SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Immunohistochemical and clinicopathological examination of possible molecular markers of progression and recurrence in meningiomas

BY DR. TAMÁS CSONKA

Supervisor: Dr. Tibor Hortobágyi

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By: Dr. Tamás Csonka MD

Doctoral School of Neurosciences, University of Debrecen

Supervisor: Dr. Tibor Hortobágyi MD, PhD, DSc

Head of the **Examination Committee**: Prof. Dr. Miklós Antal MD, PhD, Dsc

Members of the Examination Committee: Dr. Judit Boczán MD, PhD
Dr. Zsolt Kopniczky MD, PhD

The Examination takes place at Library of Institute of Pathology, Faculty of Medicine, University of Debrecen

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Head of the Defence Committee:	Prof. Dr. Miklós Antal MD, PhD, Dsc
Reviewers:	Dr. Mónika Francz MD, PhD
	Dr. Béla Kajtár MD, PhD
Members of the Defence Committee:	Dr. Judit Boczán MD, PhD
	Dr. Zsolt Kopniczky MD, PhD

The PhD Defence takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
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1 Introduction and review of the literature

Central nervous system tumours require special knowledge in routine pathological workout, and for this reason in many neuro-oncological centres specialized neuropathologists are employed. Meningioma is a tumour frequently encountered in standard neuropathological practice. In most cases, diagnosis of these tumours is not problematic, although there are challenging cases, too. In affluent countries complementary molecular studies can also be helpful and are often available as part of routine work-up. However, in Hungary, the routine use of such techniques is currently far from reality; neither financial nor technical conditions are available.

The aim of this study is to establish an easy-to-use immunohistochemical (IHC) panel from the currently available reagents in pathological institutes for routine neuropathological use, which can predict meningioma relapse/recurrence, and can help with the questionable cases. For validation we analysed the changes in immunohistochemical characteristics and expression patterns during relapse/recurrence and examined their relation to tumour grade.

In our work, we investigated proteins using conventional immunohistochemical methods that can assist in the determination of meningiomas and can provide evidence for prognosis with regards to relapse/recurrence. It is also important in the recurrence of individual cases to establish what changes the second tumours went through, how they evolved; therefore we studied how proteins change in different samples of the same patient. We selected proteins which through DNA repair can significantly influence the development of tumours such as poly(ADP-ribose) polymerase (PARP1); and on the other hand, they are significant in preserving genome integrity and have been shown to be involved in many tumours (p53); and also manifest in the active phase of cell division (Ki-67, Mib1 antibody). We also studied the widely analysed progesterone receptor (PR), which is known to be inversely related to grade, but less is currently known about its relationship to recurrence.

1.1. Meningiomas

Meningiomas are meningothelial cell originated tumours that typically stick to the inner surface of the dura mater. Generally they are benign, although rarely poor prognosis may occur.

Meningiomas are one of the most common intracranial tumours, usually sporadic, but may also be familiar. Their incidence increases with age, are found most commonly in ages 60 to 70; and they show mild female predominance (1.7: 1). Meningiomas account for almost 24-30% of intracranial tumours. Their annual incidence is about 13/100000 inhabitants.

Meningiomas are typically slow-growing tumours. Depending on localization and size they may be clinically completely asymptomatic, but may cause focal pressure symptoms and consequent neurological deficits, diplopia, headaches or epileptic episodes. Meningiomas on the MRI are typically isodense, uniform contrast material enhancing nodules, which usually adhere to the dura mater. Possible calcification can be usefully investigated with CT scan and contrast material can be used to further clarify the diagnosis.

The ethology of meningiomas is not fully understood. But full skull irradiation is a well-known risk factor, specifically for irradiation involving scalps and survivors of nuclear bombs. The effects of sex hormones are even less clear. However, the fact that they predominant in women, and in the overweight, endogenous/exogenous hormone intake, smoking, and oestrogen, progesterone and androgen hormone positivity support a possible connection. Meningiomas were one of the first solid tumours in which cytogenetic abnormalities were described, such as monosomy or deletion of the long arm of chromosome 22. The deletion, insertion or nonsense mutation of Neurofibromatosis 2 gene on the 22 chromosome is found in 60% of sporadic meningiomas. In addition, epigenetic differences may play a role in their development (promoter hyper methylation, histone modification, micro-RNAs).

Meningiomas may be intracranial, intraspinal or orbital in localisation. In the skull most of them are located on the convex plane or parasagittal. Intraspinal meningiomas are most commonly found in the thoracic region. The

metastases of anaplastic meningiomas are mainly found in the bones, the lungs, the pleura and the liver.

Macroscopically meningiomas are rubbery firm, well-demarcated, sometimes lobulated, rounded nodules. Invasion of the related dura and dural sinuses may occur. In advanced cases, through the dura mater they can infiltrate to the skull bones. The surrounding brain often shows an impression, but cerebral invasion is rare, they usually grow microscopically around the brain vessels, but true intravascular infiltration is rare. Meningiomas with immunohistochemical examinations are generally positive for EMA, Vimentin and S100, but EMA in higher gradations is weak or less pronounced. The many tissue variants of meningiomas also determine their WHO grade. The definition of the grade is based on the *2007 WHO Classification of Tumours of the Central Nervous System*: histopathologic subtype, morphological appearance and increased mitotic activity.

Meningothelial meningioma (WHO grade I.): a common classical variant in which tumour cells form lobules, with a thin collagen septum. Tumour cells are uniform with oval nuclei, and a fine chromatin structure, in which pseudo-inclusions may appear. Within the lobe, tumour cells can grow in a syncytium-like fashion because the cellular projections form fine, complex forms which cannot always be differentiated by light microscopy.

Fibroblastic meningioma (WHO grade I.): a subtype which shows spindle-shaped cells that form parallel bundles in the collagen-rich extracellular matrix, with a focally typical appearance of a meningothelial meningioma.

Transitional meningioma (WHO grade I.): the mixed appearance of meningothelial and fibroblastic subtypes.

Other WHO grade I. subtypes: angiomatous meningioma, psammomatous meningioma, lympho-plasmocyte rich meningioma, metaplastic meningioma, microcytic meningioma and secretory meningioma.

Atypical meningioma (WHO grade II.): meningiomas belonging to this group have increased mitotic activity (more than 4/10 high power field, hpf), brain invasion, or at least 3 of the following characteristics: increased

cellularity; small cells with a large nucleus:cytoplasmic ratio; a prominent nucleus; a patternless growth pattern; focal or "map-like" necrosis. Tumours that meet these criteria are eight times more likely to recur than the WHO grade I. meningiomas.

Other WHO grade II. meningiomas: chordoid meningioma and clear cell meningioma.

Anaplastic meningioma (WHO grade III.): in these cases the tumour expresses a malignant tissue pattern similar to the tissue appearance of carcinoma, melanoma, or high-grade sarcoma. It is characterized by a high mitotic division (more than 20/10 hpf), high Mib1 labelling index and extensive necrosis. These tumours are highly aggressive, with an average survival of 2-5 years.

Other WHO grade III. meningiomas: papillary meningioma and rhabdoid meningioma.

In 2016, the WHO revised the 2007 classification for central nervous system tumours, including meningiomas: cerebral invasion may occur in benign and aggressive tumours, and the rate of relapse equals atypical tumours. At present, the behaviour of the tumour is indicated by clinical factors and tissue parameters. In the case of brain invasion, tumour cells form irregular, tongue-like projections in the brain, with reactive astrocytosis. Extracranial metastases are extremely rare (1/1000 meningioma).

The rate of recurrence is different in each of the grades: 7-25% in WHO grade I., 30-50% in the WHO grade II. And 50-95% WHO grade III. For meningiomas, both progression-free survival and overall survival are closely related to WHO grade. In routine diagnostic practice, we need one or more "atypical" or "malignant" markers; and we need markers to determine the high chances of relapses/recurrences. The p53, the PARP1, the Mib1 or the PR may be such candidate.

1.2. PARP protein family

The PARP (Poly(ADP-ribose) polymerase) protein family consists of 17 members, their main function is single-stranded DNA repair and participating in programmed cell death. If a DNS error is detected, the DNS binding domain is attached to the defective DNS thereby causing a conformational change in the protein. To connect to the programmed cell death, the caspase cleavage domain is required.

PARP1 (also known as ADP-ribosyltransferase D-type 1, ARTD1) is responsible for about 90% of PARP activity, and is a 116 kDa protein found in the nucleus. Its gene is located on the long arm of chromosome 1 (1q42.12). It can be activated by exogenous and endogenous genotoxic effects, such as chemical (oxygen and nitrogen free radicals), physical (radius, heat) or metabolic (hypoxia, increased extracellular glucose concentration, hormone, steroid) factors. The activated enzyme binds to DNA and produces Poly (ADP-ribose) (PAR). PAR activates other proteins (DNA ligase III, DNA polymerase beta, XRCC1) and the base excision repair is started. Subsequently, the polymer is disrupted by the PARG. Activated PARP1 is capable of reacting with other nuclear enzymes by PARylating them, thereby increasing their negative charge, preventing interaction with other anionic molecules, like the DNA. In addition to the DNA correction function, activated PARP1 plays an important role in apoptosis by activating the 'caspase' pathway, causing the apoptotis-inducing factor to migrate from the mitochondria to the nucleus. However, in case of severe DNA damage, ATP deficiency can cause necrosis. In addition, PARP1 'knock out' cells decrease single strand breaks, the DNA replication villa stops and double strand breaks are recovered by homologous recombination. For this reason, PARP1 deficient cells exhibit hyper-recombination phenotypes.

Previous studies have shown that PARP1 plays a role in BRCA1 / BRCA2 mutant breast tumours. PARP inhibitors are capable of enhancing the effect of chemotherapeutics by inhibiting single strand DNA repair, while repairing double strand DNA fractures has also been impaired due to BRCA mutation. The role of PARP1 has been studied in many solid tumours but, as far as we know nobody has investigated them in meningiomas.

1.3. p53 protein

p53 is a 43.7 kDa weight protein. Its different homologues are important proteins in all multicellular organisms, by inhibiting the formation of tumours. p53 coded by the TP53 gene can be found on the short arm of 17 chromosomes (17p13.1), and is one of the most important tumour-suppressor proteins in humans. The physiological function of p53 is to regulate the cell cycle, preserve the stability of the genome, and prevent mutations. p53 may be activated by DNA damage, oxidative stress, osmotic shock, ultraviolet light, ionizing radiation, ribonucleotide depletion or oncogene expression. Activation causes a significant increase in the half-life and changes the conformation of the protein. Normally, Mdm2 is responsible for the low level of p53: it blocks the function and is also responsible for its degradation.

The anti-tumour activity of p53 can be accomplished in different ways: it activates DNA repair enzymes; creates a cell proliferative barrier at the G1/S regulatory site, thereby providing sufficient time for the repairing enzymes to correct the DNA defect; and it can induce apoptosis if the DNS damage is irreversible. TP53 is the most common mutant gene in human tumours (detectable in 50%, with a recessive trait). p53 resulting from genetic modification can be demonstrated by immunohistochemical methods, which correlates well with the mutation status. However, in the case of elevated p53 levels in physiological conditions, a false positive result can be obtained. In congenital p53 mutations (Li-Fraumeni syndrome), they often develop tumours in early adulthood (sarcoma, breast tumours, glioblastoma, adrenal tumours, hematologic tumours).

The role of p53 has been previously studied in meningioma: many studies yielded negative or uncertain results, while others found significant correlations between p53 status and tumour grade or recurrence.

There have been numerous publications about the relationship between PARP1 and p53, and how PARP1 regulates the function of p53 both directly and indirectly via the polymers and the promoter of MTA1.

1.4. Progesterone receptor

Progesterone receptor (PR) is a steroid receptor. PR is encoded by the PGR gene located on the long arm of 11 chromosomes (11q22). PR has two isoforms: PR-A and PR-B, which are identical except for the 165-amino acid portion of the PR-B 'N terminal, which has the function of transcription activation.

When the progesterone hormone binds to its receptor, it undergoes dimerization and transports to the nucleus where it binds to DNA and induces transcription. The resulting mRNA migrates into ribosomes to trigger protein-specific translation. PR's physiological role lies in the maintenance of female hormonal cycle regulation, ovulation, pregnancy, and breast tissue development during puberty. The progesterone hormone plays a role in breast carcinogenesis. Synthetic progesterone analogues and prolonged ovarian activity increase the chance of breast cancer, but bilateral ovarian removal can also reduce breast cancer risk by 50%.

It is known that meningiomas are positive for anti-PR antibodies and that the proportion of positive cells is inversely proportional with the WHO grade, which can help in determining the grade in questionable cases. Previous research has also revealed that the PR cellular biosynthesis is not an oestrogen-controlled process in meningiomas, as in other sex hormone dependent tissues; only some cases are oestrogen positive.

1.5. Ki-67 protein, Mib1 antibody

The Ki-67 is a nuclear-based protein, which is encoded by the MKI67 gene located on the 10th chromosome (10q25-ter). The 395 kDa and 345 kDa weight Ki-67 is a huge protein of two isoforms encoded by 30,000 base pairs in the human genome. Its exact function is still unknown. We know that the Ki-67 protein is essential for cell division; it can be found in all the active phases of the cell cycle (G1, S, G2, M), but cannot be detected in G0 phase cells. In addition, Ki-67 is essential for ribosomal RNA transcription. During the

interphase it is only found in the nucleus, but during mitosis the protein binds to the surface of the chromosomes. During the mitosis the protein has phosphorylation and dephosphorisation. Some researchers presume that the Ki-67 protein is involved in the organization of DNA. While others belive that it is binds to SNA or RNA, forming complexes with other proteins, however, Ki-67 participate in the synthesis of ribosomes during cell division.

Mib1 (Molecular Immunology Borstel 1) as a proliferative marker is the most commonly used monoclonal anti-Ki-67 antibody. The Mib1 antibody can be used on formalin fixed and paraffin embedded samples after pre-microwave treatment or heat-induced antigen recovery. In routine pathological workup Mib1 is particularly important in cases of lymphomas, melanocytic tumours and cervical intraepithelial neoplasia, in determining the dysplasia in ulcerative colitis and the differentiation of basaloid carcinoma from basal cell hyperplasia in prostate. The Mib1 labelling index (Li) has a strong correlation with tumour growth rate, chances of relapse/recurrence and survival, i.e. prognosis of hematologic and solid tumour types, including meningioma. Some authors have found that p53 positivity and high proliferative index also indicate a worse clinical behaviour even in benign tissue histology. It is now also known that the proportion of proliferative fraction is inversely related to relapses/recurrences.

2. Material and methods

During routine pathological processing, after at least 24 hours but no more than 168 hours in 10% buffered formalin fixation the macroscopic descriptions of specimens were made. The portions of the lesions for microscopic examination were put in plastic cassettes. This was followed by the dehydration phase of the samples, on the automatic *Shandon Pathcentre Tissue Processor* (Thermo Fisher Scientific Waltham, Massachusetts, USA).

The dehydrated tissue samples were embedded in paraffin, producing 'paraffin blocks'. From these, 4μm thick sections were made using "wheeled microtome" (*Leica RM2245* - Semi-Motorized Rotary Microtome, Leica Biosystems, Wetzlar, Germany) on silanized slides, followed by hematoxylin-eosin staining (H&E) in automated and standardized conditions using the *Leica ST 5020* (Leica Biosystems, Wetzlar, Germany) automatic machine. Histopathological reports from H&E stained sections were stored in the medical database of *MedSolution* (T-systems Hungary Ltd., Debrecen Support Centre) and in the archives of the institute.

The samples required for our retrospective investigations were searched in the *MedSolution* database. The FFPE blocks of the selected cases were found in the institute archive. All cases were re-screened by a neuropathologist, classified into histological subtypes and relevant WHO grading according to the 2007 *WHO Classification of Tumours of the Central Nervous System*.

Immunohistochemistry investigations were always carried out under automated and standardized conditions. 4 μm sections were made from FFPE sections. Manual method was used for detecting antigen retrieval and antibody dilution. Next we used the automated *Leica Bond Max* ™ immunohistochemical machine with the *Bond* ™ *Polymer Refine Detection Kit* on the samples. The immunohistochemical reaction was always performed according to the manufacturer's protocol under standardized conditions, with negative controls. The 4 micrometre sections were labelled with PARP1 polyclonal rabbit antibody, p53 monoclonal mouse antibody, progesterone receptor monoclonal

antibody, and monoclonal anti-Ki67 antibody. Antibodies were diluted 1: 500 for PARP1, 1: 100 for p53, 1: 100 for PR and 1: 200 for Mib1.

Our investigations were carried out in two rounds.

2.1 First experiment round

Firstly we performed PARP1 and p53 immunohistochemical reactions on a small patient group: 41 samples of 31 patients with meningioma. 100 randomly selected cells were counted in 10 fields of view at 400X magnification. We measured the intensity of the labelling as negative (0+), weak (1+), medium (2+), or strong (3+) nuclear staining.

On the basis of the staining we calculated two parameters (staining intensity, Si): ratio of 1+, 2+ and 3 + cells (Si1-3), and the ratio of highly stained 2+ and 3+ cells (Si2- 3) relative to all the counted cells.

The results were analysed with *SPSS 19.0 for Windows* statistical software. Since the samples did not show normal distribution, we used nonparametric statistical tests. The *Kruskall-Wallis H test* can be used to compare 3 or more different groups; the *Mann-Whitney U test* is suitable for comparing two non-normal distribution groups. By means of these statistical tests, we investigated whether the given samples could originate from the same population. Where the significance level was sufficiently low (<0.5; <0.1; <0.01) we were able to state that the samples were from different populations; that is they were statistically different. The non-parametric *Spearman's rank order correlation test* also reveals whether the relationship between the two parameters can be described with a monotonic function (for each X there is only one Y value in the coordinate system and vice versa). If so, it can be said that changing one value can be determined by changing the other value; that is the changes are related to one another.

First for both proteins (p53 and PARP1) we tested with *Kruskall-Wallis H tests* both Si1-3 and Si2-3 according to the three WHO grades. Then we compared the individual WHO grades (WHO grade I.-II., I.-III. and II.-III.) with *Mann-Withney U test*. Two groups were then made on the basis of the WHO grade: low-grade (WHO grade I.) and high-grade (WHO grade II. and III.) and these values were compared with *Mann-Whitney U test*. *Spearman's rank order correlation test* was performed to determine the independence of the immunohistochemical labelling of PARP1 and p53 proteins.

2.2 Second experiment round

As a second step, we performed studies on a larger patient group, with a total of 114 surgical specimens from 70 patients. Samples were selected retrospectively within a 16-year interval. Patients with one or more relapses/recurrences were classified in the R/R group. The patients in the non-R/R group had no relapsed/recurrent tumour detected by radiological studies or during autopsy.

By selecting one or two representative blocks from each case, TMA (tissue micro array) was constructed from cylinders of the most typical areas based on the morphology of the particular tissue sample. Each TMA contained 10 recipient blocks of samples: 3-3 cylinders per sample; and 2 marker cylinders. Thus, a total of 12 TMAs were built, which included all 114 specimens. Hematoxylin & eosin (H&E) staining and immunohistochemical (IHC) reactions were performed on these TMAs.

Each H&E and IHC section was digitized on the *Pannoramic Scanner*. From each of the digital samples six images were extracted (2-2 from each cylinder cross-section) on 400x magnification. Based on the intensity of the positive nuclei, 4 groups were created: 0, 1+, 2+ and 3+.

In many cases, in the beginning of our studies we calculated the number and ratio of positively staining cells (labelling index, Li) exactly, with the Cell Counter function of the *ImageJ* program, and later we used these as templates

for comparison. By multiplying the ratio and strength of staining cells, Histoscore was assigned also to each histological sample.

Results were analysed using the *SPSS 22.0 for Windows* statistical program. As samples were not normal, we used non-parametric tests*: Kruskall-Wallis H test, Mann-Whitney U test, and Wilcoxon signed rank test*. The *Wilcoxon signed rank test* is a non-parametric equivalent of the two-sample t test for examining interrelated patterns. Using this method we studied the changes in the different surgical samples from the same patient.

The number of ethics license: DEOEC RKEB: 2437-2005

3. Results

3.1 Results of the first experiment round

All examined 41 samples showed positivity for PARP1 and p53 antibody. *Kruskall-Wallis H test* showed significant correlation between the WHO grade and the PARP1 expression (Si1-3, p=0.001) as well as p53 intensive labelling (Si2-3, p=0.012). In contrast, there was no statistically significant correlation between Si2-3 PARP1 (p=0.523), Si1-3 p53 (p=0.141) staining and the WHO grade.

With *Mann-Whitney U test* PARP1, Si1-3 showed a significant correlation with WHO grade I.-II. (p=0.001) and WHO grade II-III. (p= 0.005). In addition, in the case of p53, Si2-3 showed a significant association with WHO grade I-III. (p=0.002). The peak intensity was shown by the WHO grade II. tumours for the PARP1, whereas peak intensity was shown by theWHO grade III. for the p53.

Comparisons of low grade tumours (WHO grade I.) and the high grade tumours (WHO grade II. and III.) showed a significant correlation Si1-3 (p=0.028) in the case of PARP1, while there was significant correlation for Si2-3 (p=0.018) with the p53 staining. The histopathological subgroup of tumours was as follows: WHO grade I.: 11 meningothelial, 8 transitional, 1 secretory, 1 fibroblastic and 1 microcytic; WHO grade II.: 8 atypical and 3 clear cell; WHO grade III.: all of them were anaplastic. There was no significant difference between the individual histological subtypes, neither for PARP1 nor p53. Spearman's rank order correlation study showed no relationship between PARP1 and p53 immunoassay.

3.2. Results of the second experiment round

There were 16 patients (3 men, 13 women; mean age 54 years) who had not had a relapse or recurrence of their tumours at least 5 years after their first operation (non-R/R group). In 31 patients (8 men, 23 women, mean age 53

years, average recurrence time 19.6 months), tumour recurrence was confirmed by imaging or pathological studies (R/R group). There were 23 patients (5 males, 18 female, mean age 59 years) who had had a single operation due to meningeal tumour less than 5 years prior to the end of our studies and no relapses had been confirmed. However, due to the short time window, these patients were excluded from non-R/R group.

Of the examined samples, 65 were classified as WHO grade I., 33 as WHO grade II. and 16 as WHO grade III. All non-R/R cases belonged to WHO grade I. The R/R group contained 19 WHO grade I., 9 WHO grade II. and 3 WHO grade III. during the neuropathological study from the first operation. Eight of these patients showed progression in their grade and 15 showed the same grade in both the first and last specimen. Six patients in the R/R group had only one histological sample. In their cases, the relapse was verified by the imaging test without tissue sampling. There were no significant differences in the tissue subtypes among the groups: 6 meningothelial, 5 transitional, 3 fibroblastic and 2 psammomatous in the non-R/R group; whereas 9 meningothelial, 6 transitional, 3 fibroblastic, 1 psammomatous, 1 clear cell, 8 atypical and 3 anaplastic were found in the R/R group. No particular tissue subtype of WHO grade I tumours was onstanding in terms of relapse/recurrence, nor was any histological subtype from WHO grades II. and III. recorded as outstanding (Noting that the test sample count was low for each subtype).

The individual WHO grades compared with *Kruskall-Wallis H test* showed significant correlation with the Mib1 percentage, intensity and Histoscore; p53 intensity and Histoscore; PR percentage, intensity, and Histoscore. When compared by *Mann-Whitney U test* WHO grade I. and II. showed a significant correlation with the Mib1 percentage, intensity and Histoscore; p53 intensity; PR percentage, intensity, and Histoscore. Comparison between the WHO grade II. and WHO grade III. indicate significant differences between the p53 intensity; the PR percentage, intensity and Histoscore. Among the WHO grade I. and WHO grade III. we found significant correlation between the percentage of Mib1 and Histoscore; p53 intensity and Histoscore; PR percentage, intensity, and Histoscore.

During the comparison of R/R and non-R/R groups a significant correlation between Mib1 percentage, intensity and Histoscore; p53 percentage and the WHO grade was found. However, when we compared the WHO grade I. members of the R/R group to the non-R/R group (each case was WHO grade I.), it was found that there was a significant correlation between the percentage of Mib1, Histoscore; p53 percentage and Histoscore.

Finally, we compared the first and last histological samples from the R/R group: there was a significant correlation between the Mib1 percentage, Histoscore; p53 intensity and WHO grades. With *Wilcoxon signed rank test* comparison of the first and last histological samples of each patient showed a significant differences between the WHO grades, Mib1 percentage Histoscore; and p53 percentage.

4. Discussion

As one of the most common intracranial tumours, meningioma has many morphological variants. According to the current (2016) WHO classification, 13 histological variants can be distinguished. Nine of them are among the WHO grade I., 2 belong to the WHO grade II. and 2 to the WHO grade III. The atypical and anaplastic can be diagnosed on the basis well defined morphological criteria (tissue appearance, mitosis number, brain invasion), although this is sometimes difficult. Until now, there has been no reliable immunohistochemical marker that could help to differentiate between the WHO grades. However, it is true that the Simpsons (surgical) grade can help in judging the chances of recurrence. Meningiomas are essentially non-infiltrating tumours, so surgical removal is generally curative. However, small tumour nests may appear along the dura mater, which may be a source of recurrence. Since chemotherapy is not effective in such cases, and radiotherapy increases the chance of malignant transformation, this is another pressing argument for finding simple markers that help assess the tumour progression and recurrence. Today's imaging studies provide a number of valuable information to assist the surgeon's work (localization, relationship to anatomical sites, vascularity, oedema), but also prognostic aspects: dural tail, and possible existing tumour nests along the dura. At present, the meningeal patient management at the University of Debrecen, in line with the international guidelines, contains a follow up: after a postoperative imaging study - for possible residual tumour examination – MRI examination in every six months for one year, and every year for four years to detect possible relapse. If there is no diagnostic recurrence, they will be treated as healed.

During our studies we conducted a retrospective analysis of the neurosurgically acquired histological samples from patients diagnosed with meningioma in the course of the last 16 years at the Department of Pathology, Faculty of Medicine, University of Debrecen. The available samples were classified according to the 2007 WHO recommendation. In the course of our investigations, two proteins involved in DNA repair and the preservation of genome integrity were investigated; the PARP and p53, respectively. The

relationship of these proteins with the WHO grade was also investigated. In addition, we analysed recurrent meningeal cases and sought protein markers from routine pathological practice such as Ki-67, progesterone receptor, p53, which could assist in judgement about the chance of recurrence.

PARP1, the most important and most widely studied protein from the PARP family has a well-known and important role in caspase independent apoptotic pathway and necrotic cell death. PARP1 has been studied in several tumours: breast cancer, ovarian, endometrial, pancreatic tumours, stomach tumours, certain colorectal malignancies, germinal tumours, prostate carcinomas, melanoma, Ewing's sarcoma, neuroblastomas, glioblastomas; but as far as we know no one has investigated its role in meningiomas.

p53 is one of the most important tumour suppressor proteins, it is significant in about half of the human malignancies. Numerous studies have been conducted on the p53 marker in meningiomas, but they have yielded varying results; some of them had a negative result and others showed a correlation between p53 status and WHO grade or event of recurrences.

All 41 samples tested in the first round showed positivity for both PARP1 and p53 antibodies. For PARP1, the ratio of positive cells (Si1-3) was the highest in the WHO grade II. tumour; in the case of p53, the positivity (Si2-3) showed a gradual increase and WHO grade III. tumours proved to be the highest. Compared to the low-grade (WHO grade I.) and high-grade (WHO grades II. and III.) meningiomas, higher Si1-3 was found for PARP1, while the Si2-3 value was higher for p53 in the high-grade cases. *Spearman's rank order correlation test* and linear regression analysis showed no correlation between PARP1 and p53 labelling, confirming that these two markers are not dependant to each other.

We assume that PARP1 activation increases in the WHO grade II. tumours can occur because the cells are trying to cope with DNA defects, while in the WHO grade III. tumours the PARP1 activity decrease may be due to apoptotic/necrotic cell death and lack of protein substrates. According to our results, the p53 protein plays a role in meningiomas, and this is likely to be a late occurrence of meningioma’s tumour progression. Based on our experience,

the presence of p53 protein showed a steady increase and the highest values were found in the WHO grade III. group. Interestingly, for PARP1, the significant correlation was seen in the ratio of any labelling cells (Si1-3), while in the case of p53 only in the proportion of intensive 2+ and 3+ cells (Si2-3). We assume that the reason for this is that while the PARP1 protein is practically no detectable in intact dura cells, the wild type of p53, which is also labelled with the antibody, can produce a weak reaction (1+). This also confirms our hypothesis that the more intense mutant p53 is related to the tumour grade and is not affected by the potential physiological accumulation of the wild-type protein. Since both proteins showed higher markers in the examination of 'high grade' (WHO grade II. and III) tumours, the role of both proteins can be assumed in cases of worse prognosis. Although further studies are required, our findings have shown that PARP1 and p53 may help to diagnose and determine the WHO grade in meningiomas.

In the second part of our research we were looking for an easy-to-use immunohistochemical panel for everyday neuropathological practice which could help to determine the likelihood of relapse/recurrences of meningiomas. This could be a great help with problematic localisation (such as falx cerebri) tumours.

The labelling index of the Mib1 marker may be different in each laboratory, but in standardized conditions it may help in comparing cases both for scientific and routine diagnostic testing. Earlier studies have shown that higher initial Mib1 labelling indexes more aggressive behaviour, faster growth, increased recurrence tendency, worse prognosis, and is inversely proportional to recurrence-free time in many tumour types, including meningiomas. In our studies, we have found that the Mib1 ratio and Histoscore increase with the WHO grade. In recurrent cases, higher Mib1 values were observed. These values were not only detected in the examination of all WHO grade tumours, but also in cases of only WHO grade I. meningiomas. Accordingly, tumours which are classified as WHO grade I. (but do not have the criteria for WHO grade II.) and have elevated Mib1, are more likely to show recurrence. In addition a consistent Mib1 index increase has been found during the course of

recurrent cases, which coincides with tumour progression and higher proliferation of high WHO grade tumours.

For p53, higher values were also observed in higher WHO grades. However, it was interesting to note that a higher ratio of p53 was detected in non-recurrent cases. This is probably due to the fact that the p53 antibody used in the clinical routine (and in our studies) does not distinguish between the wild type and the mutant protein. It is assumed that, in non-recurrence cases, the elevated normal p53 level is advantageously effective in repairing DNA damage. In contrast, in higher cases, the presence of mutant p53 has been shown, which is ineffective, thus contributing to tumour growth. Mutation analysis may answer this question, but it was outside the scope of our analysis; we focused on identifying immunohistochemical markers that could be used in a daily clinical practice. The intensity of the p53 protein could be seen to increase through the course of samples from the same patient with recurrent disease, and this may be due to the selective overgrosth of cells with mutant p53 protein.

Similarly to our results, previous studies have also reported that progesterone receptor expression is inversely proportional to WHO grade in meningiomas, which can assist in the determination of the WHO grade. In line with our results, other researchers have also found that although PR immunostaining is proportional to the WHO grade, it does not help to determine the probability of relapses/recurrences. In our studies there was no significant correlation with PR in relation to recurrence, and there were no differences in the presence of different tumours from the same patient. As these tumours have a hormone receptor, this raises the possibility of hormone therapy, thus the determination of a progesterone receptor may have therapeutic potential.

Briefly summarised:

- There is a significant correlation between the detection of tumour markers (PARP1, p53, Mib1 and PR) and meningioma groups based on the behaviour.

- The presence and changes of PARP1 and p53 are independent of each other.
- PARP1, p53 and Mib1 grow directly proportionally to the WHO grade.
- PR is inversely proportional to WHO grade.
- By comparing the histological slides of R/R and non-R/R patients of all WHO grades: we could confirm the previous research finding that the Mib1 index and the expression of the p53 proteins both indicate and predict the aggressive tumour behaviour.
- Examining the behaviour and immunohistochemical characteristics of WHO grade I. meningiomas only: Mib1 clearly predicts a higher probability of relapses/recurrences - while in p53 this relationship does not exist or even inverts (this may be explained by the difference in function of the wild and mutant protein variant).
- Comparing samples from patients in case of recurrence we found that the WHO grade, Mib1 percentage, Histoscore, and p53 percentage show significant differences and increase overtime

Our results in the near future could be further enhanced by digital image analysis and automation, because these methods provide standardised results that are nowadays used by researchers in common practice. In the near future, they could become part of everyday routine pathologic work.

In conclusion, we constructed a simple immunohistochemical panel that can be used for daily work to help diagnose, determine the grade, and predict the highly-recurrent meningioma cases, which need a tighter follow-up protocol.

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5. Summary

Meningiomas are one of the most frequent intracranial tumours and occur in high numbers in neuropathological practice. Although these tumours are usually benign, recurrences may occur. According to WHO criteria there are 3 grades, indicating increasing tumour aggressiveness. There are cases where the WHO grading is difficult, therefore we need immunohistochemical markers to help distinguished such cases. The DNA damage repair protein PARP1, p53 as 'the guardian of the genom', the intranuclear progesterone receptor, and the proliferation marker Mib1 (anti-Ki67 antibody) are possible candidates.

Our experiments were carried out in two phases: first on a small group of patients' samples (41 samples of 31 patients) and later on more patients (114 samples of 70 patients). We applied automated immunohistochemical methods, which are routinely used at the Institute of Pathology, University of Debrecen Clinical Centre. In the second round we performed the study on samples built into a tissue microarray (TMA) paraffin block. The samples were digitalised and the staining percentage, average staining intensity, and Histoscore (multiplying the above two readouts) were calculated. The samples were grouped according to WHO grades, and the relapse/recurrence (presence or absence) within a five-year interval was calculated and analysed with statistical tests (Kruskall-Wallis H, Mann-Whitney U, Wilcoxon signed rank test, Spearman's rank order correlation).

Our results revealed a correlation between WHO grade and PARP1, p53, Mib1 and PR expression, respectively. PARP1 has the highest value in WHO Grade II meningiomas, whereas p53 shows the highest value in WHO Grade III tumours. The expression of PARP1 and p53 are not related to each other. PARP1, p53 and Mib1 show direct proportion, while the PR shows inverse proportion to the WHO Grade. Regarding relapse/recurrence, only the Mib1 and p53 show differences.

Our findings could help the diagnostic neuropathological work-up of meningiomas, and provide information for clinicians regarding the likelihood of tumour relapse/recurrence.

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List of publications related to the dissertation

1. **Csonka, T.**, Murnyák, B., Szepesi, R., Bencze, J., Bognár, L., Klekner, Á., Hortobágyi, T.: Assessment of candidate immunohistochemical prognostic markers of meningioma recurrence
Folia Neuropathol. 54 (2), 114-126, 2016.
IF: 1.233 (2015)

2. **Csonka, T.**, Murnyák, B., Szepesi, R., Kurucz, A., Klekner, Á., Hortobágyi, T.: Poly(ADP-ribose) polymerase-1 (PARP1) and p53 labelling index correlates with tumour grade in meningiomas.
Folia Neuropathol. 52 (2), 111-120, 2014.
DOI: http://dx.doi.org/10.5114/fn.2014.43782
IF: 1.568

List of other publications

3. Murnyák, B., **Csonka, T.**, Hortobágyi, T.: A meningeomák molekuláris patológiája.
Ideggyogy. Szle. 68 (9-10), 292-300, 2015.
DOI: http://dx.doi.org/10.18071/isz.68.0292
IF: 0.376

4. Murnyák, B., Bodoki, L., Nagy-Vincze, M., Griger, Z., **Csonka, T.**, Szepesi, R., Kurucz, A., Dankó, K., Hortobágyi, T.: Inclusion body myositis: pathomechanism and lessons from genetics.
Open Med. 10, 188-193, 2015.
IF: 0.19

5. Bodoki, L., Nagy-Vincze, M., Griger, Z., **Csonka, T.**, Dankó, K., Hortobágyi, T.: Zárványteestes myositis.
Ideggyogy. Szle. 68 (1-2), 59-67, 2015.
IF: 0.376

6. Bodoki, L., Nagy-Vincze, M., Griger, Z., **Csonka, T.**, Murnyák, B., Kurucz, A., Dankó, K., Hortobágyi, T.: Inclusion body myositis - a case based clinicopathological update.
Cent. Eur. J. Med. 9 (1), 80-85, 2014.
IF: 0.153

7. Csiszkó, A., László, I., Palatka, K., Szabó, K., Kanyári, Z., Bidiga, L., **Csonka, T.**, Damjanovich, L., Szentkereszty, Z.: Primary angiosarcoma of the pancreas mimicking severe acute pancreatitis: case report.
Pancreatology. 15 (1), 84-87, 2014.
IF: 2.637

8. Bodoki, L., Nagy-Vincze, M., Hortobágyi, T., Griger, Z., **Csonka, T.**, Dankó, K.: Szignálfelismerőrészecske-ellenes autoantitest-pozitív myopathia.
Ideggyogy. Szle. 67 (9-10), 347-353, 2014.
IF: 0.386

9. Huttner, H. B., Bergmann, O., Salehpour, M., Rácz, A., Tatarishvili, J., Lindgren, E., **Csonka, T.**, Csiba, L., Hortobágyi, T., Méhes, G., Englund, E., Solnestam, B. W., Zdunek, S., Scharenberg, C., Ström, L., Ståhl, P., Sigurgeirsson, B., Dahl, A., Schwab, S., Possnert, G., Bernard, S., Kokaia, Z., Lindvall, O., Lundeberg, J., Frisén, J.: The age and genomic integrity of neurons after cortical stroke in humans.
Nat. Neurosci. 17 (6), 801-803, 2014.
DOI: http://dx.doi.org/10.1038/nn.3706
IF: 16.095

10. **Csonka, T.**, Szepesi, R., Bidiga, L., Péter, M., Klekner, Á., Hutóczki, G., Csiba, L., Méhes, G., Hortobágyi, T.: A herpesencephalitis diagnosztikájáról egy esetismertetés kapcsán.
Ideggyogy. Szle. 66 (9-10), 337-342, 2013.
IF: 0.343

11. Murnyák, B., **Csonka, T.**, Klekner, Á., Hortobágyi, T.: Alacsony malignitású gliális daganatok előfordulása és molekuláris patológiája.
Ideggyogy. Szle. 66 (9-10), 305-311, 2013.
IF: 0.343

12. Murnyák, B., **Csonka, T.**, Hegyi, K., Méhes, G., Klekner, Á., Hortobágyi, T.: Magas malignitású gliomák előfordulása és molekuláris patológiája.
Ideggyogy. Szle. 66 (9-10), 312-321, 2013.
IF: 0.343

13. Bodoki, L., Nagy-Vincze, M., Griger, Z., **Csonka, T.**, Cseri, K., Hortobágyi, T., Dankó, K.: Rituximabkezelés idiopathiás inflammatorikus myopathiákban. *LAM.* 23 (1), 16-21, 2013.

14. Méhes, G., Hegyi, K., **Csonka, T.**, Fazakas, F., Kocsis, Z., Radványi, G., Vadnay, I., Bagdi, E., Krenács, L.: Primary uterine NK-cell lymphoma, nasal-type: a unique malignancy of a prominent cell type of the endometrium. *Pathol. Oncol. Res.* 18 (2), 519-522, 2012. DOI: http://dx.doi.org/10.1007/s12253-011-9360-4 IF: 1.555

15. Nagy, E., Eaton, J. W., Jeney, V., Soares, M. P., Varga, Z., Galajda, Z., Szentmiklósi, J. A., Méhes, G., **Csonka, T.**, Smith, A., Vercellotti, G. M., Balla, G., Balla, J.: Red cells, hemoglobin, heme, iron, and atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* 30 (7), 1347-1353, 2010. DOI: http://dx.doi.org/10.1161/ATVBAHA.110.206433 IF: 7.215

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