CONTENT OF NUCLEIC ACIDS IN NUCLEI OF NEUROBLASTOMA CELLS WITH VARIOUS DIFFERENTIATION DEGREES

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Summary. In neuroblastoma with increasing of de-differentiation, reduction of the average DNA content in cancer cells has occurred; the cell range has shifted to a cells with low DNA content of the nucleus what leads to decreasing of level of tumor cellular heterogeneity. Increasing of RNA content in neuroblastomas polyploid nucleus suggests about certain degree of the functional activity preserving of the genome; it can be used as an indirect indicator of cell viability and selection of their morphological and functional types which are able, to some extent, to differentiate. Based on the availability of a direct dependence between the content of DNA in the nuclei of cells of neuroblastoma and nucleus' size, cariometric values may be used to indirectly determine the ploidy of the cells without using of complex staining techniques, and that will enhance the objectivity and reliability of tumors' assessment in routine histopathological practice.

Keywords: neuroblastoma, nucleus, the nucleic acid.

Neuroblastoma (NB) is referred to a group of embryonic tumors and is one of the largest specific solid tumors of childhood, which occurs most often, up to 8% of all pediatric cancer, taking the sixth place in the structure of pediatric cancer. In 2005 it was offered a World Class System of NB by risk groups (INRGSS) [3, 8]. It also includes individual genetic characteristics of the tumor - amplification of the gene n-myc, deletion of 11q and ploidy, and it allows to define four groups of risk at the stage of initial diagnosis: a group of extremely low-risk, low-risk, intermediate-risk group and a group of high risk [6, 16]. Based on this classification system it is proposed a new system of INRG staging (L1, L2, M, MS) in which the one of parameters is the status of n-myc gene and ploidy.

Status of n-myc gene is central stratification biological marker for determination of the risk group, regardless of the histopathological structure of NB and its degree of differentiation [4, 5]. n-myc gene amplification is clearly associated with rapid tumor progression and us poor prognosis in patients of all ages and disease stages [7]. At the same time, prognostic favorable forms of NB in almost all cases are polyploid and with absence of gene amplification [12]. Hyperploid tumors without structural changes of chromosomes are easier to be treated and sometimes are capable for spontaneous regression. [George R.E et al. (2005)]. Ploidy as mono-factor has limited value because the aneuploid tumors may also have segmental chromosomal

aberrations and gene amplification of n-myc, which always defines the unfavorable development of cancer [4]. However, numerous studies [11, 16] have proved that ploidy (DNA index) is a clear prognostic marker for children with neuroblastoma, particularly under 2 years old who have disseminated disease [11]. A significant increase in life expectancy has been observed in children with hyperploid (often triploid) tumors compared with diploid. Some authors [13, 14] found that even in children with tumors limited in one region diploid associates with increased risk of regional or distant recurrence. Moreover, despite of the fact that the amplification of n-myc gene is mostly associated with diploid DNA content, diploid is prognostic significant factor for stage 4 disease without amplification of n-myc gene in children under 2 years old.

Purpose of this work is to establish links between the content of nucleic acids in the cells' nucleus and the degree of NB differentiation and the possibility of their using in the histological test for determination of the tumor risk ratio.

Object and investigational methods

Studies have been performed based either on biopsy material, ore extracted during surgery from 39 patients with neuroblastoma: ganglioma (GN) - 3; ganglioneuroblastoma (GNB) – 11, neuroblastoma (NB – neuroblastoma as itself) - 25. Histological tumor typing was carried out using routine (hematoxylin and eosin staining) and immunohistochemical test.

The obtained material was fixed in 10% formalin buffered solution with pH 7.4 and covered in wax with using of histoprocessor "Histos-5" (Milestone, Italy). From the paraffin blocks 5 microns thick histological sections were made using microtome Microm NM325 (Thermo Scientific, Germany). Sections were stained with hematoxylin and eosin and Azur II-eosin for overall assessment of tumor, gallocyanin-chrome alum by Einarson (pH 1.62, 37 OC, 24 hours) for the detection of nucleic acids (NA) in cells [1, 2]. For each case part of the sections were treated with RNase (MACHEREY-NAGEL GmbH & Co. KG, Germany) for RNA extraction [1]. These samples were studied and photographed using Nikon Eclipse 80i microscope with camera DS-5SMc/L2 in standardized conditions.

In the images stained with gallocyanin-chrome alum (magnification power x400, 1280x960 pixels RGB) in 60 cells of each tumor using image analysis system ImageJ 1,46 it was determined: cross-sectional area of the cell nucleus (Narea), specific absorbance of the cell nucleus (NDM), integrative absorbance of the cell nucleus (NIntDen), and the calculated volume of the nucleus (NV) and the content of total number of nucleic acids (NNA) and DNA (NDNA) with formulas:

$$NV = * 3/4 * Narea2 * \sqrt{(Narea / \pi)} \quad (1)$$

$$NNA\ (NDNA) = NIntDen * 3/4 * N\ area * \sqrt{(Narea / \pi)} \quad (2)$$

As an initial starting point for assessment of the NA content in the nuclei of tumor cells,

it was used as meaning as unit, attributable for lymphocyte nuclei (2c), which were located in the tumor stroma. The cells of each particular tumor were ranked by DNA content in the nucleus. Obtained sequence was divided into the ranks with the step that is equal average DNA content in the lymphocytes' nucleus: P1 - up to 1, P2 - 1-2, P3 - 2-3, and so on. The cells of each tumor staining on NA were ranked by Narea / NV. Obtained sequence was divided into the ranks by the average Narea / NV, identified for the rank in accordance with DNA content. Within each rank it was determined: the absolute number of cells, mean values Narea, NIntDen, NV, NDNA or NNA. The content of RNA in the cells' nuclei was determined as the difference between NNA and NDNA for each pair of ranks. Obtained data was processed with standard statistical methods.

Results and discussion.

Studies have shown that based on DNA content NB cells can be divided on the ranks from P1 to P10. Sometimes in the tumors have encountered isolated cells which based on DNA content should be attributed to the higher ranks. It should be also noted that NB consisted of cells with rank P5 less than 5%, and the major of average values of their determined parameters had $p > 0.05$, so they cannot be used for further statistical analysis.

The average DNA content in the nuclei of tumor cells decreases while degree of neoplastic transformation increasing: GN (4.68 ± 0.21) - GNB (3.07 ± 0.02) - NB (2.14 ± 0.03).

NB were ranged on the average DNA content in the nuclei of their cells which allowed to divide them into three groups: diploid (D - average DNA content in the nucleus is up to 1.2), intermediate between di- and tetraploid (D + - average DNA content in the nuclei is 1.2 to 2.5), and tetra- and hyperploid (T + - average DNA content is more than 2.5) (Fig. 1). It was found that among the investigated GN and GNB, tumor T + was predominant; and there were no D in our sample. Among NB', comparing with more differentiated tumors, it was revealed significant predominance of tumors D +, and, in contrast to the above, tumors of sub-group D were in a significant proportion (Fig. 1).

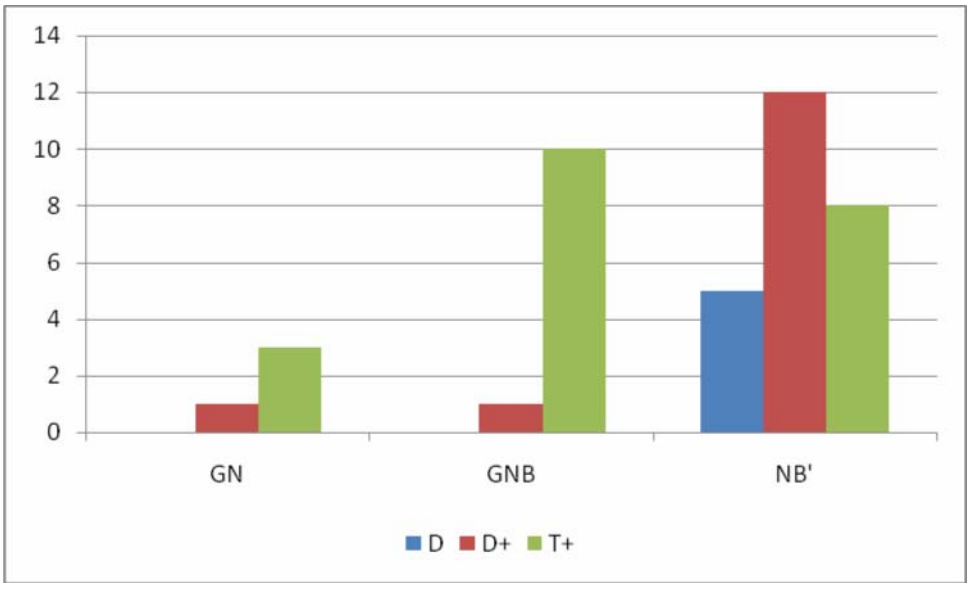


Fig. 1. Ratio (%) of NB with varying degrees of differentiation and various mean DNA content in cells (GN - ganglioneuroma, GNB - ganglioneuroblastoma, NB – neuroblastoma as itself). D - diploid, D + - intermediate between di- and tetraploid, T + - tetra-and hyperploid cells.

NB cellular structure (spectrum), determined in accordance with DNA content in their cells, shows moderate degree of dependence on the tumor differentiation degree (Fig. 2). In general, while cellular anaplasia levels increasing, tumors cellular spectrum shifted to reduce of fraction of cells with increased DNA content in the nuclei. So cell' heterogeneity decreased in the tumors with degree of anaplasia increasing.

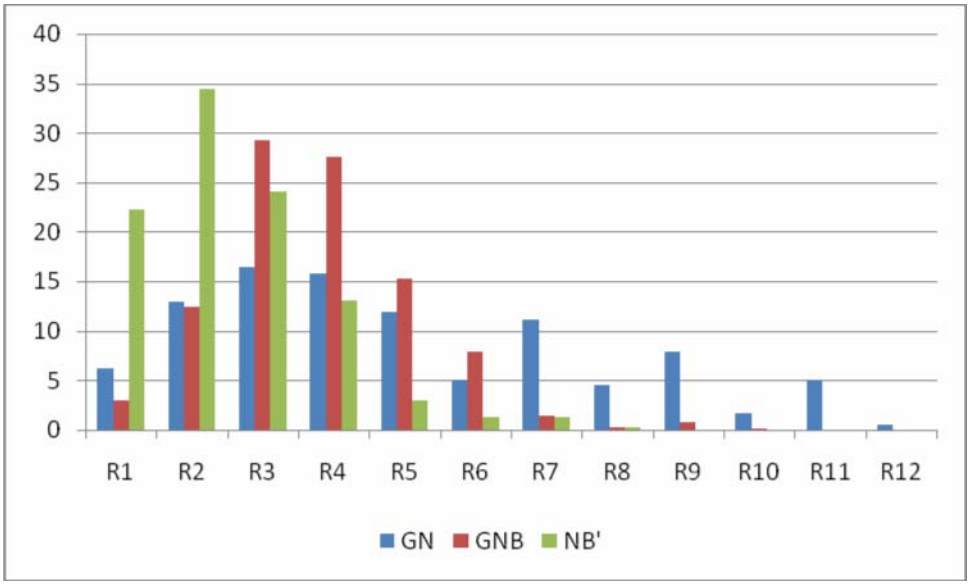
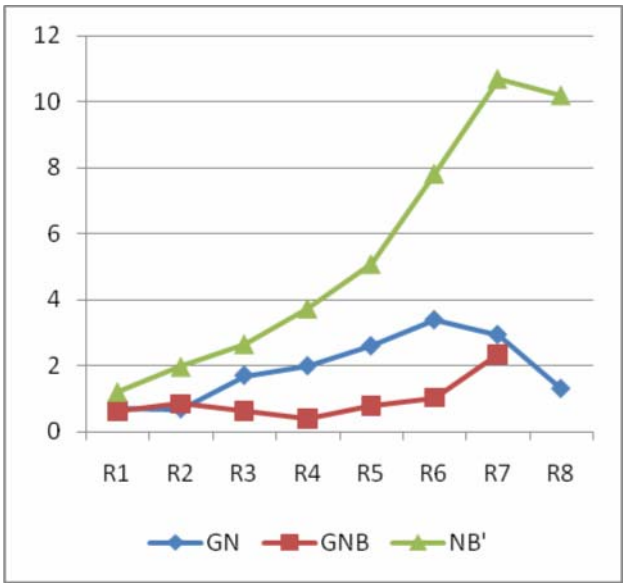


Fig. 2. Relative content (%) of different ranks cells for DNA content in NB with different levels of differentiation (GN, GNB, NB). RX - Ranks for DNA content.

The RNA content in the NB cell nuclei was reliable reduced as far as tumors differentiation reduction (Fig. 4). Absolute RNA content increased in GN and GNB as far as DNA in the nucleus growing. RNA content in GN is in range of ranks P1-P4 (P5) did not change, and then shows tendency for increasing. The relative RNA content in the nuclei of GN cells after significant reduction in in cell D +, slightly increases and remains practice at the same level in T + cells regardless of DNA content in the nuclei of their cells (Fig. 3). Clear decreasing of RNA content was happened in the nuclei of tumor cells of GNB and NB in the ranks of P1-P3 (P4), and then tendency for increasing remains.



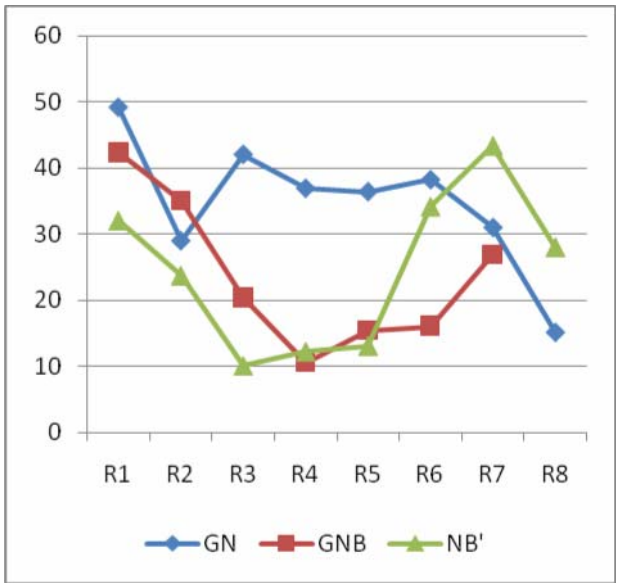


Fig. 3. Absolute (standart unit) and relative (%) RNA content in the nuclei of cells from different ranks for DNA content in NB of different differentiation levels (GN, GNB, NB). RX - Ranks for DNA content.

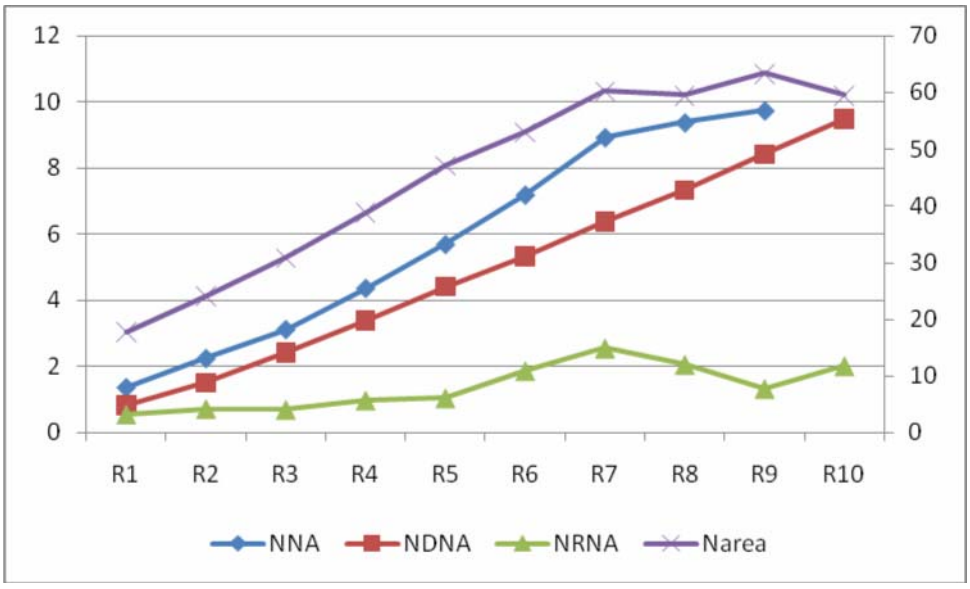


Fig. 4. Correlation between nucleus size (NArea - nuclei area, μm^2) and content of nucleic acid (NNA - total, NDNA - DNA, NRNA - RNA) in NB (all investigated cell array n = 2369).

Between DNA content, NA and tumors nuclear size (Narea, NV) (Fig. 4) direct correlation to the rank P6 is determined (correlation coefficient 0.96-0.98). For cells belonging to the higher ranks, this proportion is violated, and the correlation becomes lower than the values which showed a direct relationship between these parameters. This is due to both insignificant number of such cells in tumors and their significant variability. Such cells have a nucleus with a cross-sectional area of 55 μm^2 and volume over 320 μm^3.

The identified relationship between the nucleic acids content and size of tumor cells nuclei allowed concluding its mathematical equivalent, expressed by the equation:

NDNA = Narea * (0,0482 * lnNarea - k) (3)

Considering the significant differences regarding the RNA content in the tumor cells nuclei with different degree of differentiation, the value of variable k (under the conditions of counting at least 300 tumor cells) is: GN - 0.11, GNB - 0.1 NB - 0.0703. Comparison of the results of the empirical and indirect (calculated) determination of DNA content in NB cells showed their similarity NB - $p < 0.1$ in the range of P1-P3, GNB - $p < 0.05$ in the range P1-P6, NB - $p < 0.05$ in the range P1-P6 (Fig. 5).

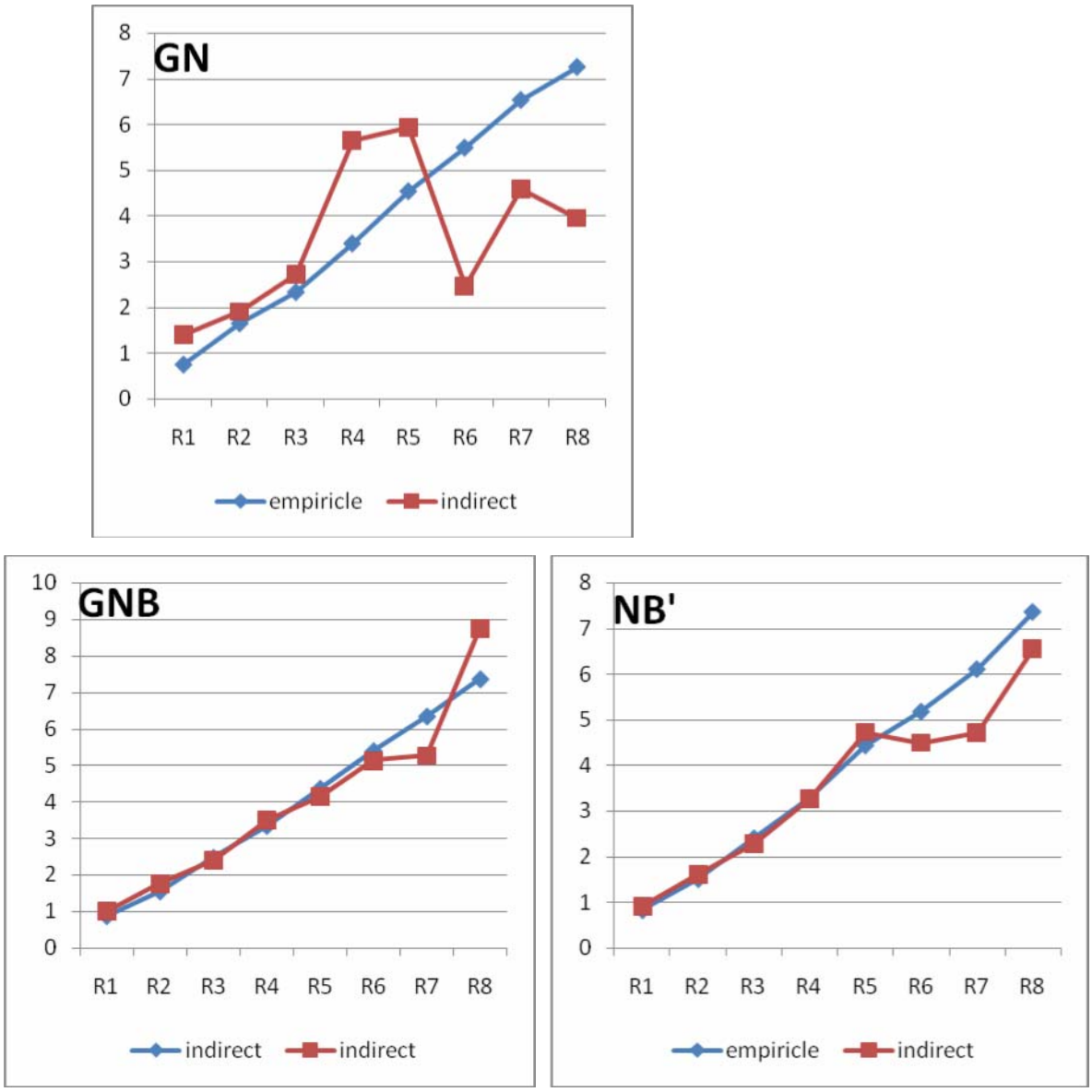


Fig. 5. DNA content in cell nuclei of GN, GNB and NB determined empirically (empir) and indirectly by the formula 3 (indirect).

Thus, studies have shown that different degrees of NB differentiation are heterogeneous. So for GN and GNB which were investigated, are characterized by the presence in their structure a significant proportion of cells in which the nuclei DNA content greater than tetraploid, what is a sign of chromosomal instability [9]. For NB, which is among to the least differentiated studied tumors, only part of the tumor is composed of polyploid cells were also found to be as polyploid. Other NB, respectively, for which there are no signs of chromosomal instability, develops with other pathogenetic mechanisms (microsatellite instability, etc.) [10], or these tumors had narrow cell range due to elimination of morphological and functional cell types with impaired life-support system.

Note that NB cells with DNA content above 4C usually did not reveal signs of degeneration (karyopyknosis, lysis of chromatin), which is quite typical for tumors from other histogenetic sources [15]. Thus an increasing of the RNA content in the nuclei has happened, as the DNA increasing. This can be seen as an indirect sign of genome activity and, therefore, the functional activity of tumor cells in general. This allows the ghost to believe that polyploid NB cells has a sufficient level of activity and life support systems is not eliminated, as is typical for tumors of other histogenetic sources with chromosomal instability [9, 15].

A variety of NB cellular DNA content is an indirect indicator of different properties and viability of tumor cells. Accordingly, it is sufficient reason for inclusion of this criterion to the comprehensive assessment of the properties of the tumor [6]. However, since the DNA content in the nuclei of tumor cells depends not only on violations of their mechanism of separation, but also on the activity of its synthesis, the ploidy and proliferative activity in tumors should be considered as a single variable. Also, in our opinion, it may be informative assessment of proliferative activity in different segments of these NB cell ranges that will point to the morphologic functional cell type that determines the potency of tumor development.

The identified relationship between the size of the nuclei of NB tumors and DNA content and mathematical description of these relationships makes it possible to determine ploidy of NB in routine histological examination, provided estimates of at least 300 cells.

Conclusions

A number of neuroblastomas, with symptoms de-differentiation increasing, the average DNA content in tumor cells is decreased; the cell range is shifted toward cells with low DNA content in the nuclei, leading to decrease of tumors cell heterogeneity.

Increase of RNA content in the nuclei of polyploid neuroblastoma cells suggests a certain degree of preservation of the functional activity of the genome can be used as an indirect indicator of cell viability and selection of morphological and functional types which are able, to some extent, to differentiate.

Based on the presence of the direct relationship between the DNA content in the nucleus of neuroblastoma cells and its size, kariometric indicators can be used to indirectly determine the cells ploidy without using of sophisticated methods of staining that increase objectivity and reliability assessment of tumors in routine histopathological practice.

Reference

1. Luppa H. (1977) Grundlangen der histochemie. Berlin: Akademie-Verlag: – 344 p.
2. Taşcă C. (1976) Introducere in morfologia cantitativa cito-histological. Bucuresti: E.S.R. – 192 p.
3. Ambros P.F., Ambros I.M., Brodeur G.M., et al. (2009) International consensus for neuroblastoma molecular diagnostics: report from the International Neuroblastoma Risk Group (INRG) Biology Committee. British J Cancer.: Vol. 100: 1471-1482.
4. Bagatell R., Beck-Popovich M., London W.B., et al. (2009) Significance of MYCN amplification in international neuroblastoma staging system stage 1 and 2 neuroblastoma: a report from the International Neuroblastoma Risk Group database. J Clin Oncol.: 27 (3): 365-370.
5. Bernardi B.D., Gerrrard M., Boni L. et. al. (2009) Высокие показатели выживаемости при использовании терапии сниженной интенсивности у детей с диссеминированной нейробластомой без амплификации гена MYCN. J Clin Oncol.: 27 (7): 1034-1040.
6. Brodeyr G.M., Fong C.T., Morita M. (1988) Molecular analysis and clinical significance of N-myc amplification and chromosome 1q monosomy in human neuroblastomas. Prog. Clin. Biol. Res.: 271: 3–15.
7. Canete A., Gerrard M., Rubie H. et al. (2009) Poor survival for infants with MYCN-amplified metastatic neuroblastoma despite intensified treatment: the International Society of Pediatric Oncology European neuroblastoma experience. J Clin Oncol.: 27 (7): 1014-1019.
8. Cohn S.L., Pearson A.D., London W.B., et al. (2009) The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. J Clin Oncol.: 27: 289-97.
9. Davoli T., de Lange T. The Causes and Consequences of Polyploidy in Normal Development and Cancer. Annu. Rev. Cell Dev. Biol. – 2011.- V. 27.- P. 585-610.
10. Holland A.J., Cleveland D.W. (2012) Losing balance: the origin and impact of aneuploidy in cancer. / // EMBO.– V. 13, № 6. – P. 501–514.
11. George R.E., London W.B., Cohn S.L., et al. (2005) Hyperdiploidy plus nonamplified MYCN confers a favorable prognosis in children 12 to 18 months old with disseminated neuroblastoma: a pediatric oncology group study. J Clin Oncol.: 23 (27): 6466-6473.
12. Ladenstein R., Ambros I.M.., Potschger U., et al. (2001) Prognostic significance of DNA di-tetraploidy in neuroblastoma. Med Pediatr Oncol.: 36: 83-92.
13. Mora J, Cheung NK, Chen L, et al: (2001) Survival analysis of clinical, pathologic, and genetic features in neuroblastoma presenting as locoregional disease. Cancer 91:435-442,
14. Oppedal BR, Storm-Mathisen I, Lie SO, et al: (1988) Prognostic factors in neuroblastoma:

Clinical, histopathologic, and immunohistochemical features and DNA ploidy in relation to prognosis. Cancer 62:772-780,

15. Park S. U., Choi E. S., Jang Y. S. et al. (2011) Effects of Chromosomal Polyploidy on Survival of Colon Cancer Cells // Korean J Gastroenterol.- Vol. 57.- № 3.- P. 150-157.
16. Schneiderman J., London W.B., Brodeur G.M., et al. (2008) Clinical significance of MYCN amplification and ploidy in favorable-stage neuroblastoma: a report from the Children's Oncology Group. J Clin Oncol.: 26 (6): 913-918.