Rīgas Stradiņa universitāte

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MORPHOLOGICAL, IMMUNOHISTOCHEMICAL AND GENETIC CHARACTERISTICS OF PAEDIATRIC RENAL TUMOURS IN LATVIA

Summary of the Doctoral Thesis

Specialty – Pathology

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The Doctoral thesis was developed at the Department of Pathology of Rīga Stradiņš University and Department of Pathology of Children's Clinical University Hospital

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ABBREVIATIONS USED IN THE PAPER

A - adenosine
CCUH - Children's Clinical University Hospital
Bcl-2 - bcl-2 (B-cell chronic lymphocytic Leukaemia) gene encoded protein
Bp - base pair
CDKN2A - cyclin-dependent kinase inhibitor 2A gene
CK - cytokeratin
CKAE1/AE3 - common cytokeratin (clone CKAE1/AE3)
CI - confidence interval
CMN - cellular mesoblastic nephroma
DMSO - dimethylsulfoxide
DNA - deoxyribonucleic acid
dNTP - deoxyribonucleotide triphosphate
DSRCT - desmoplastic small round cell tumour
EDTA - ethylenediaminetetraacetic acid (chemical formula $C_{10}H_{16}N_2O_8$)
EMA - epithelial membrane antigen
ETV6 - E-twenty six.variant 6 gene
g - gravitational acceleration ~ 9, 81 m/s2
G - guanine
H&E - haematoxylin eosin
LCA - common leukocyte antigen
NSE - neuron-specific enolase
NWTSG - National Wilms Tumour Study Group
M - the arithmetic mean of the variable
Max - the maximum value of the variable range
Min - the minimum value of the variable range
Mo - mode, the most frequent value of the variable
n - sample size (number of cases)
RCC - Renal Cell Carcinoma
nt - nucleotide
NTRK3 - neurotrophic tyrosine kinase, receptor type 3 gene
p - significance level (the probability that the null hypothesis is valid)
PCR - polymerase chain reaction
PI - proliferation index
PNET - primitive neuroectodermal tumour

r - Spearman rank correlation coefficient
SD - standard deviation
SIOP - International Society of Paediatric Oncology
Taq polymerase - thermostable thermophilic bacteria Therma aquaticus DNSpolimerase
TBS - buffered tris(hydroxymethyl)aminomethane solution
Tris - Tris (hydroxymethyl)) aminomethane (chemical formula (HOCH2) 3CNH2)
WT1 - Wilms tumour suppressor gene

1. INTRODUCTION

1.1. Topicality of the Study

The primary kidney tumours represented approximately 7% of all paediatric malignancies in the age group up to 15 years and 4.4% of all cancers in children and adults under the age of 20 years [Ries et al., 1999]. The most frequently represented in this group is the Wilms tumour or nephroblastoma (85% of the cases), followed by renal cell carcinoma (3-5%), mesoblastic nephroma (3%), renal clear cell sarcoma, rhabdoid renal tumour (2%) and rarely mixed genesis tumours (2%) [Birch et al., 1995]. Due to the relatively rare occurrence of the mentioned pathology, variable histology findings and the possibility that the histological pattern may overlap in the case of different neoplastic disorders of the group, paediatric kidney tumours have always been in the focus of interest for paediatric pathologists. Increased interest is paid to accurate histological diagnosis and tumour stage detection of paediatric kidney tumours, because the treatment and prognosis of patients significantly differ depending on the morphological type of tumour diagnosed. Globally their research is carried out within the framework of large research groups, the most significant of which are the National Wilms Tumour Study Group / NWTSG in the USA and International Society of Paediatric Oncology / SIOP Study Group in Europe. Over the past 40 years, the work of these cooperative groups in developing accurate diagnostic criteria on tumour stage and histology-based chemotherapy protocols, as well as surgical technique approaches, has been very successful. In general, as a result a significant prognostic improvement for nephroblastoma patients has been achieved. If at the beginning of the 20th century, the five-year survival rate after being diagnosed with nephroblastoma was observed in 8% of patients, in 1960 this rate already reached 50%, whereas in 2000 this rate for nephroblastoma patients increased already up to 90% [Perlman, 2005]. Similarly, in case of renal clear cell sarcoma, when initiating doxorubicin application, according to the chemotherapy protocols, the patient five-year survival rate increased from 20% to 70% [Argani et al, 2000], whereas in the cases of the rhabdoid renal tumour the prognosis remains poor, because in 80% of these tumour cases the patients die within the first two years after diagnosis [van den Heuvel-Eibrink et al., 2011]. Against the background of overall success the question of why, despite of the administered chemotherapy and surgery, poor prognosis remains unaltered for part of the

nephroblastoma patients and still up to 15% of patients die within five years of being diagnosed, remains unanswered [Malik et al., 2001]. Discussions are also initiated by the observed variety of tumour reactivity to the administered chemotherapy and a view is expressed that the blastemal component of nephroblastoma possibly consists of two types of cells with different reactivity to the administered treatment [Barocca, 2010]. In view of the mentioned above, also at present the research of paediatric kidney tumours including nephroblastomas is continued all over the world with the focus on molecular genetic abnormalities, potentially prognostically important immunohistochemical marker- and chemotherapy-induced alterations in tumours and at present a range of markers, the immunohistochemical expression level of which could relate to patient prognosis has been described [Ghanem et al., 2005].

The analysis of literature data indicates that basic research in paediatric kidney tumours is mainly carried out within NWTSG and SIOP multinational research groups, however literature on the morphologic spectrum of paediatric kidney tumour and immunohistochemical findings of these abnormalities in the Baltic States is practically unavailable. Separate local studies have been conducted in Lithuania [Jankauskiene et al., 2009]. In Latvia insight into paediatric genitourinary tumour classification is provided by the monograph published 'Bērnu uroloģija' [Paediatric Urology'] [Dobelis, 2003] and the book 'Bērnu ķirurģija' [Paediatric Surgery] [Krasts, 2005] Eds. Prof. A. Pētersons. The diagnostics and treatment of the paediatric kidney tumours in Latvia is concentrated mainly in the Children's Clinical University Hospital (CCUH). Histological tumour diagnosis is carried out in the CCUH Department for Paediatric Pathology. Starting from 2004 the immunohistochemical examination method has also been applied in practice, however so far the analysis of the incidence of primary and metastatic paediatric kidney neoplasms, their morphological spectrum and accompanying genetic pathologies, as well as the clinical, morphological and immunohistochemical data relation analysis, which could present a significant contribution to paediatric kidney tumour research has not been performed. All the above facts determine the relevance, the fundamental importance and practical value of the Promotional Thesis.

1.2. The aim of the study

To carry out research of the paediatric kidney tumour morphological spectrum of Latvia and characterize potentially important prognostic immunohistochemical marker expression, its relationship to clinical and morphological parameters, as well as genetic abnormalities in nephroblastoma cases.

1.3. The objectives of the study

1. To determine the morphologic spectrum of paediatric kidney tumours in Latvia on the basis of CCUH Department of Pathology archive data on histology results and paediatric renal tumour biopsy and surgical material repeated diagnostic histological and immunohistochemical findings.

2. To determine $p16^{INK4a}$, CD34, p53, Ki67, CKAE1/AE3, CD44, e-cadherin marker immunohistochemical expression mutual correlation and association with tumour stage, histological type, histological malignancy level, tumour progression, outcome of the disease and preoperative chemotherapy in the diagnosed nephroblastoma cases.

3. To develop recommendations for immunohistochemical diagnostics according to the data obtained.

4. To identify the alterations of the tumour suppression gene CDKN2A/$p16^{INK4a}$ present in the 9p21 chromosome segment in the cases of nephroblastoma in comparison to healthy subjects.

5. To carry out correlation analysis of alterations in the tumour suppressor gene CDKN2A/$p16^{INK4a}$ and gene encoded protein $p16^{INK4a}$ immunohistochemical expression.

1.4. Hypotheses of the study

1. The morphological range of paediatric kidney tumours in Latvia may differ from the data obtained in other regions.

2. Immunohistochemical examination is essential in the diagnostics of paediatric kidney tumours.

3. There is a statistically significant relationship between the WT1 protein, $p16^{INK4a}$ protein, CD34, p53 protein, Ki67, CKAE1/AE3, CD44, e-cadherin immunohistochemical expression and the malignancy potential of the nephroblastoma.

4. Alterations in the tumour suppressor gene CDKN2A/ $p16^{INK4a}$ localized in the 9p21 chromosome segment have an important role in the generation of nephroblastoma.

1.5. Scientific novelty of the study

1. For the first time, data on the morphological spectrum of paediatric renal cancer in Latvia have been summarized; this is also the first study of this type in the Baltic States.

2. For the first time a wide range of potentially prognostically important immunohistochemical markers in case of nephroblastoma has been determined, the mutual correlation of the expression of all these markers has been analysed, as well as its correlation with clinical and histopathological parameters.

3. The research of the tumour suppressor gene CDKN2A/ $p16^{INK4a}$ in nephroblastoma tissues is to be considered a novelty, in the internationally quoted medical literature only a few publications devoted to this theme may be detected.

1.6. Practical significance of the study

1. The data obtained are important for the research of molecular alterations of nephroblastoma, as well as the research of prognostically important immunohistochemical markers and may be used as the basis for future international studies.

2. Based on immunohistochemically verified paediatric kidney tumour morphological spectrum analysis practical recommendations for the organisation of the work of a histology laboratory for the diagnostics of blastemal type nephroblastoma, rhabdoid kidney tumour and mesenchymal genesis paediatric kidney tumours have been developed.

1.7. The structure of study

The promotional thesis is written in Latvian. It has a classical structure. The paper includes an introduction, a literature review, an overview on materials and methods, results, discussion, conclusions, practical recommendations and the list of literature. The promotional thesis has been written on 181 pages, it contains 71 tables and 97 figures. 293 literature sources have been used in the paper.

2. MATERIALS AND METHODS

2.1. The design of the study

The promotional work has been done at basis of Children's Clinical University Hospital Paediatric Pathology Department. The target population of the study were children and young people aged from the moment of birth to 18 years with surgically treated primary kidney tumours, or their metastases in CCUH. Analysed tumours were diagnosed during the period from January 1997 to July 2012. The tissue material from the tumours, the morphological diagnoses determined in the CCUH Paediatric Pathology Department and the patients' clinical medical record data (including identity) have been obtained from the CCUH archive. Initially, for the detection of the morphological spectrum of primary kidney tumours and establishing of a research group for advanced nephroblastoma studies, a review of all the examined tumour material H&E (hematoxylin-eosin) microspecimens and immunohistochemical verification of the diagnosis carried out in the CCUH Paediatric Pathology Department was done. For diagnostic purposes immunohistochemical examination of 31 tumour or 63.26% of all diagnosed formations was carried out. The expression of potentially prognostically significant immunohisto-chemical markers in the case of primary nephroblastoma has been analysed for 26 primary nephroblastoma, 8 remote metastases and one recurrence tissue samples. In the case of primary tumours research has been carried out by grouping formations depending on the stage, histological type, histological grade of malignancy, chemotherapy administered in the preoperative period and clinically observed responses to the mentioned treatment. Separately the group of deceased and surviving patients as well as patients in whom progression of malignancy process was observed on the background of the administered therapy has been analysed. The expression of immunohistochemical markers in the group of primary nephroblastomas and metastases/recurrence group has been mutually compared as well. $P16^{INK4a}$ expression both in the cases of primary and metastatic tumours has also been analysed according to the alterations of tumour suppressor gene $CDKN2A/p16^{INK4a}$ detected in DNA sequencing. Research of genetic alterations of the $CDKN2a/p16^{INK4a}$ locus of 9p21 chromosome was carried out also in the Biomedical Research and Study Centre of the University of Latvia in cooperation with the Doctor of Biology D.Pjanova. In the case of genetically

analysed nephroblastomas DNA was isolated from paraffin embedded tissues, DNA amplification and sequencing was performed by the PCR (polymerase chain reaction) method. DNA isolation from paraffin embedded tissue samples, the subsequent propagation and sequencing for the research of 1α and 2 exon alterations was carried out for 14 tissue samples in total. In 11 cases (78.57%) primary tumour material, in 2 cases (14.29%) lung metastases, whereas in one case (7.14%) recurrence tissues have been used. In addition, microsatellite marker analysis of the mentioned locus has been carried out in 14 primary tumour specimens. In the paper only the material used for diagnostic needs from Children's Clinical University Hospital was analysed. All the provisions related to the Helsinki agreement have been observed. The permit from Riga Stradiņš University Ethics Commission has been obtained (decision nr. E-9 (2), 24.11.2011).

2.2. The immunohistochemical examination of the analysed material

2.2.1. Histological and immunohistochemical tissue processing methods

All tissue samples for at least 24 hours were fixed in 10% buffered formalin and, after rinsing the tissue samples in running water for 1 hour and 24 hours of dehydration in 96% alcohol solution, embedded in paraplast blocks (Diapath S.r.l. Bergamo, Italy). For the examination of tumours both in the H&E preparations and for immunohistochemical visualization 3 μM thick sections were obtained. The sections were exposed to antigen structural retrieval in the microwave for 15 minutes 97^0 C in Tris/ EDTA buffer solution at pH 9.0 (Target Retrieval Solution, pH 9, Dako, Denmark) and cooled for 20 minutes to the temperature of 65^0 C. After subsequent rinsing in TBS buffer (0.05 mol/L Tris-HCl, 0.15 mol /L NaCl, pH 7,6, Dako, Denmark), endogenous peroxidase blocking incubating tissue in 3% hydrogen peroxide liquid for 5 minutes was carried out. For diagnostic purposes primary antibodies have been used for detection of WT1 protein, vimentin, CKAE1/AE3, EMA, Ki67, bcl-2, actin, desmine, CK7, LCA, CD99, NSE, chromogranin, synaptophisin, S100, myoglobin, MyoD1 miogenin MyoD1, CD34 antigen (all antibodies Dako, Denmark) and INI1 protein detection (Santa Cruz Biotechnology, USA). The expression of potentially prognostically important immunohistochemical markers has been analysed using antibodies for WT1 protein CKAE1/AE3, p53, e-cadherin, Ki67 and CD34, CD44s (all antibodies Dako, Denmark) and

$p16^{INK4a}$ protein antigen (Abcam, Cambridge, MA, USA) detection. Immunohistochemical staining was performed manually, by using polymer conjugate system EnVision for further visualization. On all stages adequate positive and negative control responses have been used.

2.2.2. Assessment methodology for immunohistochemical responses in the group of the analysed nephroblastomas

All the researched material has been examined with the light microscope. During the analysis of CD44s, Ki67, p53 and $p16^{INK4a}$ protein expression responses positivity has been independently evaluated in all three components of the tumour, stromal, epithelial and blastemal visual fields with the maximum visual response intensity. The e-cadherin, WT1 protein, CKAE1/AE3 and CD34 expression was evaluated in the visual fields with the maximum visual response intensity, regardless of the histological tumour component, in which the mentioned visual field is located. During the analysis of CD44s expression, membranous response is considered to be positive. In the stromal and blastemal component CD44s expression has been evaluated by counting positive cells per visual field (magnification x400) and expressing it as a percentage of positive responder cells over all the cells located in the visual field. The intensity of the mentioned above immunohistochemical response in the epithelial component has been determined by counting CD44s positive epithelial structures per visual field (magnification x100). P53 protein, Ki67 and $p16^{INK4a}$ protein positivity has been evaluated by counting the positive cells per visual field (magnification x400) and expressing it as a percentage of positive responder cells over all the cells located in the visual field. In assessing the response for determining of p53, Ki67, and $p16^{INK4a}$ protein antigen, response in tumour cell nuclei was considered positive. CD34 expression has been determined by counting the number of the vascular structures with the positivity of the mentioned marker in endothelial cells per one visual field (magnification x100). The e-cadherin expression has been determined by counting the number of positive structures per visual field (magnification x100). Membranous response is considered too be positive. WT1 protein expression has been determined by counting the positive cells per one visual field (magnification ×400) and expressed as a percentage of positive responder cells over all the cells existing in the given visual field. The response in the nuclei of tumour cells is considered to be positive. The number of epithelial structures was determined per visual field (magnification ×100) with the largest

response intensity. The epithelial structures are visualized immunohistochemically identifying the antigen CKAE1/AE3. Tubular and glomerular structures with positive cytokeratin expression in tumour cell cytoplasm are considered to be epithelial components of full value.

2.3. Methodology for determinations of the tumour suppressor gene CDKN2A/p16^{INK4a} genetic aberrations in nephroblastoma tissue

2.3.1. DNA extraction from paraffin embedded tissues

DNA extraction from paraffin embedded tissues was carried out applying methodology, described in 1999 by the American scientist M. D. Mailman [Mailman et al., 1999], introducing only minor changes to it. In particular, for the examination of tumour DNA from paraffin-embedded material 10 mm thick sections were obtained, which were inserted into 1.5 mL microcentrifuge tube. The prepared tissue sections were depharaffinized by immersing them into 1 ml of xylene, incubating for 10 minutes at room temperature and centrifuging them for 5 minutes at 13000g.This process was repeated twice. Afterwards gradual rehydration of tissue by repeated centrifugation in 1 mL 100% (twice), 80% and 50% ethanol liquid was carried out. In order to complete the rehydration 1 ml of water was added and the tube was placed into the refrigerator for 24 hours at the temperature of 40^0 C. Cell lysis was carried out by heating the material to room temperature, centrifuged for 5 minutes at 13000g, replacing the water with 700μL nuclei lysis buffer solution (10 mM Tris, 400 mM NaCl, 2 mM Na2EDTA, pH=8.4), and mixing of the tube contents. In order to carry out the degradation of membranes and proteins, 70 μL proteinase K (1 mg/ml) and 30 μL 200 g/L sodium dodecyl sulphate solution was added to the tissue samples. The tube contents was mixed and incubated at 44^0C for 24 hours. Further DNA extraction had already been carried out using standard phenol-chloroform DNA extraction method. Tissue samples were incubated for deproteinisation during 10 minutes at room temperature in 700 μL phenol solution and afterwards centrifuged for 10 minutes at 13000g. Separated, DNA-containing aqueous phase was inserted into a new microcentrifuge tube, 700 μL chloroform solution was added to it and the material was centrifuged for 5 minutes. After centrifugation the created bottom layer was separated from the examined material at 13000g. This procedure was repeated twice. For DNA precipitation, 700 μL 96% ethanol was

added to the material, and it was incubated at the temperature of 20^0C until the next morning. In order to obtain DNA the tissue samples were centrifuged for 10 min at 14000g. The resulting liquid was separated, whereas the DNA-containing sediment was rinsed in 1mL of 75% ethanol. The rinsing was repeated twice. After that the residue was dried at room temperature and dissolved in TBE buffer solution. The concentration of the obtained DNA was determined spectrophotometrically by NanoDrop ND 1000 (NanoDrop Technologies).

2.3.2. Amplification of the CDKN2A/p16^{INK4a} gene by application of the polymerase chain reaction

In Polymerase chain reaction (PCR) two axons of CDKN2A/ p16^{INK4a} gene (1α (126 nt) and 2 (307 nt)) were propagated.The response was carried out in 25 μL volume containing 2.5 μL 10xPCR buffer, 3 μL of dNTP (200 μM each deoxyribonucleotide), 1.5 μL MgCl2 (25 mM) 4μM forwarded primer, 4μM reverse primer, 1.25 μL of dimethyl sulfoxide (DMSO), 0.25 U Taq polymerase and 50 ng genomic DNA (all reagents from MBI Fermentas, Lithuania). Polymerase chain reaction conditions were as follows: initial DNA denaturisation at the temperature of 95^0 C for 10 minutes, followed by 35 cycles consisting of a denaturisation step at 95^0 C for 30 seconds, primer binding at 55^0 C for 30 seconds, and synthesis at72^0C for 1 minute followed by the final phase of synthesis for 7 min at the temperature of 72^0 C.The list of primers used in the study has been previously described by D. Pjanova [Pjanova et al., 2007]. All primers synthesized in Metabion International GA.

To verify the existence of the product obtained from PCR the material was analysed in 1.5% agarose gel electrophoresis and visualized with the help of ethidium bromide (1μg/ml).

2.3.3. Sequencing of CDKN2A/ p16^{INK4a} gene

Sequencing of DNA was performed using the reagent kit ABI PRISM Dye Terminator Cycle Sequencing Kit according to the manufacturer's guidelines. The conditions of PCR sequencing were as follows: 25 cycles were performed, with each cycle consisting of denaturation the temperature of 94^0 C for 30 seconds, binding of primers at the temperature of 53^0 C for 30 seconds and synthesis at the temperature of 60^0 C for 4 minutes. Afterwards samples

were purified by 3M sodium acetate and ethanol precipitation. The sequenced samples were analysed by genetic analyser 3100 (Applied Biosystems).

2.3.4. The analysis of the loss of heterozygosity in CDKN2A locus using microsatellite markers

Using primers available in the Gene Bank and synthesized by Applied Biosystems from the tumour and the patient's own tissue not affected by tumour seven (D9S942, D9S1604, D9S974, D9S1748, D9S1870, D9S171 and D9S736) CDKN2A locus microsatellite markers were amplyfied. In each set of primers used one primer was labelled with a fluorescent dye 5 at the 'end (PET, 6-FAM, VIC, and NED). The location of the applied microsatellite markers in the CDKN2A locus is shown in the Fig.2.1.

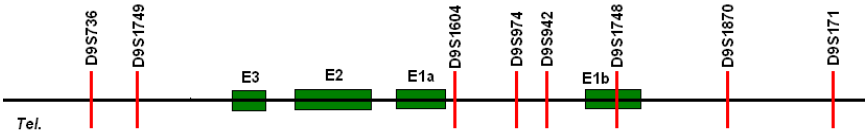


Fig. 2.1 The structure of the CDKN2A locus in schematic representation with presented location of the analysed microsatellite markers.

Reaction was performed in a 15 µL volume, containing 1.5 µL 10xPCR buffer, 2.5 µL MgCl2 (25 mM), 1.5 µL dNTP (200 µM each deoxynucleotide), 1 µL primer mix, 0.15 U Taq polymerase and 50 ng genomic DNA. Amplyfication conditions were as follows: initial denaturation of the material for 10 minutes, followed by 35 cycles of response, with each cycle consisting of a denaturing step at 95^0 C for 30 seconds, primer binding at 55^0 C (D9S942, D9S1604, D9S974, D9S1748, D9S7369) or at 50^0 C (D9S1870),) or at 50^0 C (D9S1870), or at 58^0 C (D9S171) for 30 seconds, and synthesis at the temperature of 72^0 C for 1 min. The procedure was completed with the final extension for 7 min phase of 7 min at the temperature of 72^0 C.The product obtained as a result of PCR was analysed by the genetic analyser 3100 (Applied Biosystems, England).

2.4. Statistical methods of data processing

The data were analysed on a computer program Microsoft Excel and the software package SPSS (Statistical Package for the Social Sciences) version 17, standard descriptive statistical methods were applied in their processing. The expression of potentially prognostically important immunohistochemical markers in primary nephroblastoma groups, as well as metastasis/recurrence group has been characterized by determining the arithmetic mean for each variable, the standard deviation of the variable, the confidence interval of the arithmetic mean and the variable range. The obtained data were examined using the Kolmogorov-Smirnov test for normal distribution. Distribution of variables did not correspond to the normal; therefore in further analysis non-parametric statistical data processing methods were used. For the comparison of the expression of immunohistochemical markers between primary tumour and metastasis/recurrence groups, as well as between different primary tumour groups Mann-Whitney test was used. Significance level (p), which was less than or equal to 0.05 was considered to be statistically significant. In order to analyse the intensity of immunohistochemical responses, depending on the clinical stage of the tumour, the data were divided into two groups, respectively, low-stage (stage I and II) and high stage (III, IV and V stage) tumour groups were compared. Immunohistochemical marker expression in low and medium malignancy stage tumour group was compared with high malignancy level tumours. When analysing the response intensity in cases of different histological types, the marker immunohistochemical expression for each of the types was compared to all other types of material. Analysing the data obtained in the examination of the primary tumour group in order to determine the mutual relationship between the marker immunohistochemical expression Spearman nonparametric correlation analysis was used. To evaluate the role of the alterations in nephroblastoma development detected in the CDKN2A/$p16^{INK4a}$ a gene analysis, their incidence in the patient group was compared to the incidence in the control group using Fisher's test. The control group consisted of 203 practically healthy participants form the State genome database for whom the analysis of the CDKN2A gene has been described before [Pjanova et al., 2007].

3. RESULTS

3.1. Description of the morphological spectrum of paediatric kidney tumours in Latvia

3.1.1. The morphological characteristics of the spectrum of primary kidney tumours according to the initial histological examination data

Due to clinically diagnosed primary renal tumours during the period from January 1997 to July 2012 50 nephrectomies and 3 partial kidney resections were performed in CCUH. The tumour diagnosis was confirmed morphologically in 49 or 92.45% of the examined cases. The breakdown of tumour diagnosis is shown in the Figure 3.1.

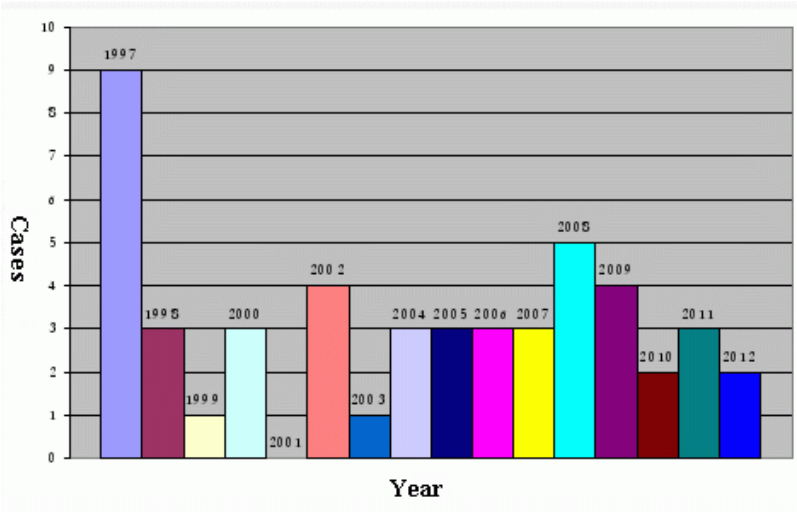


Fig. 3.1 Number of primary kidney tumours in CCUH (from January 1997 to July 2012)

When analysing histological diagnosis available in the CCUH Paediatric Pathology Office, it was detected that the following histological tumour types were diagnosed in morphological examination: 1) nephroblastoma - 77.55% (n=38), 2) clear cell sarcoma - 2.04% (n=1),

3) rhabdoid tumour – 4.08% (n=2), 4) angiomiolipoma – 4.08% (n=2), 5) embryonal rhabdomyosarcoma - 2.04% (n=1), 6) mesoblastic nephroma - 4.08% (n=2), 7) multicystic nephroma - 4.08% (n=2), 8) angiosarcoma - 2.04% (n=1). In the nephroblastoma group, 34.21% (n=13) consisted of blastemal type tumours, whereas 23.7% (n=9), were mixed, 21.05% (n=8), epithelial, 10.52% (n=4) stromal, 7.89% (n=3) regressive type tumours. In 2.63% of the cases (n=1) cystic nephroblastoma was detected. Anaplasia was not found in any of the primary nephroblastoma. The results obtained in the morphological examination performed in the CCUH Pathology Department are displayed in Figure 3.2.

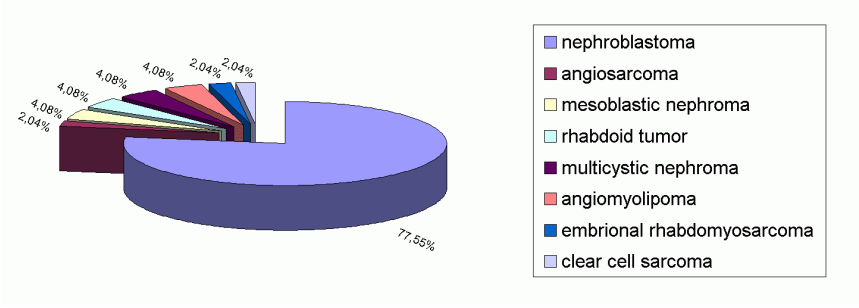


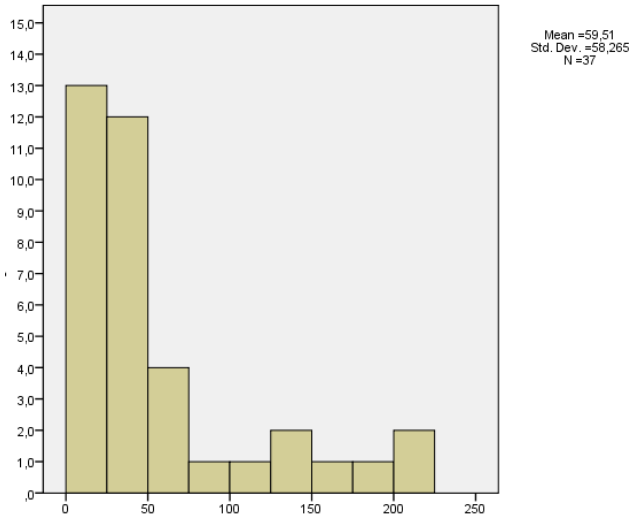
Fig. 3.2 Morphological distribution of tumours according to the data of the examination performed at the CCUH Paediatric Pathology Department (from January, 1997 to July 2012).

Over the analysed period the remote metastase material from 9 primary renal tumours and the diagnosis of metastatic process was confirmed in 6 patients who had already undergone resection of the tumour and subsequent chemotherapy. Histologically in one case rhabdoid tumour metastasis were detected, in other cases, nephroblastoma was primarily detected. In 5 cases metastasis were localized in the lungs, in 1 case intraperitoneally, in 1 case in the upper pole of the spleen and intraperitoneally, in 1 case in the diaphragm dome. Recurrence material was histologically examined also in two primary kidney tumours.

3.1.2. The primary kidney tumour morphological spectrum characteristics according to repeated morphological and immunohistochemical examination data

In repeated H & E preparation and immunohistochemical examination 37 of the 38 nephroblastoma diagnoses were approved. One of the cases was interpreted as neuroblastoma. Histologically the tumour cells were small, monomorph with a round, basophile nucleus and negligible cytoplasm. Isolated Homer Wright rosettes were detected. In immunohistochemical examination completely negative response in WT1 antigen detection was established, tumour tissue had no EMA and CKAE1/AE3 positive epithelial structures, but neuronal marker NSE, synaptophisin and chrormogranin expression was positive. Other paediatric small round cell tumours markers as LCA, desmine, vimentin, muscle-specific actin, myoglobin, miogenin and CD99 were negative. Using Ki67 antigen identification the proliferation index was 40%. According to histological and immunohistochemical pattern, neuroblastoma was diagnosed and the tumour was excluded from the examined nephroblastoma groups.

Separate analysis after repeated examination in the newly created nephroblastoma group demonstrates that 20 out of 37 or 54.05% of the patients were boys, and 17 or 45.95% were girls. The age of the patients ranged from 8 months to 17 years 11 months (M 58.29 months, SD 58.26, mode 36 months). 70.27% (n=26) were patients younger than 4 years, while 86.48% (n=32) pertained to the age group under the age of 10 years (see figure 3.3).



Vertically - No. of cases, horizontally – Age (in months)

Fig. 3.3 The distribution of nephroblastoma patients according to age at the time of diagnosis.

The breakdown of the diagnosed nephroblastomas per annum was as follows: in 1997 seven cases were detected, three in 1998, one in 1999, three in 2000, none in 2001, one in 2003, one in 2002, three in 2004, three in 2005, none in 2006, one in 2007, four in 2008, four in 2009, two in 2010, three in 2011 and two tumour cases in 2012 (until July). When re-defining histological subtypes according to the currently used SIOP 2001 classification [Vujanic et al., 2002] the nephroblastoma breakdown detected was as follows: blastemal type nephroblastomas comprised 32.43% (n=12) of the analysed nephroblastoma, epithelial - 24.32% (n=9), mixed - 18.925 % (n=7), mesenchymal type - 8.1% (n=3), regressive type - 13.51% (n=5), cystic partially differentiated nephroblastoma - 2.7% (n=1). Anaplasia has not been detected in any of the primary tumour tissues. Diffuse anaplasia was detected only in one case, in blastemal type nephroblastoma recurrence tissue. Breakdown by stage at the time of the detection of the primary process was as follows: first stage tumours detected in 8.11% (n=3) cases, whereas second stage tumours detected in 45.95% (n=17) cases, third stage tumours in 21, 62% (n=8) cases, fourth stage tumours in 13.51% (n=5) cases, fifth stage tumours in

2.7% (n=1) cases. For three or 8.11% of the diagnosed nephroblastomas the exact tumour stage could not be determined. In the course of the study (the most recent data verification was carried out on July 10, 2012), 86.49% (n=32) nephroblastoma patients were alive, 10.81% (n=4) had passed away, whereas it was not possible to obtain accurate data on patient survival in 2.7% (n=1) cases. During the period from 1997 to 2007 twenty-three tumours were diagnosed 17,39% (n=4) of the patients had passed away, accurate data on the survival of the patients was impossible to obtain in 4.35% (n=1) cases, therefore, the five-year survival rate has been detected in 78.26% (n=18) cases.

Over the analysed period two rhabdoid kidney tumours were detected in morphological examination performed by the Pathology Department of CCUH. The patients were an 18 months old boy and a 27 months old girl. In one case, the diagnosis did not present difficulties in terms of differential diagnostics; in the second case the histological picture after the primary process tissue examination indicated a blastemal type nephroblastoma. The diagnosis was altered in the Pathology Department after morphological examination of lung metastasis tissue. In both cases, in order to approve the diagnosis, repeated immunohistochemical examination was carried out, during which vimentin positivity, EMA and CKAE/AE3 positivity (see figure 3.4, page 40) was detected, INI1 protein immunohistochemical expression was also determined and a negative response in the nuclei of tumour cells was detected, while the response with preserved renal tissue was positive (see Figure 3.5, page 40) corresponding to the kidney tumour rhabdoid immunoprophile [Coffin et al., 2006].

Clinically, the aggressive course typical for the mentioned formation was observed in both patients. One of the patients died 2 months, and the other 9 months after the excision of the primary tumour.

During the given period, also such a rare paediatric primary kidney tumour as clear cell sarcoma was diagnosed. The patient was an 8 months old boy. During the operation, the metastatic process was detected in the liver tissue. Immunohistochemically vimentin and bcl-2 cytoplasmic positivity was detected, while the response to determining of WT1 protein, CD34, muscle-specific actin EMA, desmin CKAE1/AE3, LCA was negative. The Ki67 proliferation index was 30%. The detected histological picture and immunohistochemical profile was consistent with the findings described in literature in the case of renal clear cell sarcoma (Argani 2004.). Currently, 4 years after being diagnosed, tumour remission has been detected.

The revision of histological material has been carried out also for the case of embryonal rhabdomyosarcoma diagnosis in the primary morphological examination. The patient was a 25 day-old girl with congenital, focally cystic macroscopically yellow kidney tumour. In H&E slides a high cellularity tumour consisting of round, spindle-like cells similar to myofibroblasts infiltrating renal tissue was detected. During repeated immunohistochemical examination vimentin positivity was detected. Suspecting embryonic rhabdomyosarcoma, muscular marker MyoD1, muscle-specific actin, desmin, and miogenin and myoglobin detection was repeated. All the reactions were to be interpreted as negative. WT1 protein, EMA, CKAE1/AE3, CD34, CD31, LCA antigens were not identified immunohistochemically as well bcl-2 positivity was detected in some rare cells. Ki67 proliferation index was 44.44%. The clinical data, as well as histological and immunohistochemical picture was more appropriate to the diagnosis of cellular mesoblastic nephroma (infantile renal fibrosarcoma). Currently, 6 years after the initial diagnosis, the patient has a tumour remission.

3.1.3. The comparison of primary kidney tumour morphological spectrum before and after repeated morphological and immunohistochemical examination

When analysing the spectrum of primary renal tumours diagnosed in CCUH before and after repeated morphological and immunohistochemical examination it can bee seen that in both cases, the most common neoplasm is nephroblastoma. According to the histological examination data, carried out at the CCUH Pathology Department it represented 77.55% (n=38) of all kidney tumours. In turn after the re-examination 37 cases of nephroblastomas, 75.51% of the analysed formations were detected. A neuroblastoma, undetected during the first examination, was detected, which accounted for 2.04% of all tumours. Embryonic rhabdomyosarcoma diagnosis made in the Pathology Department was altered. The tumour was considered to be cellular mesoblastic nephroma (infantile renal fibrosarcoma), thus mesoblastic nephroma accounted not for 4.08%, but already for 6.12% of the analysed tumours. During repeated examination renal clear cell sarcoma, kidney rhabdoid tumour, cystic nephroma, angiomiolipoma and angiosarcoma diagnoses were confirmed (see figure 3.6). In general, diagnostic inaccuracies have been detected in 2 cases, or 4.08% of all the examined tumours.

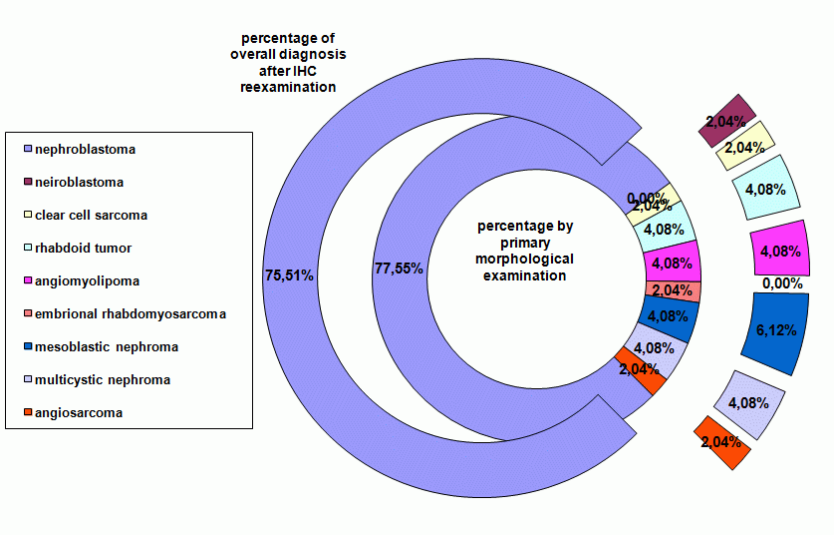


Fig. 3.6 The comparison of the results between primary morphological examinations carried out in the CCUH Pathology Department and immunohistochemical reexamination (January 1997 to July 2012).

3.2. The expression of immunohistochemically detected, potentially prognostically important markers in the case of nephroblastoma

3.2.1. The characteristics of the clinical and histological parameters in the examined group of patients

For further work in order to detect potentially prognostically important immunohistochemical markers, material from the 26 primary nephroblastomas, in the case of which the stored tissue quality was sufficient for full immunohistochemical examination, was used. In addition immunohisto-chemical examination of 8 remote metastases and 1 recurrence tissue also was carried out. In total, 35 cases were analysed. Patients researched in the primary tumour group ranged in age from 8 to 215 months (M=53.92, SD=55.36, Mo 36). 3.85% (n=1) had stage I tumours, 57.69% (n=15) stage II, 23.08% (n=6) stage III, 11.54% (n=3), stage IV, and 3.85% (n=1) stage V tumours. Most of the material analysed 80.77% (n=21) consisted of intermediate malignancy tumours; high-grade malignancy tumours were detected in 15.38% (n=4), but the low-grade in 3.85% (n=1) cases. Depending on the histological type the

tumours examined were divided as follows: blastemal type tumours 38.46% (n=10), epithelial type 19,23% (n=5), mixed type 15.38% (n=4), regressive type 15, 38% (n=4), stromal type 7.69% (n=2), whereas in 3.85% of the cases (n=1) cystic partially differentiated nephroblastoma was detected. In all primary tumours favourable histology was detected. In 57.69% of the cases (n=15) chemotherapy was administered before surgery, for 42.31% of the patients (n=11) kidney and tumour excision was performed first. In the group of tumours treated before surgery in 10 cases, or 66.67% clinically good tumour response to chemotherapy carried out was observed, whereas in 5 cases, or 33.33%, a small size reduction or even an increase in the size of the formation was observed on the background of the administered treatment, 92.31% (n=24) of the analysed nephroblastoma patients were alive, 7.69% (n=2) patients had passed away. In both cases, the deceased patient survival period after diagnosis was less than two years. Progressive course of the disease (recurrence or metastasis development after removal of the primary tumour and subsequent chemotherapy) was observed in 11.54% (n=3) of the cases, while in 88.46% (n=23) of the cases the progression of the malignancy was not detected. In one of the cases of the tumour progression the patient has passed away at present, in the other case the patient is in palliative care, while in the third case tumour remission is detected and the patient is now, 11 years after the detection of metastatic process, alive. In the metastase and recurrence group material from 9 cases (8 metastases and 1 recurrence) in total for 6 patients was examined. The patients at the time of the first metastatic process diagnosis ranged in age from 120 to 14 months. For 3 or 50% of the patients currently tumour remission has been detected, 2 or 33.33%, have passed away. In 3 cases or 50% of the cases also the primary tumour tissue material is available and included into the researched group. In 3 cases the primary tumour has been diagnosed before 1998 and the tissue material could not be used for repeated examination. In the single recurrence case diffuse anaplasia was detected, which was not present in the primary tumour material.

3.2.2. The expression of CD44s glycoprotein isoform

During analysis of all primary nephroblastoma cases regardless of clinical and histological parameters the mean CD44s positive cell number in the blastemal component was 3.76% (0,00%-17.41%; SD 4.85%), in the stromal component M=34.54% (8.93%-78.49%, SD 19.93%), while the average number of positive epithelial structures per visual field was 7 (0-44; SD 14.2).

In the metastase and recurrence group in the blastemal component M=3.39% (0.76%-9.98%; SD 3.16%), the stromal component M=48.69% (16.62%-97.42%; SD 28.22), but in the epithelial component M=3.75 (0-9; SD 4.11). The highest response rate was detected in the stromal component, which essentially corresponds to the norm and is usually explained by the existing positive CD44s expression in fibroblasts (see Figure 3.7, page 40). In the epithelial component mainly uneven response rate was detected, where the part of epithelial structures are entirely negative, whereas part is completely positive. Statistically significantly increased expression of CD44s in metastatic tumour stroma in comparison to primary nephroblastoma stroma has not been detected (p=0.186). Similarly, there were no statistically significant differences between the response rate in both groups in blastemal and epithelial component of the tumour (p=0.664 and p=0.488).

Examining the CD44s expression depending on the stages, statistically significant difference in response rate in the case of low and high stage tumours has not been detected either in the blastemal, stromal or epithelial component (p=0.13, p=1, p=0.35).

When examining the expression of CD44s in relation to the nephroblastoma histological malignancy, statistically significant difference between CD44s expression in the blastemal, stromal and epithelial component in the low and intermediate malignancy grade tumour group compared with the high malignancy grade tumour group was not detected (respectively p=0.16, p=0.25, p=0.75).

Regarding histological type of tumours, statistically significant difference between CD44s expression in the blastemal, stromal and epithelial component of the blastemal type tumours, compared to the other histological types, was not observed (p=0.093; p=0.2; p=0.791). No statistically significant difference was detected also between the immunohistochemical response intensity in the stromal and epithelial component of the stromal type tumours, compared to the other histological versions (p=0.676; p=0.84). CD44s expression in the blastemal and stromal components of the epithelial type formations was not significantly different from the response intensity in the case of other tumours (p=0.125, p=0.749), but was significantly reduced in the epithelial component (p=0.043). In the regressive type tumour blastemal, stromal and epithelial components response intensity was not significantly different from CD44s expression in the case of other histological types (p=0.257; p=0.58; p=0.172). When comparing mixed type tumours to other

formations, significant difference between CD44s expression in the blastemal and epithelial component was not detected (p=0.482; p=0.428), but the expression of CD44s in the stromal component was significantly increased (p=0.027).

A statistically significant difference between CD44s expression in blastemal, stromal and epithelial components of chemotherapy affected and unaffected material was not observed (p=0.53; p=0.14; p=0.62). When analysing separately the nephroblastoma group treated before surgery an increased expression of CD44s in all histological components was observed in those tumours, the response of which to chemotherapy was poor compared to the cases where marked reduction of the size was clinically detected, however, this difference was not statistically significant (p=0,13; p=1; p=0,35).

A statistically significant difference between CD44s expression in the blastemal, stromal and epithelial component for the deceased and surviving groups of patients is not detected (p=0.182; p=0.56; p=0.307).

In the group of those nephroblastomas, for which chemotherapy was administered preoperatively and progressive malignant process was identified, in two cases out of the existing three, primary tumours belonged to the epithelial type and measurable blastemale component was not detected in them. In both cases, the epithelial component did not display any positive CD44s structures. In the single formation, in which the blastemal component was detected, CD44s positive cells accounted for 11.88% of its cells. In turn, the epithelial component was virtually absent in this tumour. In the stromal component in the case of progressing course M=36.52% (26.76%-48.61; SD 11.1, 95%CI 8.93-64.11). In cases where the development of metastases or recurrence are not observed on the background of the administered treatment, in the blastemal component M=3.31% (0.00%-17.41%; SD 4.57; 95% CI 1.03-5. 58), in the stromal component 34.26 % (8.93%-78.49%; SD 21.07; CI 95% 26.44-43.85) an average of 7.77 CD44s positive epithelial structures (0-44, SD 14.8, 95% CI 0.41-15.13) were detected. A statistically significant difference between the CD44s expression in the blastemal, stromal and epithelial components of the formations in the two groups was not observed (p=0.19, p=0.6; p=0.17).

3.2.3. The CDKN2A/p16^{INK4a} tumour suppressor gene encoded p16^{INK4a} protein expression

In the primary tumour group, the median number of p16^{INK4a} positive cells in the blastemal component M=76.91% (0.94%-100.00%; SD 26.31%), in the stromal component M=78.30% (0.80%-100%; SD 27.42%), in the epithelial component M=86.86% (0.00%-100.00%; SD 24.65%). In the metastatic and recurrence group in the blastemal component M=92.73% (81.09%-99.23%, SD 6.66%), in the stromal component M=93.47% (82.75%-100.00%; SD 6.54%), in the epithelial component M=91.4% (86.53%-97%; SD 4.37%). Overall response rate is to be evaluated as high. The number of positive cells exceeding 80% in the blastemal component has to be noted in 66,66% (n=12) of the cases. (See Figure 3.8, page 40). A statistically significant difference in the reaction intensity of the metastatic tumours in the blastemal, epithelial and stromal component compared to p16^{INK4a} expression in these histological components in the case of primary nephroblastoma was not detected (respectively p=0.276, p=0.391; p=0.177).

When analysing p16^{INK4a} expression, depending on the stages of the formation, by analogy to what was observed when researching the reactivity of CD44s, statistically significant difference between p16^{INK4a} expression in the blastemal, stromal and epithelial component in the case of low and high stage tumours has not been detected (p=0.77; p=0.22; p=0.21).

When examining the p16^{INK4a} expression in relation to the histological degree of malignancy of nephroblastoma, a statistically significant difference between p16^{INK4a} expression in the blastemal, stromal and epithelial component of low and intermediate malignancy tumour group compared to the high malignancy tumour group was not detected (p=0.75; p=0.46, p=0.42).

Regarding histological type of tumours, statistically significantly decreased expression of p16^{INK4a} in the stromal and epithelial component of blastemal type tumours, compared to other histological types (p=0.007; p=0.03) has been discovered, whereas the response rate in the blastemal component did not differ (p=0.075). Comparing the response rate in the case of stromal and other histological types of tumours a statistically significant increase in p16^{INK4a} expression in the stromal component was detected, whereas no significant differences in respect to the epithelial component (p=0.03; p=0.18) were found. P16^{INK4a} expression in the blastemal, stromal and epithelial component of the epithelial type formations did not significantly differ from the response rate in other tumours (p=0.14; p=0.33; p=0.47). In the blastemal, stromal and

epithelial component of the regressive type tumours the response rate was not significantly different from that of $p16^{INK4a}$ expression in the case of other histological types (p=0.77; p=0.33; p=0.81). Comparing mixed type tumours to other formations a significantly increased expression of $p16^{INK4a}$ in the blastemal and epithelial component (p=0.003; p=0.024) was detected, whereas no differences were found in respect to the stromal component (p=0.057).

A statistically significant difference in $p16^{INK4a}$ expression of the blastemal, stromal and epithelial component between tumours after chemotherapy and tumours unaffected by treatment was not detected (p=0.85; p=0.18; p=0.15), as well as statistically significant difference in $p16^{INK4a}$ expression of the blastemal, stromal and epithelial component between the tumours with good un poor response to chemotherapy was not detected (p=0.77; p=0.22; p=0.21).

Statistically significant differences in the blastemal, stromal and epithelial component $p16^{INK4a}$ expression between the deceased and surviving patients have not been detected (p=0.48; p=0.81; p=0.18).

In the nephroblastoma group, on the background of the administered surgery and chemotherapy, with detected progressive malignant course of the process, in two cases from the existing three, the primary tumours belonged to the epithelial type and no measurable blastemal component was detected. In the single formation, in which the blastemal component was detected, the $p16^{INK4a}$ positive cells accounted for 89% of its cells. In the epithelial component the average number of positive cells, M=96.31% (92.63%-100.00%; SD 5.21, CI 95% for mean 49.49-143.13), whereas in the stromal component M=86.09 % (77.46%-91.93%; SD 7.62, CI 95% for the mean 67.14-105.03). In cases where the development of metastases or recurrence was not observed on the background of the administered treatment, in the blastemal component M=81.68% (35.86% -100.00%; SD 18.97; CI 95% for the mean 71.92-91.43), in the stromal component M=81.10% (25.00%-100.00%; SD 23.04, 95% CI 70.31-91.88), in the epithelial component M=85.81 (0.00%-100.00%; SD 25.8; 95% CI 72.97- 98.64). A statistically significant difference between $p16^{INK4a}$ expression in the blastemal, stromal and epithelial component of the two groups of tumours was not detected (p=0.92; p=0.58; p=0.65).

3.2.4. The expression of nuclear antigen Ki67 associated with cell proliferation

In the primary tumour group, the mean proliferation index (PI) determined by means of Ki67 antigen in the blastemal component was 37.04% (3.57%-78.57%; SD 16.82), in the stromal component 13.67% (1.12%-37.66%, SD 10.31), in the epithelial component 23.5% (2.33%-38.55%, SD 12.55). In the metastatic/recurrency group the proliferation index in the blastemal component was 34.78 (9.70%-62.29%; SD 17.59), in the stromal component 6.93% (1.60%-17.52%; SD 5.8), in the epithelial component 12.14% (5.00%-28.57%; SD 11.2). In this case the wide range of response intensity has to be noted, because even in the case of tumours of the same type PI was different (see Figures 3.9 and 3.10, page 41).

Statistically significantly increased size in the blastemal, epithelial and stromal component of the metastatic tumours compared to primary nephroblastoma cases was not detected ($p=0.931$; $p=0.75$; $p=0.89$).

Comparing the PI in the low and high malignancy stage tumours, in the blastemal, stromal epithelial component a statistically significant difference between the two groups has not been detected ($p=0.56$; $p=0.38$; $p=0.13$).

When analysing the Ki67 expression depending on tumour histological grade of malignancy , statistically significant difference between the PI value in the blastemal, stromal and epithelial component of low and intermediate malignancy tumour group compared with the high malignancy tumour group was not detected ($p=0.48$; $p=0.63$; $p=0.09$).

Regarding histological type of tumours, a statistically significant difference between the PI blastemal tumour type blastemal, stromal and epithelial component, compared to other histological types was not detected ($p=0.74$; $p=0.57$; $p=0.75$). There was also no statistically significant difference detected between the immunohistochemical reaction intensity in the stromal and epithelial component of the stromal type tumours, compared to other histological versions ($p=0.53$; $p=0.54$). The size of PI in the epithelial type and other formation blastemal, stromal and epithelial component was not significantly different ($p=0.79$; $p=0.27$; $p=0.62$). In the blastemal, stromal and epithelial component of the regressive type tumours, and response rate was not significantly different from the Ki67 expression in the case of other histological types ($p=0.28$; $p=0.64$; $p=0.59$). Also, when comparing mixed tumours with other formations, a significant difference between Ki67 expression in the

blastemal, stromal and epithelial component was not detected (p=0.072; p=0.053; p=0.68).

A statistically significant difference in PI values in the blastemal, stromal and epithelial components between tumours after chemotherapy and tumours unaffected by treatment was not detected (p=0.41; p=0.43; p=1), as well as statistically significant difference in PI in the blastemal, stromal and epithelial component between tumours with good un poor response to chemotherapy was not detected (p=0.8; p=0.61; p=0.52).

A statistically significant difference between the PI values of deceased and surviving patient groups in the blastemal, stromal and epithelial component has not been detected (p=0.18; p=0.4; p=0.32).

In the case of the single progressing tumour, in which a blastemal component was detected, PI was 53.33%, while in the epithelial component in the case of tumour progression the mean PI was 20.25% (13.84%-26.66%, SD 9.06, CI 95% for the mean 0.00-101. 69), in the stromal component M=10.32% (2.36%-22.22%; SD 10.49, 95% CI for the mean 0.00-36.4). In the cases where the development of metastases or recurrence on the background of the administered treatment is not observed, the average PI in the blastemal component is 36.14% (3.57%-78.57%; SD 18.97, 95% CI for the mean 27.77-44.51), in the stromal component M=14.15% (1.12% -37.66%; SD 10.45, 95% CI for the mean 9.4-18.91) in the epithelial component M=23.84% (2.33%-38.55%; SD 13, CI 95% for the mean 17.57-30.11).A statistically significant difference between the PI values of both groups in the blastemal, stromal and epithelial component was not detected (p=0.57, p=0.63, p=0.63).

3.2.5. P53 protein expression

In the primary tumour group, the mean number of p53 positive cells in the blastemal component was 9.71% (0.00%-37.37%; SD 11.44), in the stromal component 1.74% (0.00%-12.77 %; SD 2.73), in the epithelial component 6.35% (0.00%-25.38%; SD 9.03). In the metastatic/recurrence group in the blastemal component M=27.58% (1.92%-88.46%; SD 29.03), in the stromal component 3.84% (0.00% -17.05%; SD 5.95), in the epithelial component 9.1% (3.03%-20.89%; SD 8.25). A statistically significantly increased response rate in the epithelial and stromal component of metastatic tumours, compared to p53 expression in the mentioned histological components in the case of the primary nephroblastomas was not detected (p=0.179; p=0.269). Increased response rate in the blastemal component of the metastase/recurrence group (see Figure 3.11,

page 41) compared to the primary tumours was statistically significant (p=0.03).

Comparing p53 expression in the blastemal, stromal and epithelial component in the groups of low and high stage tumours a statistically significant difference in both groups was not observed (p=0.93; p=0.58; p=0.97).

In relation to the nephroblastoma degree of malignancy, statistically significant difference between p53 expression in the blastemal, stromal and epithelial component of the low malignancy level tumour group compared with the high malignancy level group was not detected (p=0.16; p=0.68; p=1).

Researching p53 positive cells , depending on the histological type of tumours, statistically significant difference between the expression of p53 in the blastemal tumour type in the blastemal stromal and epithelial component, compared to other histological types was not detected (p=0.62; p=0.73; p=0.09). No statistically significant difference was detected in the immunohistochemical reaction intensity of the stromal and epithelial component in the stromal type tumours compared to other histological versions (p=0.71; p=0.67). P53 expression in the blastemal and stromal component of the epithelial type formations was not significantly different from the response rate in the case of other tumours (p=0.89; p=0.32), but was significantly increased in the epithelial component (p=0.02). In the stromal and epithelial component of regressive type tumours the response rate was not significantly different from that of p53 expression in other histological types (p=0.79; p=0.75; p=0.34). Similarly, when comparing the mixed type tumours with other formations, a significant difference between p53 expression in the blastemal stromal and epithelial component was not detected (p=0.19; p=0.6; p=0.44).

A statistically significant difference between p53 expression in tumours after treatment with chemotherapy and in the material unaffected by treatment in the blastemal stromal and epithelial component between was not detected (p=0.46; p=0.92; p=0.38).

A statistically significant difference between the expression of p53 in the formations with positive or negative response to treatment in the blastemal, stromal and epithelial component was not detected (p=0.8; p=0.26; p=0.64).

A statistically significant difference between p53 expression in the blastemal, stromal and epithelial component between the deceased and surviving patient groups was not detected (p=0.79; p=0.52; p=0.21).

In the case of the single progressing tumour, in which a blastemal component was detected, the number of p53 positive cells was 15.26%, while in the epithelial component in the case of the progressing tumour M=22.69% (20.00%-25.38%; SD 3.8, 95% CI for the mean 0.00-56.86) in the stromal component M=1.47% (0.00%-2.85, SD 1.42, 95% CI for the mean at 0.00 - 5.01). In the cases where the development of metastases or recurrence on the background of administered treatment was not observed, in the blastemal component M=9.41% (0.00%-37.37%; SD 11.7, CI 95% for the mean 3.59-15. 22), in the stromal component M=1.78% (0.00%-12.77%; SD 2.89, CI 95% for the mean 0.46-3.10), in the epithelial component of M=4.63% (0.00%-23.68%; SD 7.55; CI 95% for the mean 0.98-8.27). When comparing p53 expression in both groups it was established that the response rate in the blastemal and stromal component did not significantly differ (p=0.27;p=0.72), whereas it was significantly increased in the epithelial component in the case of the tumour progression (p=0.039).

3.2.6. E-cadherin expression

In the primary tumour group, the average quantity of e-cadherin positive structures in the visual field was 22.74 (0-86; SD 23,21). In the metastase/recurrence group, M=9.88 (0-40, SD 14, 86). Both in the case of the primary tumours and the metastases positivity was detected in well-differentiated tubular structures (see Figure 3.12, page 41). The response rate in the case of a metastatic process was statistically significantly reduced compared to the primary nephroblastomas (p=0.046).

Comparing the response rate for the groups of low and high stage tumours, a statistically significant difference between the two groups was not detected (p=0.36), but In the group of high malignancy tumours e-cadherin expression was significantly decreased compared to the low and intermediate malignancy tumour group (p=0.049).

Comparing e-cadherin expression between the tumour group in total and that of different histological types, it has been detected that the response rate in the case of none of the histological types was significantly different from the total response group (p=0.24; p=0.74; p=0.14; p=0.86; p=0.53).

In tumours with chemotherapy administered prior to surgery the mean e-cadherin positive structure number M=24.13 (0-86; SD 28.36; CI 95% for the mean 8.43-39.84), while in chemotherapy undisturbed material M=31.86 (0-66; SD 17.23; CI 95% for the mean 19.79-42.94). A statistically significant

difference between the e-cadherin expression in both groups has not been detected (p=0.15). If the tumour response to chemotherapy was clinically considered as a good M=28.2 (0-86; SD 30.26, CI 95% for the mean 6.55-49.85). In the insufficient tumour regression case, M=16 (0-60, SD 25.11, CI 95% for the mean 0.00-47.18). The difference between the two groups is not statistically significant (p=0.34).

In the deceased patient group, the mean quantity of e-cadherin positive structures M=13.5 (0-27; SD 19.09; CI 95% for the mean 0.00-185.00). In the group of the surviving patients, M=28.33 (0-86; SD 24.49; CI 95% for the mean 17.99-38.67). A statistically significant difference between the response rate of the groups of surviving patients and the deceased patients has not been found (p=0.46). In cases where there is evidence of tumour progression, the mean e-cadherin number of positive structures detected in the primary formations M=4.33 (0-13; SD 7.5; CI 95% for the mean 0.00-22.97). If the tumour progression was not detected, then M=30.17 (0-86; SD 24; CI 95% for the mean at 19.79-40.55). The mentioned difference of expression was statistically significant (p=0.05).

3.2.7. CK AE1/AE3 expression

In the group of primary tumours, the mean number of CKAE1/AE3 positive structures per visual field was 94.7 (0-480; SD 128.61). The largest number of CKAE1/AE3 positive structures has been found in the case of an epithelial type glomerular differentiation nephroblastoma (see Fig. 3.13, page 42). In the metastase/recurrence group, M=9,88 (0-40; SD 14,86). The quantity of immunohistochemically positive epithelial structures in the case of a recurrent process was statistically significantly reduced compared to the primary nephroblastomas (p=0.005). In primary tumour group statistically significant difference between the quantity of positive structures in the case of low and high stage tumours as well as in the group of low and intermediate malignancy tumours compared to the group of high malignancy tumours has not been detected (p=0.36; p=0,11).

Comparing the number of CKAE1/AE3 positive structures between the total group of tumours with different histological types, it has been detected that the response rate in the case of blastemal, epithelial, stromal and mixed type tumours is not significantly different from the response in the group in total (p=0.09, p=0.69; p=0.92, p=0.74), but there is a significant increase in the case of regressive type tumours (p=0.015).

In tumours with chemotherapy administered prior to surgery the average number of CKAE1/AE3 positive structures M=121.73 (0-480; SD 158.97; CI 95% for the mean 33.69-209.77) and chemotherapy undisturbed material M=58 (0-199; SD 58.27; CI 95% for the mean 18.84-97.15), but the described difference was not statistically significant (p=0.49). If the tumour response to chemotherapy was clinically interpreted as a good M=159.9 (0-480; SD 182.66; CI 95% for the mean 29.22-290.57). In the case of insufficient tumour regression M=45.4 (0-114; SD 49.58; CI 95% for the mean 0.00-106.96). The difference between the described two groups was not statistically significant (p=0.21).

In the group of deceased patients, the mean number of CKAE1/AE3 positive structures M=24 (0-48; SD 33.94; CI 95% for the mean 0.00-328.94). In the group of the surviving patients, M=100.66 (0-480; SD 132.13; CI 95% for the mean 44.87-156.46). A statistically significant difference between the groups of the deceased and the surviving patients was not detected (p=0.31). In cases where there is evidence of tumour progression the estimated average number of CKAE1/AE3 positive structures in primary formations M=160 (0-480; SD 277.12; CI 95% for the mean 0.00-848.42). If the tumour progression was detected, then M=86.26 (0-476; SD 105.64, CI 95% for the mean 40.57-131.94). A statistically significant difference between the response rates in both groups was not found (p=0.44).

3.2.8. CD34 positive vascular proliferation

In the primary tumour group, the mean number of CD34 positive microvessels per visual field was 123.46 (25-374; SD 87.84). In the metastase/recurrence group, M=116.44 (24-266; SD 98.3). A statistically significant difference between the number of microvessels in the case of the primary and recurrent process has not been detected (p=0.439). In all cases both already formed vascular structures with lumen and CD34 positive cell structures without lumen formation were detected (see Figure 3.14, page 42).

When comparing the response rate for low and high stage tumour group a statistically significant difference between the two groups has not been established (p=0.51).

A statistically significant difference between the response rate of low and intermediate malignancy tumour groups when compared to high malignancy tumour group has not been detected (p=0.16).

When comparing the response rate between the general tumour group and different histological types, it has been detected that the number of CD34 positive blood vessels in any of the tumour histological versions (blastemal, stromal, epithelial, regressive and mixed) is not significantly different from the number in the general group (p=0.19; p=0.12; p=0.58; p=0.095; p=0.3).

In tumours with chemotherapy administered before surgery, the average number of CD34 positive microvessels M=125.26 (25-270; SD 89.07; CI 95% for mean 75.93-174.59) and in chemotherapy undisturbed material M=121 (40-374, SD 90.4, CI 95% for the mean 60.26-181.70). A statistically significant difference between the two groups of tumours has not been detected (p=0.9). If the tumour response to chemotherapy is clinically considered as a good M=135 (25-270; SD 89.66, CI 95% for the mean at 70.85-199.14). In the case of insufficient tumour regression, M=105.8 (40-270; SD 94.7, CI 95% for the mean 0.00-223.39). A statistically significant difference between tumours with good and inadequate response to chemotherapy has not been detected (p=0.75).

In the group of deceased patients, the mean number of CD34 positive blood vesels M=92.5 (56-129; SD 51.61; CI 95% for mean 0.00-556.27). In the group of the surviving patients, M=126.04 (25-374; SD 90.45; CI 95% for the mean 87.84-164.23). In the cases where there is evidence of tumour progression, the average number of CD34 positive blood vessels estimated in primary formations M=100.33 (25-220; SD 104.7; CI 95% for the mean 0.00-360.63). A statistically significant difference between the response rate for the deceased and the surviving patients has not been detected (p=0.73) If the tumour progression is not detected, then M=126.47 (32-374; SD 87.68, CI 95% for the mean 88.56-164.39). A statistically significant difference between the two mentioned groups has not been detected (p=0.44).

3.2.9. Wilms tumour suppressor gene protein expression

In the primary tumour group, the mean quantity of the Wilms tumour suppressor gene (WT1) protein positive cells M=44.13% (0.00%-99.47; SD 44). In the metastatic/ recurrence group, M=63.23% (0.00%-99.23%; SD 47.55). A statistically significant difference between the WT1 expression in the metastatic and primary process has not been detected (p=0.525). A positive response has been detected in the cell nuclei of the blastemal and primitive epithelial components, although also a missing immunoreactivity has been

discovered in the blastemal component cells of the histologically classic nephroblastoma (see Figure 3.15 and Figure 3.16, page 42).

Comparing the response rate for low and high stage tumour group a statistically significant difference between the two groups has not been detected (p=0.62), as well as statistically significant difference between the response rate of low and intermediate malignancy tumour group compared with the high malignancy group has not been detected (p=0.94).

Comparing the response rate between the total tumour group and different histological types, it has been detected that the number of WT1 protein positive cells in any of the tumour histological variants (blastemal, stromal, epithelial, regressive and mixed) does not significantly differ from the number in the total group (p=0.62; p=0.23; p=0.078; p=0.66; p=0.27).

In tumours with chemotherapy administered prior to surgery the mean quantity of the WT1 positive cells, M=35.70% (0.00%-99.00%; SD 41.76; CI 95% for the mean 12.57-58.82) and in chemotherapy undisturbed material M=55.63% (0.00%-99.47%; SD 46.3; CI 95% for the mean 24.50-86.76). A statistically significant difference between the response rate of chemotherapy affected and undisturbed material has not been detected (p=0.41). If the tumour response to chemotherapy has been clinically considered as a good M=52.86% (0.00%-99.00%; SD 41.55, CI 95% for the mean 23.14-82.59). In the case of insufficient tumour regression M=1.37 (0.00%-6.87%; SD 3.07; CI 95% for the mean 0.00-5.18). This difference is statistically significant (p=0.009).

In the deceased patient group, the mean quantity of WT positive cells M=46.52% (0.00%-93.04%; SD 65.78; CI 95% for the mean 0.00-637.61). In the group of the surviving patients, M=43.93% (0.00%-99.47%; SD 43.77, CI 95% for the mean 25.45-62.42). A statistically significant difference between the response rate of the deceased and surviving patients has not been found (p=0.76). In the cases where there is evidence of tumour progression the mean quantity of WT1 positive cells in primary formations M=35.21% (0.00%-63.33%; SD 32.25; CI 95% for the mean 0.00-115.33). If the tumour progression has not been detected, then M=45.29% (0.00%-99.47%; SD 45.75; CI 95% for the mean 25.51-65.00). The immunohistochemical response rate between the two groups is not statistically significantly different (p=0.65).

3.2.10. The correlation of the expressivity of immunohistochemical markers

With the help of non-parametric Spearman correlation analysis for determining of the mutual correlation of immunohistochemical marker expression in the primary tumour group a statistically significant positive correlation between the number of blood vessels immunohistochemically visualised with CD34 and the number of CKAE1/AE3 positive epithelial structures in the tissue of the formations (r=0.551, p=0.004) was detected. A statistically significant positive correlation was found between p53 expression and CD44s in the blastemal component (r=0.596, p=0.007). In turn, CD44s expression in the stromal component had a positive correlation to $p16^{INK4a}$ positivity in the blastemal and stromal component (r=0.595, p=0.15; r=0.45, p=0.031). A statistically significant relationship has been detected between CD44s expression in the epithelial component and the number of CKAE1/AE3 positive structures (r=0.514, p=0.02). When analyzing $p16^{INK4a}$ protein expression a positive correlation between $p16^{INK4a}$ and p53 protein expression in the blastemal component (r=0.513, p=0.029) has been detected. The number of $p16^{INK4a}$ positive cells in the epithelial component had a negative correlation with WT1 expression (r=-0.514, p=0.02). Statistically significant relationship between CKAE1/AE3 and e-cadherin positive structure quantity (r=0.768, p=0) has been detected. A statistically significant correlation has been observed between p53 and Ki67 positive cells in the stromal component (r=0.654, p=0.001), between e-cadherin positive structure number and the proliferation index in the stromal component (r=0.404, p=0.024).

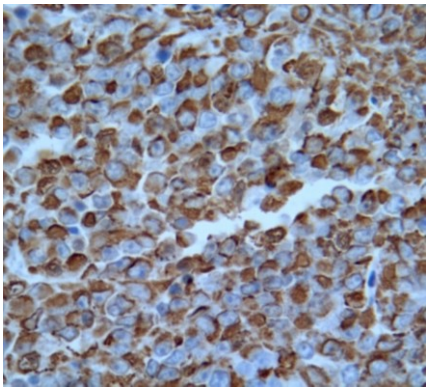


Fig. 3.4. Rhabdoid tumour of kidney, positive staining with vimentin in tumour cell cytoplasm. Magnification x400.

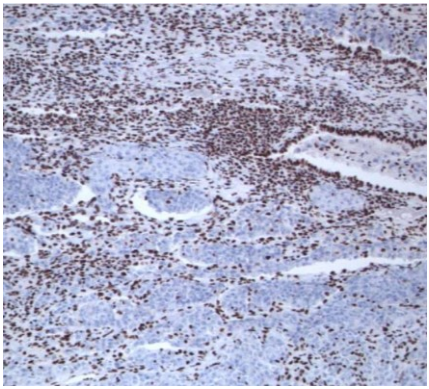


Fig. 3.5 Rhabdoid tumour of kidney extensive loss of staining with INI1 in tumour cells, whilst the nuclei of adjacent normal cells retain their pattern. Magnification x200.

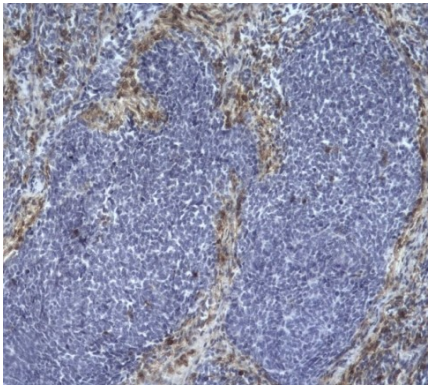


Fig 3.7 Positive CD44s expression in the blastemal type nephroblastoma stroma, negative reaction in blastemal component cells. Magnification x100.

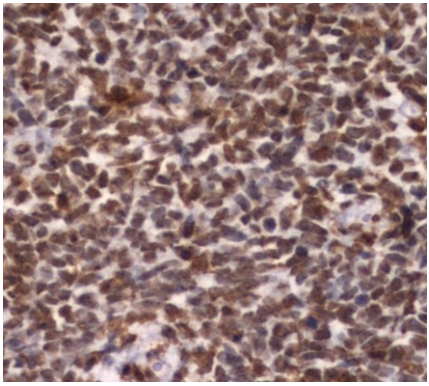


Fig. 3.8 Positive $p16^{INK4a}$ expression in the nephroblastoma blastemal component cell nuclei. Magnification x200.

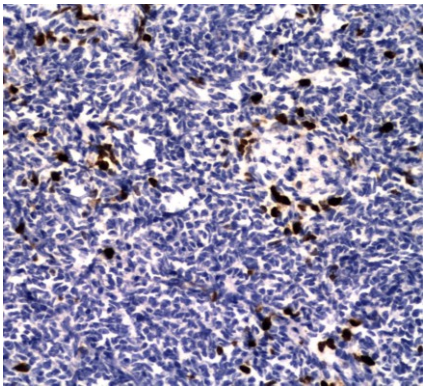


Fig. 3.9 Low Ki67 expression in the blastemal type high malignancy nephroblastoma blastemal cell nuclei in the case of chemotherapy administered in the preoperative period. Magnification x200.

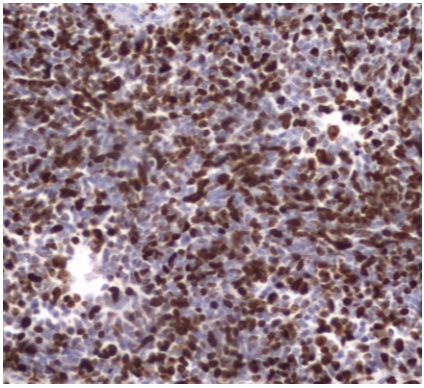


Fig. 3.10 High Ki67 expression in the blastemal type intermediate malignancy snephroblastoma blastemal cell nuclei in the case of primary nephrectomy. Magnification x200.

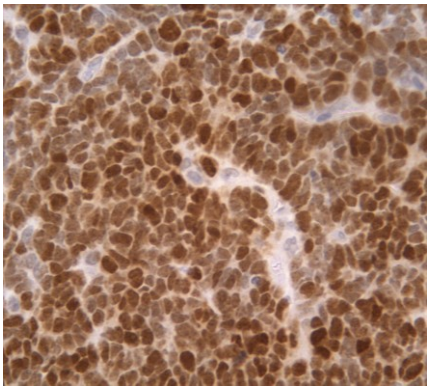


Fig. 3.11 P53 expression in the blastemal component cells of nephroblastoma lung metastasis. Magnification x400.

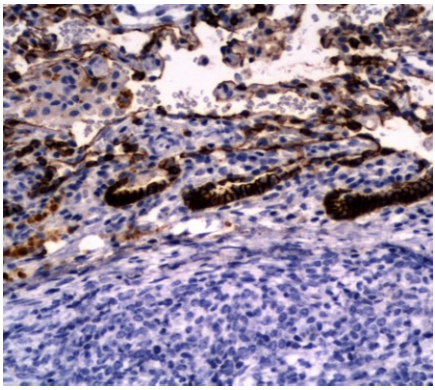


Fig. 3.12 E-cadherin expression in the tubular structures of the nephroblastoma metastasis at the border with lung tissue. Magnification x200.

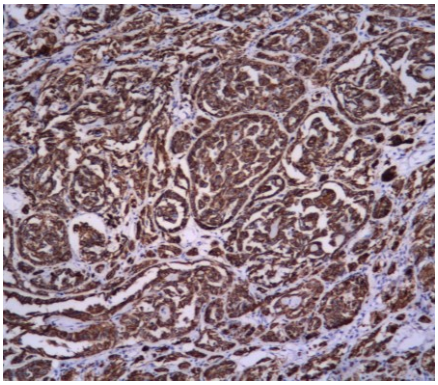


Fig 3.13 CKAE1/AE3 expression in the primitive tubular and glomerular structures in case of epithelial type nephroblastoma. Magnification x200

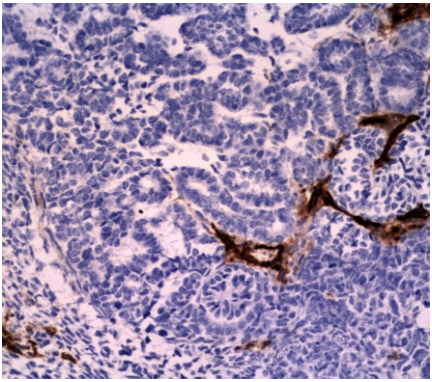


Fig. 3.14 CD34 positive structures with and without lumen formation in the case of epithelial type intermediate malignancy nephroblastoma. Magnification x200

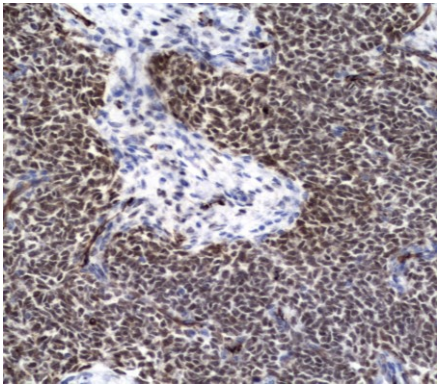


Fig. 3.15 Positive WT1 protein expression in the blastemal component cell nuclei in the case of blastemal type nephroblastoma. Magnification x200

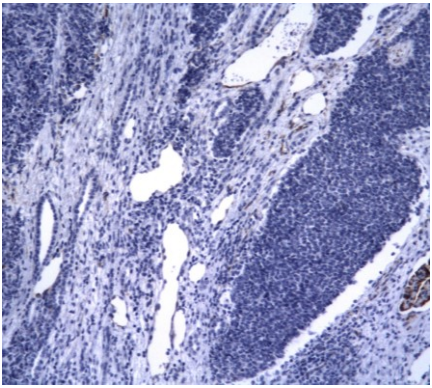


Fig. 3.16 Negative WT1 protein expression in the blastemal component cell nuclei in the case of blastemal type nephroblastoma, positive reaction in the glomerular cells of the kidney. Magnification x100

3.3. Genetic alterations in the tumour CDKN2A/p16 ^{INK4a} suppressor gene in the case of nephroblastoma

3.3.1. The characteristics of the researched tumour group

DNA isolation from paraffin embedded tissue samples, the subsequent propagation and sequencing for the research of 1α and 2 exon alterations was carried out for 14 tissue samples in total. In 11 cases (78.57%) primary tumour material, in 2 cases (14.29%) lung metastases, whereas in one case (7.14%) recurrence tissues have been used. Microsatellite markers were analysed in 14 primary tumour specimens.

3.3.2. The analysis of the genetic alterations obtained from CDKN2A locus DNA sequencing

When summarizing the obtained DNA sequencing results in 12 cases or 85.71% of the analyzed tissue samples gene CDKN2A/p16^{INK4a} 1α exon wild type form, which corresponds to the norm, was detected. In respect to the 2nd exon, the wild type form has been detected in 4 (28.57%) cases. In seven or 50% of the analyzed tissue samples it was detected that the 442nd nucleotide guanine (G) was replaced by adenosine (A) (hereinafter referred to as the c.442G> A) or p16^{INK4a} protein 148th amino acid alanine replaced by threonine (hereinafter A148T). A148T incidence in the nephroblastoma patient group was compared to the incidence in the control group (see Section 2.4). In this group of the 203 cases analyzed in 200 samples (98.52%) a wild type 2nd exon was detected, while the described alteration A148T (c.442G> A) was detected only in 3 or 1.48% of cases. By the means of Fisher's test it was detected that in the nephroblastoma group the replacement of the 148th amino acid alanine with threonine (A148T) had a statistically significantly higher incidence than in the control group ($p< 10^{-7}$), which indicates the role of the detected alteration in the development of nephroblastoma.

In CDKN2A locus microsatellite marker analysis a loss of heterozygosity was detected in three cases in total (21.43%), in four different microsatellites (see Table 3.1). A complete loss of one allele or reduction of at least 50% compared with normal tissues was regarded a loss of heterozygosity (see Figure 3.17). In one case, or 7.14% of the samples analysed loss of heterozygosity was detected in the analysis of the marker D9S736, which is located behind the last third exon of the CDKN2A gene, indicating that potentially genetic alterations related to the development of nephroblastoma in

this locus should be sought behind the CDKN2A gene. In one case (7.14%) of the samples analyzed loss of heterozygosity was detected in the analysis of the marker D9S1604 situated before CDKN2A/p16^{INK4a} gene, indicating a possible association of nephroblastoma development with this gene. It was not possible to detect the exact size of the deletion, because the rest of the analysed microsatellite markers were uninformative (see Table 3.1). CDKN2A gene A148T alteration was detected in this tumour as well. In one case the loss of heterozygosity was detected both in the marker D9S942, and at the same time in the D9S171 marker. These markers are located respectively in the intron between the exons 1α and 1β and before CDKN2A gene exon 1β (see Section 2.3.4 Figure 2.1) indicating a deletion in a larger region. Unfortunately, similarly to the previous case, it is impossible to detect the exact size of the deletion, since the rest of the analyzed markers were uninformative. However, similarly to the previous, also in these tissues in addition to deletion alterations in the CDKN2A gene A148T were detected as well.

Table 3.1

The results of CDKN2A locus microsatellite marker analysis

Nr.	Marker D9S736	Marker D9S1604	Marker D9S974	Marker D9S942	Marker D9S1748	Marker D9S1870	Marker D9S171
1.	NI	LOH	—	—	NI	—	—
2.	—	—	normal	normal	—	normal	NI
3.	NI	normal	normal	normal	normal	normal	normal
4.	LOH	normal	normal	normal	NI	NI	normal
5.	NI	NI	norma	norma	norma	norma	norma
6.	NI	NI	—	LOH	NI	NI	LOH
7.	NI	NI	NI	normal	normal	—	NI
8.	normal	NI	NI	normal	normal	—	normal
9.	normal	normal	normal	normal	normal	normal	normal
10.	normal	NI	NI	normal	normal	normal	normal
11.	normal	NI	NI	normal	normal	normal	normal
12.	—	NI	NI	—	—	NI	—
13.	normal	—	normal	normal	normal	normal	normal
14.	normal	normal	NI	—	—	—	—

"—" Uninformative result, because the material does not contain normal tissue or the response product cannot be interpreted.
NI- uninformative marker (homozygosity)
LOH - Loss of heterozygosity.

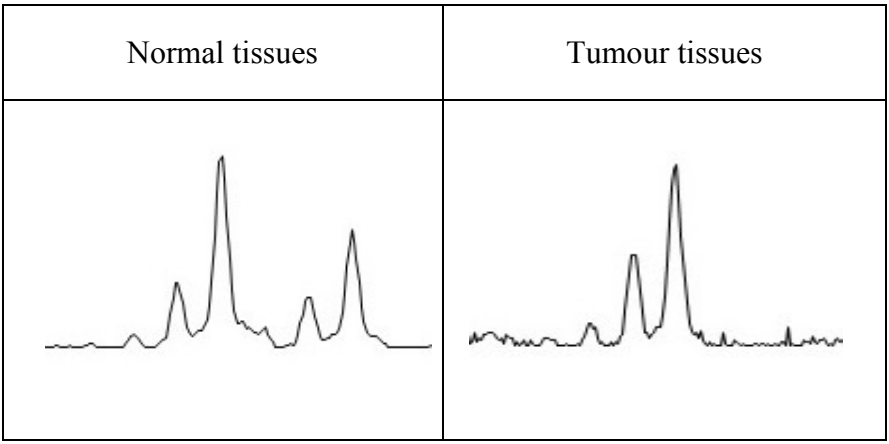


Fig. 3.17 Loss of heterozygosity in the CDKN2A locus microsatellite marker D9S171 in the case of nephroblastoma in comparison to the same patient's own normal tissues.

3.3.3. Comparison of the result obtained in CDKN2A locus DNA sequencing and $p16^{INK4a}$ protein immunohistochemical expression

In patients with detected A148T alteration the mean number of $p16^{INK4a}$ positive cells in the blastemal component is 84.30% (70.00%-98.57%; SD 11.69), in the stromal component 93.58% (88.88%-100.00%; SD 5.19) and in the epithelial component 97.10% (92.63%-100.00%; SD 3.05). In patients without the mentioned alteration number of $p16^{INK4a}$ positive cells in the blastemal component was 78.06% (51.85%-92.77; SD 22.7), in the stromal component M=71.15% (41.66%-92.47%; SD 22.58), in the epithelial component 77.63% (42.66%-96.5%; SD 30.3). A statistically significant difference between the immunohistochemical reaction intensity in the blastemal, stromal and epithelial component of patients with 2nd exon wild type and patients with detected A148T alteration in the second exon of the gene, was not detected (p=1, p=0.086, p=0.177).

In the cases where in the microsatellite analysis both alleles were detected the mean quantity of $p16^{INK4a}$ positive cells in the blastemal component was 80.55% (51.85%-100%; SD 17.02), 79.87% in the stromal component (37.97%-100%; SD 24.46), in the epithelial component 87.25% (42.66% -100%; SD 18.97). If loss of heterozygosity was detected, the mean

quantity of $p16^{INK4a}$ positive cells in the blastemal component was 84.28% (70.00%-98.57%; SD 20.2), in the stromal component 93.51% (88.88%-98.14%; SD 6.54); in the epithelial component the number of $p16^{INK4a}$ positive cells in all cases was 100%. A statistically significant difference between the protein expression in any of the histological components in both groups was not detected (respectively p=0.88; p=0.75; p=0.091).

4. DISCUSSION

4.1. Morphological spectrum analysis of paediatric renal tumours in Latvia

Analysing the results of the research carried out it is necessary to take into account the fact that all the examined paediatric kidney tumours are relatively rare both in the world and in Latvia. In the currently presented work only tumour material, diagnosed in Latvia, in the CCUH has been used, therefore the number of cases studied is small. In the total primary tumour group 49 tumours have been analyzed. The study material used has been obtained through data collection and histological material pooling from the detected tumours for the period of nearly 15 years (from January 1997 to July 2012). Therefore sometimes there is a situation, in which not all tumour histologic material quality has been sufficient for complete immunohistochemical examination. Immunohistochemical diagnosis verification has been carried out in 63.26% of all primary renal tumour cases. At the same time, repeated examination of the H&E material did not provide sufficient reasons for replacement of the existing histological diagnosis and exclusion of the tumour from the primary renal tumour group. Thus, individual mesoblastic nephroma cases, as well as angiosarcoma were included in the total spectrum of kidney tumours without additional immunohistochemical examination.

In respect to the morphological spectrum of the analyzed primary renal tumours, the study indicates that the most commonly diagnosed in CCUH paediatric renal neoplasm is nephroblastoma. According to the data of the morphological examination carried out in CCUH Pathology Department nephroblastoma accounts for 77.55% of all kidney tumours in children under the age of 18 years, whereas repeated immunohistochemical examination proved the mentioned diagnosis for 75.51% of all cases. The result is broadly consistent with the global data. Depending on the literature source nephroblastoma in different age groups accounts for 80% to 85% of all paediatric renal tumours [Sebire et al., 2010], and is the most common renal neoplasm in children. The study results show that in Latvia nephroblastomas account for less than 80% of the cases, but it is possible that this deviation from the tumour spectrum presented in literature is related to the small total number of cases examined. In respect to the rest of the CCUH diagnosed paediatric

kidney tumour morphological types, it is interesting that such rarely observed tumours as renal clear cell sarcoma and rhabdoid tumour of kidney, as well as angiomiolipoma, which is rare in the early age were observed, however neither in morphological abnormalities diagnosed in the CCUH Pathology Department examination, neither in the repeated immunohistochemical preparation review no renal cell cancer (RCC) cases were detected. Clinically and radiologically RCC was suspected in 3 cases, or 5.66% of the entire morphologically examined nephrectomy and partial kidney resection material, whereas histologically xanthogranulomatous pyelonephritis was detected. In comparison the Lithuanian colleagues in Vilnius University Children's Hospital during the period from 1997 to 2008 have diagnosed 30 primary renal tumours. 93.7% of them were nephroblastoma, while 6.3% were renal cell cancer [Jankauskiene et al., 2009]. According to the research carried out by the U.S. National Cancer Institute in children below 15 years of age renal cell cancer has been detected in 2.6%, in the patients up to 20 years of age, 5.4% of all kidney tumours, whereas in the age group of 15 to 19 years kidney RCC was detected in 63% of the cases [Bernstein et al., 1999]. In general, it has to be noted that the data from the study on renal cell cancer in the total paediatric spectrum in Latvia differ from those in the world literature, however it would not be proper to talk about Latvia as a country where renal cell cancer in children occurs less frequently than in other parts of the world. To confirm this idea it would be necessary to continue the research analyzing kidney tumours diagnosed in CCUH in the future as well and to carry out a statistical analysis of the data comparing the obtained findings with the data in the database of the Centre for Health Economics.

When analyzing the cases, where during repeated immunohisto-chemical examination the existing histological diagnosis was altered, it is seen that overall diagnostic inaccuracies were detected in 2 cases, or 4.08% of all the examined tumours. These results also highlight some of the issues that should be addressed by practicing paediatric pathologists. Usually nephroblastoma is a tumour with a characteristic histological structure, which consists of blastemal stromal and epithelial component in variable proportions, but there may be cases where it consists of only one or two histological components. Diagnostic problems are usually generated by a differential diagnosis between blastemal type nephroblastoma and other paediatric 'small, blue, round cell' tumours like neuroblastoma, PNET, renal lymphoma, as well as the difficulties in differentiating between nephroblastoma, renal clear cell sarcoma, and renal

rhabdoid tumours, described in the present section above [Sebire, 2010]. In the current study, in the immunohistochemical examination an immunophenotype more appropriate to neuroblastoma was discovered in one of the cases initially diagnosed as blastemal type nephroblastoma. According to the literature data, for determining of accurate diagnosis extensive immunohistochemical examination plans would be applicable, which include determining of the WT1 protein, neuroendocrine and epithelial markers, as well as LCA and CD99 antigens. In the case of renal neuroblastoma, WT1 protein negativity has the main role, in combination with the positivity of such variable neuroendocrine markers as NSE, synaptophisin and chromogranin [Coffin et al., 2006; Sebire, 2010], therefore the issue of intrarenal primary neuroblastoma incidence is complicated in general. On the initial stage of the study all diagnosed cases of neuroblastoma were deliberately excluded from the collected material, because it was detected that in this type of retrospective study it is practically impossible to accurately identify whether the researched formation has primarily developed the kidneys or grown in from the retroperitoneal space. Also in this case of neuroblastoma detected during repeated immuno-histochemical examination, the issue of the genesis of the formation is controversial. Although currently the formation is regarded to be a primary kidney tumour, in the primary computer topography examination a retroperitoneal tumour grown into the kidney was suspected. In this case, the tumour cannot be excluded from the common primary renal tumour spectrum, although there remains some doubt as to its origin. The final results also indicate that in the primary renal tumour spectrum created after the repeated immunohistochemical examination neuroblastoma constitutes 2.04%, whereas the literature describes only a few cases of primary intrarenal neuroblastoma [Sellaturay et al., 2006]. It is possible however that the mentioned difference is related to the small total number of cases, which may cause certain statistical deviations.

The second case, in which the morphological findings of the Pathology Department of CCUH were finalized, is related to embryonic rhabdomyosarcoma. According to literature data, primary renal rhabdomyosarcoma cases are very rare. In separate surveys only eight published cases have been found [Grignon et al., 1998]. The majority of renal tumours, which under light microscopy provide the impression of rhabdomyomatous genesis, are usually tumours of a different origin [Eble et al., 2007]. In our case, detailed immunohistochemical examination displayed

vimentin positivity, while the response for the detection of muscular markers MyoD1, muscle-specific actin, desmin, myogenin and myoglobin, WT1 protein and bcl-2 were negative. The mentioned immunophenotype and clinical anamnesis of congenital tumour in a 25 days old patient is more consistent with cellular mesoblastic nephroma (CMN), or infantile internal fibrosarcoma [Argani et al., 2004]. In the final results of the study the formation was included in the mesoblastic nephroma group, whereas the detected highly proliferative activity could be a source for discussion. The proliferation index was 44.44% compared to the findings of 20% -15%, described in literature. [Whittle et al., 2010], which also explains the choice of the pathologist to determine the diagnosis of a high malignancy level primary tumour during morphological examination. In the essence, in order to determine a more precise genesis of the formation, a cytogenetic examination would be necessary for the CMN characteristic translocation (12, 15) (p13, q25) and ETV6-NTRK3 gene convergence diagnostics. From the practical point of view the case under discussion indicates that in the future it is necessary to evaluate the paediatric primary mesenchymal genesis renal tumour diagnosis critically and to apply in the examination extensive muscular marker immunohistochemical examination plans taking into account the existence of cellular mesoblastic nephroma.

4.2. The correlation of the immunohistochemical and molecular genetic findings with the clinical morphological data in the case of nephroblastoma

4.2.1. The evaluation of the expression of potentially prognostically important immunohistochemical markers

When summarizing the data presented in the result section of the present study it can be seen that in the group of the primary tumours there is a significantly decreased CD44s expression in the epithelial type nephroblastoma epithelial component (p=0.043) compared to the expression of the mentioned marker in the histological component of other types of tumours. Interestingly, at the same time a statistically significant positive correlation between the expression of CD44s epithelial component and CKAE1/AE3 (p=0.02) and e-cadherin positive structure quantity (p=0.019) has been discovered. These findings can be explained by the fact that high CKAE1/AE3 and e-cadherin positive structure quantity has been found also in the regressive type nephroblastomas and the maximum number of CD44s positive structures - 44

was found precisely in the case of the regressive type tumour. In turn in the mixed type tumours a significantly increased expression of CD44s in the stromal component (p=0.027) has been detected compared to nephroblastomas of other histological types. However, taking into account that the CD44s is expressed in fibroblasts, and the detection of the mentioned response in the stroma in the presented work rather serves to separate its reactivity from the immunohistochemical response in the epithelial and blastemal component, it is considered that this correlation is not significant. Similarly, it is likely that there is no essential prognostic significance in the identified positive stromal correlation of CD44s expression with $p16^{INK4a}$ protein immunohistochemical reactivity in the blastemal and stromal component (p=0.015 and p=0.031), moreover because the positive $p16^{INK4a}$ expression, by using $p16^{INK4a}$ clone 2D9A12 has to be considered as normal. There could be a significant scientific importance for the research of prognostically essential markers in the detected positive relationship between p53 and CD44s expression in the primary blastemal component (p=0.007) of all tumours, the prognostically poor significance of high p53 expression in the case of nephroblastomas has been proven, which however pertains more to tumours with unfavourable histology. For example, pronounced p53 expression in anaplastic tumours compared to the observed ($p < 0.001$) in case of favourable histology and significantly reduced survival of p53 positive patients compared to negative cases ($p < 0.01$) has been described [Jadali et al., 2011]. Comparing the results obtained to the data available in publications, it appears that the literature data regarding the expression of CD44s in the case of nephroblastoma are controversial. In one of the studies an increase in the expression of CD44s epithelial cells with the increase in tumour stage and a positive correlation of the mentioned marker blastemal reactivity with tumour stage has been detected [Ghanem et al., 2002]. In turn when researching the patients, untreated in the preoperative period, the same group of authors has not found any correlation between the CD44s expression and the stage of nephroblastoma or clinical course [Ghanem et al., 2011]. Other researchers describe the positive immunoreactivity association of the mentioned markers with tumour histological type and degree of histological malignancy, regardless of whether or not chemotherapy has been applied prior to surgery [Taran et al., 2008]. Although in the study presented major differences between CD44s expression depending on the stage of nephroblastoma, histological risk level or tumour progression were not detected and response rate did not significantly differ in the surviving and deceased

patient groups, however, the established positive correlation between p53 and CD44s expression in the blastemal component of primary tumours could be indicative of prognostic significance of this marker in evaluation of immunoreactivity in the blastemal component, which has to be confirmed in future studies. The rest of the results are associated with alterations in CD44s expression depending on the histological type of tumour. The detected reduced CD44s expression in the epithelial component of epithelial type nephroblastomas possibly reflects to a greater extent the lowest possible epithelial type nephroblastoma malignancy level rather than the prognostic significance of CD44s antigen detection.

In the evaluation of the immunohistochemical expression of $p16^{INK4a}$ protein the interpretation of the performed reaction is essential. The literature describes various possible options for $p16^{INK4a}$ expression in normal tissues, which depend on the applied antibody clone. Most authors have established that the response rate in the tissue unaffected by tumour is very low or not detectable at all and that corresponds to the normal basal $p16^{INK4a}$ protein level. Currently, in part of the studies and in clinical practice, it is accepted as a standard for evaluation of immunohistochemical response [Natrajan et al., 2008; Zhao et al., 2012]. Also in the research carried out in the case of nephroblastoma, part of the authors use antibodies, the intensity of immunohistochemical reactivity of which in normal tissues is low. In the presented study for $p16^{INK4a}$ protein detection the clone 2D9A12 was used (Abcam, Cambridge, MA, USA). Positive external control in the case of brain astrocytoma was used as positive control according to the manufacturer's instructions, applying a positive control in normal human brain tissue, or brain tumours. The results of the performed control reaction, in addition to the established $p16^{INK4a}$ protein expression in renal tubular epithelial cell nuclei of the kidney unaltered by tumour and literature data on the reduced expression of the mentioned antibody clone in those brain tumour cases, where CDKND2A/$p16^{INK4a}$ allele loss was detected, whereas in the unaltered brain tissue positive expression (Royds et al., 2011) indicates that a positive nucleus reaction in this case should be considered normal physiological condition. The study data are also in accordance with $p16^{INK4a}$ protein immunohistochemical expression in unaltered human kidney tissue reflected in individual publications [Basta-Jovanović et al., 2008]. The analysis of the results of the performed immunohistochemical reactions indicate that, overall the expression can be evaluated as high, which in this case corresponds to the norm. Changes in

$p16^{INK4a}$ expressiveness depending on such prognostically important criteria as tumour stage and histological malignancy level were not found. Similarly, the immunohistochemical reaction rate did not change significantly, depending on chemotherapy administered in the period prior to surgery and tumour response to chemotherapy. There was no difference in the groups of surviving and deceased patients; there was also no statistically significantly increased or decreased expression of $p16^{INK4a}$ in the case of tumour progression. Consequently, the study results suggest that $p16^{INK4a}$ expression is likely to have no prognostic significance in nephroblastoma cases, which corresponds to the results of a study made by Natrajan and other authors [Natrajan et al., 2008], but is contrary to the association detected by Gordana Basta-Jovanović in $p16^{INK4a}$ expression with tumour stages [Basta - Jovanović et al., 2008]. In respect to $p16^{INK4a}$ protein expression in various nephroblastoma histological groups it can be seen that the response rate was significantly reduced in the stromal and epithelial component of the blastemal type tumours, compared to other histological types (p=0.007, p=0.03). In stromal type tumours the stromal component response rate was significantly increased compared to the reaction in the same histological component of different type tumours (p=0.03). In turn, when comparing the mixed type tumours with other formations a significantly increased expression of $p16^{INK4a}$ in the blastemal and epithelial component (p=0.003, p=0.024) was detected. It is difficult to understand whether these relations have any effect on the prognosis for the patient. It is possible that they reflect more pronounced response intensity in the prevailing component of each type of tumour, which may indicate a histologic type rather than a specific marker expression impact on prognosis. The statistically significant increased expression of $p16^{INK4a}$ blastemal type tumours stated in the publications of other authors [Basta-Jovanović et al., 2008], which could indicate a relationship with a higher degree of histological malignancy has not been detected. In this case, as in the analysis of CD44s expression, a positive correlation between $p16^{INK4a}$ and p53 expression has been detected in the blastemal component of primary tumours (p=0.029), but this fact is not considered clinically important, because high $p16^{INK4a}$ protein expression has to be considered physiological. The quantity of $p16^{INK4a}$ positive cells in the epithelial component had a negative correlation with WT1 expression (p=0.02), however in the interpretation of this fact it should be taken into account that the WT1 protein has been immunohistochemically determined independently of the histological

types in the blastemal and primitive epithelial component, therefore the expression may be reduced in tumours with a marked epithelial component.

The fact that while studying the expression of such an important prognostic marker widely used for other tumours as Ki67, significant differences in the immunoreactivity of this marker between any of the studied groups of primary tumours was not revealed. Similarly, Ki67 expression was not increased in metastatic tumours. Also with the non-parametric Spearman correlation analysis the only positive correlation between p53 and Ki67 positive cells was found within the stromal component of the primary tumour ($p=0.001$), as well as between e-cadherin positive structure number and the proliferation index in the stromal component ($p=0.024$), however these findings do not have a significant prognostic importance. The average number of Ki67 positive cells in the stromal component of primary nephroblastoma however was 13.67% (range 1.12%-37.66%; SD 10.31), even though it is higher than the proliferation index of 1% to 3% detected in the kidney unaffected by tumour [Ghanem et al., 2004], the average number of p53 positive cells in the stromal component of primary tumours was 1, 74% (range 0,00%-12.77%; SD 2.73). In the essence p53 expression in the stromal component is practically negative, in most publications the number of p53 positive cells of less than 5% is considered to be a negative result [Govender et al., 1998], thus the observed correlation clinically cannot be considered significant. Literature data in respect to the prognostic role of Ki67 expression in cases of nephroblastoma is extremely controversial. In addition, it is difficult to compare the data, because different researchers analyze the obtained results in different histological components in different groups depending on the administered chemotherapy, histological stage of malignancy or other clinical parameters. Results reported in some of the studies suggest a possible prognostic significance of Ki67/MIB1 detection. Thus in the group of patients with preoperatively applied chemotherapy the increased MIB1 expression found both in the epithelial and the blastemal component was associated with poor patient prognosis [Juszkiewicz et al., 1997], similar data on the impact of MIB-1 positive cell count in the blastemal component on patient survival are published in another study, while the reactivity of the epithelial component did not have a significance [Ghanem et al., 2004]. Analyzing the primary nephrectomy cases in the single-factor dispersion analysis blastemal MIB-1 association with progressive course of the disease emerged, but in the Cox regression analysis a correlation between this parameter and clinical parameters was not found

[Ghanem et al., 2011]. In another study the critical Ki67 value in the blastemal component predicting metastases in cases of intermediate malignancy tumours was determined [Berrebi et al., 2008]. However, some scientists also concluded that Ki67 immunohistochemical detection does not provide a significant contribution to the evaluation of the possible prognosis in cases of nephroblastomas treated in the preoperative period [Juric et al., 2010] and the identification of this immunohistochemical marker in prognostic purposes has not been implemented in clinical practice. In the study currently presented, the results are more indicative of the fact that identifying the Ki67 expression in order to determine the prognosis is not reasonable. However, the fact that all the present results apply only to favourable histologic type nephroblastomas represented in the studied group has to be taken into account.

As already noted above, the role of p53 immunoreactivity in the case of nephroblastoma is well researched. Overall, most part of the researchers note the association of p53 expression with tumour anaplasia level and poorer prognosis [Govender et al., 1998]. Immunohistochemically analysing the p53 expression in case of different histological patterns, a significant difference was detected (p=0.001) between nephroblastomas with favourable histology or nuclear atypia, and anaplastic tumours: namely the quantity of p53 positive cells in the respective groups is 8.3%, 4% and 76% [Hill et al., 2003]. In the presented promotional work all the analysed primary nephroblastoma cases belonged to the group of favourable histology formations. Therefore in essence the average number of p53 positive cells 9.71% in the blastemalcomponent, 1.74% in the stromal component and 6.35% in the epithelial component detected in the study does not contravene to the one reported in publications. According to the literature data, in this group p53 expression is not as important as in the case of anaplastic tumours. For instance, when researching separately only the favourable histology nephroblastomas, in the case of primarily administered nephrectomy a statistically significant correlation between p53 expression and tumour stage (p> 0.3) or the prognosis for the patient (p> 0.3) was not detected [D'Angelo et al., 2003]. At the same time, other researchers do not detect a correlation of p53 expression with the tumour anaplasia degree, however it was more frequent in blastemal type nephroblastomas compared to stromal and epithelial type tumours. A correlation between the stage of the tumour and the number of p53 positive cells and less frequent (p=0038) nephroblastoma metastasis development in the low intensity response group compared to the high intensity response group was detected as well [Sredni

et al., 2001]. The results of the study currently presented suggest that the increased expression of p53 is of importance in the case of favourable histology. A statistically significant increase in the response rate in the blastemal component of the metastases/recurrence group compared to primary tumours (p=0.03) was detected in the study. Of course, in this case, it is a debatable issue whether the result is to be attributed to the favourable histology tumour group, because primary tumour material was not available in all the cases of metastasis in order to determine the stage of anaplasia. Comparing p53 expression in those cases where for both the primary tumour and metastases material is available; it is evident that high expression in the metastatic tissue can be found. However, in this case it would be more appropriate to talk about histological changes in the structure of the tumour during the increase of the aggressiveness of the process. It can be seen that in one of the primary tumour cases the formation belongs to the epithelial type and it practically does not contain a blastemal component. The mentioned patient does not have a blastemal component in the tissue of the first metastasis, whereas the second metastasis consists of blastemal cells. In one more of the analyzed cases in the primary tumour there are practically no blastemal cells. They have been found only in metastatic tissues. In respect to the analyzed primary tumour group there was a significant increase in p53 expression in the epithelial component of epithelial type formations (p=0.02) compared to the same response in the epithelial component of another type of formations. Also a significant increase in p53 expression in the epithelial component in the case of tumour progression (p=0.039) was detected. This suggests that in the case of favourable histology, attention should be paid to the positive p53 expression in the prevailing component of the specific histological type also in the case of relatively low level of the immunohistochemical reaction. There was the average expression level of the p53 in the epithelial component of those primary tumours, where the detected tumour progression was 22,69%, which is usually also interpreted in research as a low response rate [Govender et al., 1998]. In those cases, where the progression has not been found in turn, the mean number of p53 positive cells was 4.63%, which can be considered to be a negative reaction.

When researching the number of CKAE1/AE3 positive tubular structures in different nephroblastoma groups a statistically significant reduction in the number of immunohistochemically positive epithelial structures in the case of a recurrent process in comparison with the primary nephroblastomas is detected (p=0.005). It is also in line with the previously

described alterations in the histological structure of the tumour with the increasing aggressiveness of the process, when the proportion of the epithelial component in the metastatic tissue decreases, while the proportion of the blastemal component compared with the primary tumour material in the same patient increases. Findings in this case possibly indicate that the epithelial component is less aggressive and they generally do not contradict to literature data on the relation of the intense epithelial structure generation in the tissue of the tumour with better patient prognosis [Lawler et al., 1977]. At the same time, the findings obtained in the study are not complete in order to validate the idea expressed by other researchers that the epithelial type nephroblastomas are less aggressive and should be separated out from intermediate malignancy tumour group [Verschuur et al., 2010], as significant differences in the number of tubular structures between the groups of surviving and deceased patients, as well as in tumours with and without clinically detected tumour progression have not been discovered. The findings also contradict the increased p53 expression in the epithelial component of the epithelial formations, which may indicate a poorer prognosis. The result however is significant enough to continue further studies, because currently in the case of tumours untreated before surgery, both epithelial and blastemal type nephroblastomas are in the intermediate malignancy tumour group. The established positive relationship ($p=0$) between CKAE1/AE3 and e-cadherin number of positive structures does not have a scientific value, because both of them are epithelial markers. Rather, the mentioned findings could serve as a kind of internal control for statistical data analysis. The detected increase of CKAE1/AE3 positive structures in regressive type tumours ($p=0.015$) compared to other types in turn causes interest. These findings cause certain doubt, because logically the number of CKAE1/AE3 positive structures should have been highest in the case of epithelial type nephroblastomas. It should be noted, however, that the regressive type nephroblastomas with a high number of CKAE1/AE3 positive structures have been found specifically by performing excision of the material during the study. In this case, in more than a third of the material the tumour tissue was not retained, whereas in the rest of it formation of intense mature epithelial tubular structures was not observed. Thus there is the issue of further research of regressive type tumours and it cannot be excluded that the currently existing intermediate malignancy nephroblastoma group should be subdivided further, making a separate distinction for the regressive type nephroblastomas.

In terms of the established correlation of the number of CD34 positive microvessels in the epithelial structure (p=0.004), it should be noted that also in this case the highest mean number of microvessels per visual field was detected in regressive type tumours. This finding could affect the result and may indicate the need for further study of regressive type tumours and the impact of the number of epithelial structures on the overall results. At the same time, when separately researching the CD34 expression no difference was detected in tumour tissue between the primary tumour groups analysed or when comparing primary and metastatic tumours. The mentioned result does not confirm the literature data on the possible impact of the number of microvessels on the prognosis [Abramson et al., 2003], but correspond to the research results of those authors, who do not confirm the correlation of the microvessel count on the prognosis or association with histological types [Ghanem et al., 2011].

When researching e-cadherin expression, a statistically significant difference was discovered when comparing low and intermediate malignancy tumours with high malignancy tumours (p=0.049), which corresponds to the decreased immunohistochemical e-cadherin expression in the case of high stage nephroblastoma in comparison with low stage formations described in literature [Safford et al., 2005]. Also lower response rate in the metastatic tumour tissue compared to primary formations presented in the publications [Alami et al., 2003] has been detected, (p=0.046). Both of these findings could nevertheless be associated with the impact of the histological type of the tumour on the result, because blastemal type tumours after preoperative chemotherapy prevail among the high malignancy tumours. In the case of metastatic formations, increase of the blastemal component in the tumour tissue in relation to other components is also observed. However, it is interesting that lower e-cadherin expression in primary tumour tissue has been found also in those cases where progression of the formation was observed on the background of chemotherapy (p=0.05). Similarly, there was no statistically significant relationship between any of the histological tumour types including epithelial and regressive. The described results also suggest that e-cadherin immunohistochemical expression could also have a prognostic significance independent of the histological type. However, significant alterations in response intensity in the groups of deceased and surviving patients were not detected, which corresponds to the findings of other authors in which no relationship between e-cadherin immunohistochemical expression, tumour stage and patient survival was detected. In the quoted publication however a statistically significant increase

in response rate (p=0.003) in the case of preoperatively administered chemotherapy was established [Ramburan et al., 2006], which was not found in the presented study.

In the analysis of WT1 protein expression no significant differences in intensity of immunohistochemical response, depending on the tumour stage, histological malignancy level or type were established. Significant alterations were not observed in the expression in surviving and deceased patient groups either, as well as in the cases, when tumour progression was established. This finding did not correspond to the correlation of the WT1 protein expression with higher tumour stage, advanced nephroblastoma course and tumour-induced death detected by other authors in the case of nephroblastomas treated during the preoperative period [Ghanem et al., 2000]. However it has to be said that the same group of authors, when examining intermediate malignancy tumour tissue, untreated before surgery, have not found a correlation between WT1 protein expression in any of the histological components and tumour stage, at the same time detecting a correlation of the blastemal component response with progressive course of nephroblastoma [Ghanem et al., 2011]. It should be noted, that in respect to the histologically determined tumour malignancy levels a Czech researcher Kateržina Taran has not detected a relationship between them and the WT1 protein expression in the epithelial and blastemal tumour component [Taran et al., 2008]. In the presented study a statistically significant difference with a p level of 0.009 between the WT1 protein expression in cases where the tumour response to chemotherapy has been clinically considered as good and in insufficient regression cases was detected. It must be said that the prognostic significance of this finding is very disputable. The tumour group with clinically detected poor response to chemotherapy was very heterogeneous. Of the five examined tumours two were of high malignancy stage with retained prevailing blastemal component even after administered chemotherapy. In these cases, poor response to therapy should indeed be associated with increased malignancy potential of the formation. In 1 case a cystic partially differentiated nephroblastoma was detected, the malignancy potential of which was low. In this case absence of reduction of tumour size is rather attributable to the fact that well-differentiated tumour elements are less sensitive to the administered treatment. In one case regressive type nephroblastoma was detected, whereas the histological type was determined in the review of preparations without a full review of all macroscopic material, and in one case mixed nephroblastoma was detected.

Thus, the issue whether the reduced WT1 protein expression in this case could be attributed to the increase or decrease in tumour malignancy potential, remains open.

4.2.2. The evaluation of the genetic alterations of the tumour suppressor gene CDKN2A/p16^{INK4a}

In assessing 442nd nucleotide G replacement to A (c.442G> A) obtained in the result of sequencing CDKN2A locus DNA in the 50% of the analyzed samples, which is significantly more frequent in the analyzed group than in the control group ($p<10^{-7}$), the issue of clinical significance of this alteration exists. In the research of genetic changes the fact that there are many human genome 'structural variations' is significant. These changes can manifest both on the microscopic and submicroscopic level as deletions, duplications, and DNA segment copy number variations or insertion, inversion and translocation. The amount of evidence that these structural variants within the genomic heterogenity can involve millions of nucleotides in each genome rapidly increases. Thus separate nucleotide polymorphisms are found in more than 1% of the human population and it is possible that more than approximately 10 million of such single nucleotide polymorphisms exist [Feuk et al., 2006]. The alteration 442G> A mentioned in part of the literature in respect to the CDKN2A locus is also considered to be a gene polymorphism [Puig et al., 2005]. In the study carried out in Latvia in 2007 the mentioned CDKN2A locus second exon polymorphism 442G> A was detected in melanoma patients, and its frequency was statistically significantly increased in melanoma patient group compared to the control group [Pjanova et al., 2007]. Talking of the researched group of nephroblastomas the mentioned alteration could be interpreted not as a gene mutation, but as a polymorphism. The result obtained in the p16^{INK4a} protein immunohistochemical detection is also more indicative of that. Comparing the DNA sequencing results with the p16^{INK4a} protein immunohistochemical expressiveness, it has been established that a statistically significant difference between the mentioned immunohistochemical reaction intensity in the histological components of patients with the second exon wild type and in patients with detected 442nd G nucleotide replacement with A in the second gene exon, has not been detected (p=0.086, p=0.177). Therefore the mentioned alteration has no effect on p16^{INK4a} protein synthesis and most probably does not have a relevant significance. However, the study results do not completely exclude genetic influence of CDKN2A locus on the

origin of nephroblastoma, because when analyzing the results obtained in microsatellite research LOH is detected in the case of four analyzed microsatellites D9S736, D9S1604, D9S942 and D9S171, which may suggest the influence of different genes existing in this locus. In one more case loss of heterozygosity was detected when analysing the marker D9S1604, which is located in the intron before CDKN2A/p16^{INK4a} gene, in one case, loss of heterozygosity was detected in the analysis of marker D9S736, which is located behind the CDKN2A gene last third exon indicating that potentially nephroblastoma development related genetic changes in this locus would be searched behind the CDKN2A gene. In one case, loss of heterozygosity was found in the marker D9S942, as well as in the marker D9S171 at the same time. The mentioned markers are located respectively in the intron between exons 1α and 1β and before CDKN2A gene exon 1β, indicating the deletion of a larger region. However a statistically significant difference between the protein expression in any of the histological components in both groups with and without the loss of heterozygosity was not detected (p=0.88; p=0.75; p=0.091), which is more indicative of the fact that these alterations are not associated with the CDKN2A/p16^{INK4a} gene. In a similar study, the authors, when researching the CDKN2A locus alterations in nephroblastoma tissue, detected heterozygosity loss in chromosome 9 in CDKN2A/2B locus between microsatellites D9S932 and D9S265 in 35% (12 of 34) of the analyzed tumour cases, heterozygosity loss between D9S286 and D9S775 microsatellites in 24% (8 of 34) cases and heterozygosity loss when analysing microsatellite marker D9S1748 in 12% of the cases (4 of 34), which suggests involvement of other genes of the locus in the origination of nephroblastoma. No relationship between the result obtained in microsatellite analysis and p16^{INK4a} protein expression or clinical data was detected in the study either [Natrajan et al., 2008].

5. CONCLUSIONS

1 During the period from January 1997 to July 2012 the most frequently diagnosed paediatric kidney tumour in Latvia is nephroblastoma, which corresponds to the world data.

2 In Latvia rare paediatric tumours, such as renal clear cell sarcoma and rhabdoid tumour of kidney have been diagnosed and they occur in the percentage of 2.04% and 4.08%, respectively.

3 In the analyzed group renal cell cancer cases have not been detected, which may indicate a lower incidence of this tumour in Latvia for children less than 18 years of age, however the data should be confirmed in additional statistical research.

4 In the course of repeated immunohistochemical examination diagnostic inaccuracies in 4.08% of cases indicate the importance of this method in diagnosing paediatric renal tumours.

5. Detection of p53 immunohistochemical expression has a prognostic importance also in the case of favourable histology nephroblastomas, because there is a statistically significant relationship between the intensity of the mentioned response in the epithelial component of the tumours and the tumour progression.

6 The detection of e-cadherin immunohistochemical expression has a prognostic importance in the case of favourable histology nephroblastomas, because a statistically significantly decreased expression of e-cadherin in the metastatic tumours has been detected compared to the primary ones, the response rate in the case of high malignancy tumours compared to low and medium malignancy tumours is significantly reduced and e-cadherin expression in the case of tumour progression is decreased.

7. The expression of CD44s glycoprotein isoform in the blastemal component can be used for future research of favourable histology nephroblastoma malignancy potential, as there is a statistically significant positive correlation between CD44s and p53 expression in the blastemal component of nephroblastomas.

8 Intermediate malignancy favourable histology nephroblastoma of different histological types, especially epithelial, stromal and regressive, with a different malignancy potential which is indirectly indicated by significantly reduced CD44s expression in the epithelial component of epithelial type nephroblastoma, increased p53 expression in the epithelial component of

epithelial type formations, decreased CKAE1/AE3 positive epithelial structure count in the case of recurrent process in comparison with the primary nephroblastomas, CKAE1/AE3 positive structure count increase in the case of regressive type tumours, cannot be ruled out.

9. In the nephroblastoma group a statistically significantly more frequent c.442G> A CDKN2A/p16^{INK4a} gene alteration than in the control subjects has been found, however gene polymorphism can not be excluded, as indicated by the unaltered immunohistochemical expression of p16^{INK4a} protein.

10. Absence of heterozygosity in four of the analyzed microsatellite markers (one in the case of D9S736, D9S1604, D9S942, and D9S171) was detected, which means that the CDKN2A locus may be involved in the genesis of nephroblastoma, whereas the gene attributable to development of cancer yet has to be identified.

6. PRACTICAL RECOMMENDATIONS

When analyzing problems in differential diagnosis of paediatric tumours reflected in study results, for practicing children pathologists the following recommendations arise:

1) extend the immunohistochemical examination panel with a compulsory WT1 protein, neuroendocrine epithelial marker, as well as LCA, Ki67 and CD99 antigen detection, in differentiating blastemal type nephroblastomas from other paediatric 'small, round, blue cell tumours',
2) interpret a negative result only in a complex manner, taking into account the WT1 protein immunohistochemical positivity detected in the study only in 61.54% of the cases, by analyzing the obtained findings from immunohistochemical examination and using a sufficiently broad examination plan,
3) expand the immunohistochemical usage of mesenchymal markers, including the muscular ones in diagnosing of cellular mesoblastic nephroma,
4) engage in the work of inter - institutional paediatric kidney tumour study groups, in order to obtain advice on the diagnostics of rare tumours and to add material for research purposes,
5) develop cooperation between several national laboratories for the immunohistochemical detection of INI1 protein in the diagnostics of rhabdoid tumour of kidney and cytogenetic detection of (12, 15) (p13;q25) translocation for the diagnostics of cellular mesoblastic nephroma.

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8. LIST OF PUBLICATIONS AND REPORTS ON THE STUDY THEME

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