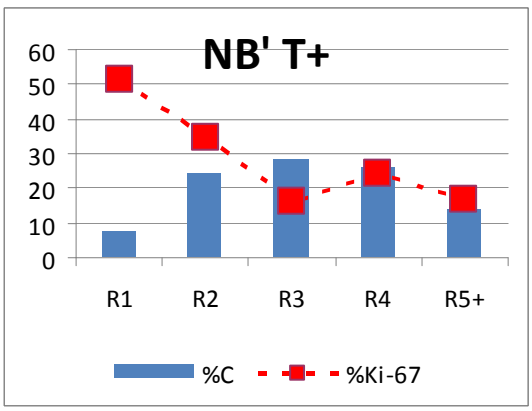


Fig.. 4. The relative content (percentage C) different ranks cells by DNA content and mitotic activity (percentage Ki-67) in NB' with different average DNA content (D - diploid, D + - intermediate between di-and tetraploid, T + - tetra-and hyperploid tumor. RX- Ranks by DNA content).

Correlation assessment between the average DNA values in cells' nuclei and mitotic activity in the HNB and NB found no direct relationship between them. However, the assessment of this indicator in each individual tumor showed that it varies from +1 to -1. Herewith 2/3 of tumors both HNB and NB (no matter what is the average DNA content) tumors have a negative correlation index, while others 1/3 - positive.

The RNA content in the tumor cells nuclei showed inverse dependence of DNA - that is, with increasing DNA content decreases its relative content. [2]. However, the estimation of percent RNA content in separated tumors gives an opportunity to distinguish the tendency that tumors with positive correlation index between DNA content and mitotic activity have less expression of this proses then tumors with negative correlation index have.

As a result, the carry out studies have shown that dedifferentiation level rise in NB (in a line HN – HNB – NB') we saw the decrease of average DNA content [2] and the proliferative activity increase. Nevertheless, huge ranges of mitotic activity vibration and their blocking in HNB and NB do not allow using them during identifying the malignant potential of specific separate tumor. Definite interpretation mitotic activity can get only in regional parts of these diapasons (approximately, while expression Ki-67 is less than 10% and more 35% of nucleus).

Among HNB and NB we can select tumors which have opposed vectors of their nucleus range forming. The first characterized by a decrease in mitotic activity, and the second, contrariwise, by its increase, with increased cells' DNA content.

The correlation between DNA and proliferative activity among tumors' cells allow selecting two vectors: the tumors that have the rise of average DNA contents in cell's nuclei. This rise happens due to high proliferative activity (big amount of cells where DNA synthesis happens). Moreover, there are tumors where this index is caused in cells range with high DNA level (polyploid). Up to this, tumors NBD+ can be mostly related to the first vector, and NBT+ can be mostly related to the

second one.

There is also a difference in RNA content level decrease within nuclei on the background of polysemantic divide of mitotic activity in tumors nuclei with the measure of DNA contents rise [2], it is additional inconstant that gives its contribution into the NB heterogeneity.

The variety of NB at DNA/proliferative activity and the RNA content within the nuclei is the index of qualities variety and tumor cells vitality that is connected with different patogenetic oncogenesis mechanisms [8, 9]. Complex estimation of these parameters, first without amplification of *n*-myc gene and 11q aberation [5, 7] can become additional high-informative histological determination criteria of NB development potential.

Conclusions

The anaplasia grade rise in NB (in line HN-HNB-NB') followed by decrease of average DNA contents and increase of proliferative activity. However, wide proliferative activity variation range in HNB and NB do not allow using it for determining the malignant potential of a single specific tumor (except with extreme values).

Among the HNB and NB' we can distinguish tumors that have opposite vectors of forming their cellular spectrum: at the expense of decrease or, on the contrary, the increase in mitotic activity as the DNA content rise within tumor cells. On the other hand, the NB also can be divided into the tumor, where the average DNA content rise within the cell's nuclei takes place due to high proliferative activity (large share of cells where DNA synthesis is done). And such, where this index is due to increase of interest in the cellular spectrum cells with a high DNA content (polyploid).

Various RNA reduction grade within the nuclei on the background of unclear mitotic activity distribution in tumor cells as the DNA content rise is an additional variety which contributes to the NB' heterogeneity.

A variety of NB for DNA content / proliferative activity and RNA content in the nucleus are not only heterogeneity indicators of these tumors on their cell

composition, but also reflect to a certain extent pathogenetic mechanisms of their formation and may become quite informative criteria for the determination their growth.

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