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# Number of Mitotic Figures in Microscopic Picture of Nephroblastoma Histologic Types – Estimation of the Value of the Oldest Known Prognostic Factor

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**There are two main widely accepted prognostic factors in nephroblastoma group of tumors – histological type of tumor and clinical stage of disease. However nuclear anaplasia, one of the elements of Wilms' tumor microscopic picture was found as a new prognostic marker in this group of tumors. Estimation of nuclear anaplasia is an obligatory procedure in currently used therapeutic protocols. In our work we compared another element of nephroblastoma microscopic picture - number of mitoses as the oldest known indicator of activity of tumor cells with prognostic markers estimated in routine histologic examination according SIOP Protocols (histological risk, nephroblastoma type and the presence of diffuse anaplasia) and with CD44 expression – widely known marker of discussed prognostic value. We found statistically important correlation between number of mitoses and all the examined features. We believe that mitotic figure counting may become in future a helpful tool in the qualification of prognosis for individual patients in doubtful cases.**

## Introduction

Mitotic figures counting in tumor tissue sample is one of the oldest methods of the estimation of the degree of tumor malignancy. However number of visible mitotic figures reflects only to the most shorter part of the proliferating phase in the cell cycle [13]. Utility of the calculation of mitoses in currently done routine histologic examination of malignant tumors in children is an interesting problem and maybe requires standardization. The number of mitotic figures in tumor tissue sample is estimated in fields of the greatest mitotic activity or in fields chosen by chance. Those results are given as the number of mitoses per number of investigated fields, the number of mitoses per number of tumor cells or the number of mitoses per square millimeter. Despite the fact that estimation of mitotic activity is relative to many factors (e.g. preparation and type of tissue sample, type of stain, material thickness), is still helpful in routine examination of malignancy of some tumors e.g. sarcomas. In addition modern immunohistochemistry allows to evaluate the proliferating activity of cells much more precisely by investigation of the expression of the Ki-67 protein and the presences of the PCNA antigen. Whatever is the way of estimation, mitotic activity is very good indicator of the proliferating potential of the tumor cells and one of the widely used prognostic factors [13, 14]. In routine histologic examination of nephroblastoma tissue samples mitotic divisions are typically observed, however in some cases gigantic polypoid, multipolar figures appeared. The presence of those large, atypical mitoses became one of the elements of the diagnosis of nuclear anaplasia. Term 'nuclear anaplasia' and its division into 'focal' and 'diffuse' was used in National Wilms' Tumor Study (NWTS) Protocols and now became widely accepted marker of worse prognosis in nephroblastoma group of tumors. According European SIOP Protocols estimation of the presence of nuclear anaplasia is also an obligatory procedure in routine examination of Wilms' tumor tissue samples [6, 7, 23]. Current data point that nuclear anaplasia is present in 4-5% of nephroblastoma cases and the frequency of anaplasia increases with the patients' age. Nuclear anaplasia is rare among the youngest patients, and is found in 10% of

tumor tissue samples taken from the patients over 6 years of life [5, 10]. The most important morphological expression of anaplasia is the presence of multipolar mitotic figures and large hyperchromatic nuclei (at least of three times greater in every axis from nuclei of blastemal cells). For the recognition of nuclear anaplasia is necessary to find all mentioned above microscopic features, and criteria are identical for cells of every Wilms' tumor components. It is proved that chemotherapy does not call out microscopic features of anaplasia, and only allows to identify its foci originally difficult to observe in the tumor mass. Due to this observation criteria of nuclear anaplasia in preoperatively treated tumors and tumors without primery chemotherapy are the same. The diagnosis of nephroblastoma with anaplasia leads to essential clinical implications – first of all intensification of the treatment [17, 23].

CD44 is one of the particles expressed on the surface of various cells, mostly monocytes, granulocytes, erythrocytes, lymphocytes B, mature lymphocytes T, epithelial cells, fibroblasts and oncocytes. It was proved that CD44 molecule plays an essential part in interactions between cells, and between cells and intercellular matrix, either. High CD44 expression was found in many neoplasms and the presence of CD44 on the surface of tumoral cells was usually connected with the ability for dissemination. However, CD44 particle is included in many other mechanisms e.g: embryonic development, or inflammations and its real function in both physiologic and neoplastic processes remains unknown [24, 25].

## Materials and Methods

39 formalin-fixed and paraffin-embedded nephroblastoma tissue sections from the files of the Department of Pathology of the Age of Development and Department of Pathology Konopnicka Memorial Hospital Medical University of Łódź were selected for our study. From these tissues samples paraffin blocks about the thickness 3-4 of μm were prepared and stained with hematoxylin and eosin (H&E). For the purpose of our study all of the previously diagnosed tumors became reclassified according current criteria for this group (based on SIOP Classification of Renal Tumors of Childhood).

Tumor tissue samples were examined with computer image analysis system (Multi Scan Base v. 8.08 - Computer Scanning System, Ltd.). All microscopic pictures (Nikon Microphot FXA) was transferred to the computer by camera (CC2OP).

We counted the number of mitoses and classified for statistical analysis as: 'low' – less than 20 per 10 high power field (400x, h.p.f.), or 'high' –20 mitotic figures or more per 10 h.p.f. In estimation of nuclear anaplasia we followed SIOP criteria.

We performed immunohistochemical stains with commercial mouse antibody, CD44 (H-CAM), F10-44-2 (Novocastra), and with use of immunoperoxidase reaction according to Hsu (EnVision+the System, Peroxidase - DAB (DAKO).

In research of CD44 expression we accepted cytoplasmic type of the reaction - the brown color of cytoplasm of neoplastic cells and we rated CD44 expression as: weak (less than 10% of positive tumor cells), of intermediate degree (from 10% to 60%) or strong (more than 60%). In every case 1000 of tumor cells were counted.

We compared the number of mitoses with routine histologic examination data (histologic risk, histologic type and the presence of anaplasia) and results of immunohistochemical research – expression of CD44 particle. For the analysis we used the statistical pack SYSTAT for Windows (Version 5.03, SYSTAT, Inc, Evaston, Illinois, USA , the license No: DA021594).

## Results

According to SIOP Classification we diagnosed: 2 low risk, 18 intermediate risk and 19 high risk nephroblastomas. In two cases Wilms' tumors with diffuse anaplasia was diagnosed. The subgroups appeared as follow: cystic partially differentiated neproblastoma - 2 cases ; nephroblastoma epithelial type - 12 cases; nephroblastoma stroma predominance - 2 cases; nephroblastoma mixed type – 4 cases; nephroblastoma blastemal type - 17 cases; nephroblastoma diffuse anaplasia - 2 cases. Details of histologic examination are presented in Table 1.

In evaluation of the prognostic value of microscopic picture elements ( histologic risk, nephroblastoma type, presence of anaplasia and number of mitoses) we found statistically significant correlation between 'high' number of mitoses and the level of histologic risk and with histologic type, in both cases, $p<0.001$. 'High' number of mitoses we observed exclusively in intermediate or high risk nephroblastomas. Those tumors belonged to following subtypes: epithelial type (2 cases), stroma predominance (1 case) and blastemal type (4 cases). In microscopic picture of nephroblastoma with diffuse anaplasia (2 cases) we never observed 'high' number of mitoses, $p<0.001$. CD44 expression was found in 18 cases of investigated nephroblastomas and classified as: strong - 1 case, the intermediate degree - 9 cases, and as weak - 8 cases. We found statistically significant correlation between 'high' number of mitoses and CD44 expression, $p=0.004$. Details of statistic analysis represents Table 2.

**TABLE 1**
Results of histologic examination in nephroblastoma group of tumors

| No | SIOP Classification | Number of mitoses |
|---|---|---|
| **1** | IIIA | 20 mitotic figures or more per 10 h.p.f. |
| **2** | IIB | 20 mitotic figures or more per 10 h.p.f. |
| **3** | IIA | 20 mitotic figures or more per 10 h.p.f. |
| **4** | IIIB | less than 20 per 10 h.p.f. |
| **5** | IIIB | less than 20 per 10 h.p.f. |
| **6** | IIIA | 20 mitotic figures or more per 10 h.p.f. |
| **7** | IIIA | 20 mitotic figures or more per 10 h.p.f. |
| **8** | IIA | 20 mitotic figures or more per 10 h.p.f. |
| **9** | IA | less than 20 per 10 h.p.f. |
| **10** | IA | less than 20 per 10 h.p.f. |
| **11** | IIIA | less than 20 per 10 h.p.f. |
| **12** | IIIA | less than 20 per 10 h.p.f. |
| **13** | IIIA | less than 20 per 10 h.p.f. |
| **14** | IIIA | less than 20 per 10 h.p.f. |
| **15** | IIC | less than 20 per 10 h.p.f. |
| **16** | IIA | 20 mitotic figures or more per 10 h.p.f. |
| **17** | IIIA | less than 20 per 10 h.p.f. |
| **18** | IIIA | less than 20 per 10 h.p.f. |
| **19** | IIC | less than 20 per 10 h.p.f. |
| **20** | IIA | 20 mitotic figures or more per 10 h.p.f. |
| **21** | IIC | less than 20 per 10 h.p.f. |
| **22** | IIIA | less than 20 per 10 h.p.f. |
| **23** | IIA | less than 20 per 10 h.p.f. |
| **24** | IIC | less than 20 per 10 h.p.f. |
| **25** | IIIA | less than 20 per 10 h.p.f. |
| **26** | IIA | less than 20 per 10 h.p.f. |
| **27** | IIIA | 20 mitotic figures or more per 10 h.p.f. |
| **28** | IIA | less than 20 per 10 h.p.f. |
| **29** | IIA | less than 20 per 10 h.p.f. |
| **30** | IIIA | less than 20 per 10 h.p.f. |
| **31** | IIA | less than 20 per 10 h.p.f. |
| **32** | IIA | less than 20 per 10 h.p.f. |
| **33** | IIIA | less than 20 per 10 h.p.f. |
| **34** | IIB | less than 20 per 10 h.p.f. |
| **35** | IIIA | less than 20 per 10 h.p.f. |
| **36** | IIA | less than 20 per 10 h.p.f. |
| **37** | IIA | less than 20 per 10 h.p.f. |
| **38** | IIIA | less than 20 per 10 h.p.f. |
| **39** | IIIA | less than 20 per 10 h.p.f. |

**IA** – cystic partially differentiated nephroblastoma**; IIA** – nephroblastoma - epithelial; **IIB** – nephroblastoma – stroma predominate; **IIC** – nephroblastoma – mixed; **IIIA** – nephroblastoma – blastemal; **IIIB** – nephroblastoma – diffuse anaplasia

**TABLE 2**
Statistically significant correlations in examined Wilms' tumors group

| Feature I | Feature II | *P* | Pearson Chi$^2$ | Spearman Rho |
|---|---|---|---|---|
| 20 or more mitotic figures per 10 h.p.f. | Histologic risk | 0.001 | 38.606 | 0.414 |
| | Histologic type | 0.001 | 43.433 | - |
| | Diffuse anaplasia | -0.001 | 48.455 | 0.568 |
| 20 or more mitotic figures per 10 h.p.f. | CD44 expression | 0.004 | 15.276 | 0.321 |

## Discussion

Modern pediatric oncology based on estimation of chosen independent prognostic markers. Nephroblastoma group of tumors is an excellent example how useful is routine histologic examination. Result of macro- and microscopic examination of Wilms' tumor tissue sample become an independent prognostic factor and the most important marker together with stage of the disease. Except based on histologic examination division into risk groups (which follows examination of nephroblastoma type) microscopic picture of tumor tissue sample is a source of one more information – absence or presence of nuclear anaplasia – new prognostic factor for this group. National Wilms Tumor Study found nuclear anaplasia as an independent prognostic factor [1, 2, 17]. This new microscopic feature was divided into two categories: focal and diffuse. Focal anaplasia is defined as one or few easy to delimited foci visible only within of the primary tumor, without other abnormalities of nuclei in the remaining part of the tumor. Patients with nephroblastoma in II-IV; stage of disease with the presence of focal anaplasia are treated as patients with tumors without anaplasia in the same stage. Diffuse anaplasia does not fulfill criteria mentioned above – it is a presence of anaplasia features in every investigated area of the tumor or foci of anaplasia without distinct separation and with nuclear abnormalities in remaining tumor cells. The presence of diffuse nuclear anaplasia makes the prognosis worse. According NWTS research among patients in IV stage of the disease with features of focal anaplasia in microscopic examination of tumor tissue sample deaths or relapses were never observed, and all the metastases underwent chemotheraphy. Opposite, 96% of patients in the same stage but with diffuse anaplasia died without any positive results of the performed treatment [4, 10, 11, 12]. In Europe SIOP Protocols point that the diagnosis of nuclear anaplasia automatically includes the tumor into high risk group and leads to the intensifications of the treatment [8, 13, 20].

In current pediatric oncology there is no widely accepted standardization of the estimation of mitotic activity of tumor cells – one of the oldest, however easy to estimate feature of tumors microscopic picture. The number of figures is examined in fields of the greatest mitotic activity or in accidental areas. Results of investigation are described as the number of mitoses per number the field of vision, per number of tumor cells or per square millimeter. Despite those differences calculation of mitotic figures appear simple and very useful diagnostic method and in some group of tumors e.g. sarcomas method of the greatest importance.

Modern immunohistochemistry resolved the main problem of quantification cells activity due to investigation of the expression of Ki-67 antibody and the presences of PCNA antigen, much more useful for the estimation of proliferation potential than mitotic figure counting. It is necessary to point that every information of cells activity may be one of the factors included in the investigation of the degree of the malice of tumors (and at the same in planning treatment) in all those cases where doubts appear in prognoses estimated by using widely accepted risk factors [9, 14, 21, 22]. In our opinion it is useful to search for correlation between already known prognostic factors and sometimes forgotten elements of microscopic picture of tumor tissue samples [4, 15, 18, 19]. Investigation of mitotic activity in our research brought some new information about nephroblastoma biology. We found the correlation between high number of mitoses and both, histologic risk and histologic type in nephroblastoma group of tumors. High number of mitoses appeared exclusively in intermediate and high risk Wilms' tumors. The absences of multiple mitoses in anaplastic nephroblastomas needs further explanations. Maybe in this most aggressive tumors group 'the quality' of mitoses (multipolar, polypoid) is much more important for tumor biology than their number. It is known from some type of neoplasms biology that high number of mitoses is not always connected with malignancy when mitotic figures are 'normal'. Surprising result of our study was the presence of correlation between number of mitoses and CD44 expression. Mechanisms of CD44 function still remain unknown. It was proved that CD44 expression

permits tumoral cells to metastasize e.g, in stomach, colon and prostate cancers, however according our knowledge there is no widely accepted data about its part in cell cycle. The process of alternative RNA splicing and modifications of CD44 protein lead to formation of variants with different adhesive proprieties. Till now 30 CD44 isoforms were identified and maybe some of them are included in progression of neoplastic disease and others in cell division mechanisms [16, 26].

We believe that correlations between mitotic activity of tumor cells and widely accepted prognostic factors in nephroblastoma and maybe in other childish malignancies needs further investigations and that standardized estimation of the number of mitotic figures in nephroblastoma tissue sample may be taken into consideration as a helpful tool in estimation of the prognosis in individual doubtful cases.

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