Assessment of candidate immunohistochemical prognostic markers of meningioma recurrence

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Abstract

Although tumour recurrence is an important and not infrequent event in meningiomas, predictive immunohistochemical markers have not been identified yet. The aim of this study was to address this clinically relevant problem by systematic retrospective analysis of surgically completely resected meningiomas with and without recurrence, including tumour samples from patients who underwent repeat surgeries. Three established immunohistochemical markers of routine pathological meningioma work-up have been assessed: the proliferative marker Ki-67 (clone Mib1), the tumour suppressor gene p53 and progesterone receptor (PR). All these proteins correlate with the tumour WHO grade, however the predictive value regarding recurrence and progression in tumour grade is unknown.

One hundred and fourteen surgical specimens of 70 meningioma patients (16 male and 54 female) in a 16 years' interval have been studied. All tumours had apparently complete surgical removal. On Mib1, PR and p53 immunostained sections, the percentage of labelled tumour cells, the staining intensity and the multiplied values of these parameters (the histoscore) was calculated. Results were statistically correlated with tumour WHO grade, (sub)type, recurrence and progression in WHO grade at subsequent biopsies.

Our results confirmed previous findings that the WHO grade is directly proportional to Mib1 and p53 and is inversely proportional to the PR immunostain. We have demonstrated that Mib1 and p53 have a significant correlation with and predictive value of relapse/recurrence irrespective of the histological subtype of the same WHO grade. As a quantitative marker, Mib1 has the best correlation with a percentage of labelled cells, whereas p53 with intensity and histoscore.

In conclusion, the immunohistochemical panel of PR, p53, Mib1 in parallel with applying standard diagnostic criteria based on H&E stained sections is sufficient and reliable to predict meningioma recurrence in surgically completely resected tumours.

Key words: immunohistochemistry, Ki-67, meningioma, p53, progesterone receptor, prognostic markers, tumour recurrence.

Introduction

Meningioma is one of the most frequent brain tumours [9]. According to the World Health Organization (WHO) classification, there are several subtypes like meningothelial, fibrous, transitional, psammomatous, angiomatous, microcystic, secretory, lymphoplasmacyte-rich, metaplastic, choroid, clear cell, rhabdoid, papillary and other rare morphological phenotypes [6,21]. The assigned WHO grade I-III reflects the probable prognosis which is

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determined by the subtype and/or specified morphological features such as mitotic rate, presence or absence of small geographic necrosis, nucleus-cytoplasm ratio an others [21]. Although tumour recurrence is an important and not infrequent event, our knowledge on predisposing factors is rather limited. The risk of recurrence increases with the WHO grade being 7-25% in WHO grade I, 30-50% in WHO grade II and 50-95% in WHO grade III [27,28,32,33]. The extent of resection assessed by the Simpson Grading System influences recurrence rates which is one reason of the wide range of probability [30]. The Simpson Grading System classifies the completeness of removal in a 5-tier scale ranging from macroscopically complete removal (grade I) to simple decompression with or without biopsy (grade V). Skull irradiation, inherited mutation of the NF2 gene (neurofibromatosis type 2) and epigenetic factors may also predispose to recurrence [22,23].

Another important phenomenon is tumour progression to a higher WHO grade. However, the risk and probability of progression remains rather unpredictable – even less so than tumour recurrence. Hence, there is a growing clinical need to identify additional and better predictors for recurrence and tumour progression than the currently used histological grade and extent of resection. Because immunohistochemistry has been routinely used in the pathological diagnostic practice for decades, the search for predictive immunohistochemical markers is of importance. In our study we focussed on 3 well-known immunohistochemical markers in routine pathological work-up of meningioma: the proliferative marker Ki-67 (clone Mib1), the tumour suppressor gene p53 and progesterone receptor. All these proteins have been studied in meningioma and the correlation with tumour grade has been confirmed by several studies. However, the predictive value regarding recurrence and progression in tumour grade remains unknown. The aim of this study is to address these clinically relevant questions by a systematic retrospective analysis of meningiomas with and without recurrence, with special emphasis on tumour samples from patients who underwent repeat surgeries due to tumour recurrence.

p53 is one of the major tumour suppressor proteins. The physiological functions of p53 are cell cycle regulation and conservation of the stability of the genome by preventing mutations, therefore it is called 'the guardian of the genome' [17]. More

than 50 percent of human tumours carries a deletion or mutation of the p53 genes (TP53) [13]. p53 can be activated by DNA damage, oxidative stress, osmotic shock, ribonucleotide depletion or oncogene expression. The activation is marked by an increase in the half-life of p53 and a change of its conformation [16], therefore shows increased Labelling Index (LI) with immunohistochemistry with the polyclonal antibodies routinely used in tumour diagnostics. The anticancer activity of p53 is through several mechanisms: it activates DNA repair proteins, induces growth arrest at the G1/S regulation point through p21 or initiates apoptosis if the DNA damage is irreversible [12]. It has been investigated also in meningioma and several studies showed a positive correlation with the grade and tumour recurrence [4,7,8,14,15,24,26], whereas authors reported the grade as an independent predictive factor of recurrences with high Mib1 and p53 LI being a supportive marker helpful in borderline cases [31].

Ki-67 is necessary for cellular proliferation; it is present during all active phases of the cell cycle, and absent from the G0 phase. Mib1 is the usually applied clone of the Ki-67 antibody which is widely used as a proliferative marker in the routine diagnostic work-up. The Mib1 LI shows a strong correlation with tumour growths, relapse/recurrence, length of disease free survival in various tumours [2,3,34] including meningioma [18,19].

Progesterone receptor (PR) is a steroid hormone receptor. It has been demonstrated that meningioma cells show positivity for PR; the ratio of the positive cells is inversely proportional to the WHO grade [18,27]. Also described earlier that the cellular biosynthesis of PR in meningioma is not oestrogen regulated as it is other sex steroid in tissues [5,7]. PR is encoded by the *PGR* gene on the long arm of chromosome 11. In a physiological situation after binding the progesterone hormone, the receptor undergoes a dimerization and is transported to the nucleus to bind to the DNA and induce transcription. Both forms (progesterone receptor A and progesterone receptor B) have a regulatory domain, a DNA binding domain, a hinge section and a ligand binding domain, but only the PR-B form possesses a transcription activation function.

The Mib1 antibody, p53 and PR are widely used immunohistochemical markers in meningioma diagnosis. In high-grade meningioma, the Mib1 LI is higher [1,4,28,29]. In our previous study we have reported
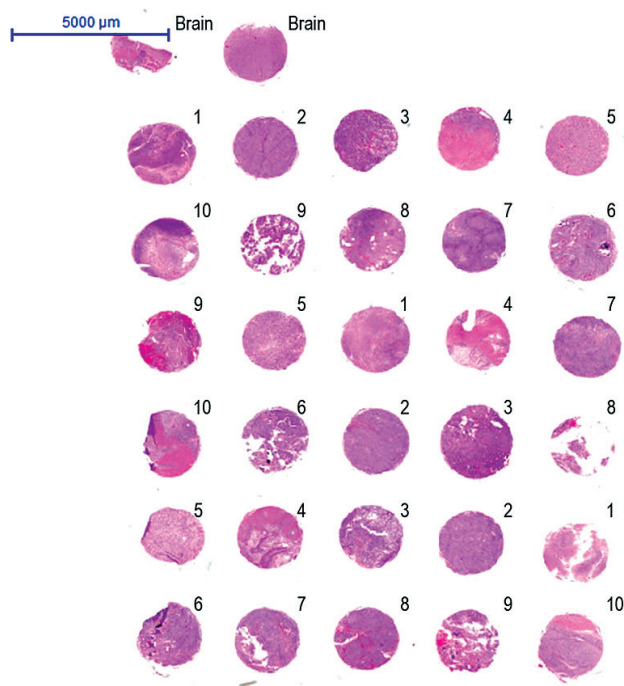
Fig. 1. Low magnification image of histological slide stained with haematoxylin-eosin, made from tissue microarray (TMA) paraffin block. In the upper left corner there are two tissue (brain) samples for guidance regarding localization. The numbers from 1 to 10 represent the individual cases. Every 'donor' case have 3 different samples in the 'recipient' block. Scale bar 5 mm.

a significant correlation between the frequency and intensity of p53 immunostaining and WHO grade [10]. The reduced of PR immunoreactivity is another known feature in the high grades of meningioma [18,20,25].

The aim of this study is to establish an easy-to-use immunohistochemical panel for the routine neuropathological use, which can predict meningioma relapse/recurrence. For validation we analysed the changes in immunohistochemical characteristics and expression patterns during relapse/recurrence and examined their relation to tumour grade.

Material and methods

One hundred and fourteen surgical specimens of 70 meningioma patients (16 male and 54 female) in a 16 years' interval have been retrospectively studied. All cases were revised by a consultant neuropathologist (TH) and divided into three grades and histological subtypes according to the WHO classification [21].

We established two study groups: patients with one or more recurrence/relapse(s) (R/R group) and patients with meningioma without any radiological or post mortem evidence of recurrence/relapse (non-R/R group). Only cases with apparently complete surgical removal and no evidence of residual tumour on post-operative MRI were included.

After the surgical removal tissue samples were processed to generate sections from formalin fixed and paraffin embedded (FFPE) blocks which were stained with haematoxylin-eosin (H&E). One representative tissue block was selected per case. From these blocks tissue micro arrays (TMAs) were built. Each TMA contained samples from 10 cases (three samples from each cases) plus 2 normal brain tissue samples in the left upper corner as a reference to enable specimen identification in the TMA (Fig. 1). In total 12 TMA were built, containing tissue samples from 114 neurosurgical interventions.

Immunohistochemistry (IHC) was performed according to standardized methods. In brief, 4 µm thick sections from TMA blocks were stained with p53 mouse monoclonal antibody (clone DO-7, M7001, DAKO, Glostrup, Denmark); PR antibody (NCL-PGR-312, clone 16, Novocastra, Newcastle, UK) and anti-Ki-67 antibody (clone Mib1, M7240, DAKO, Glostrup, Den-
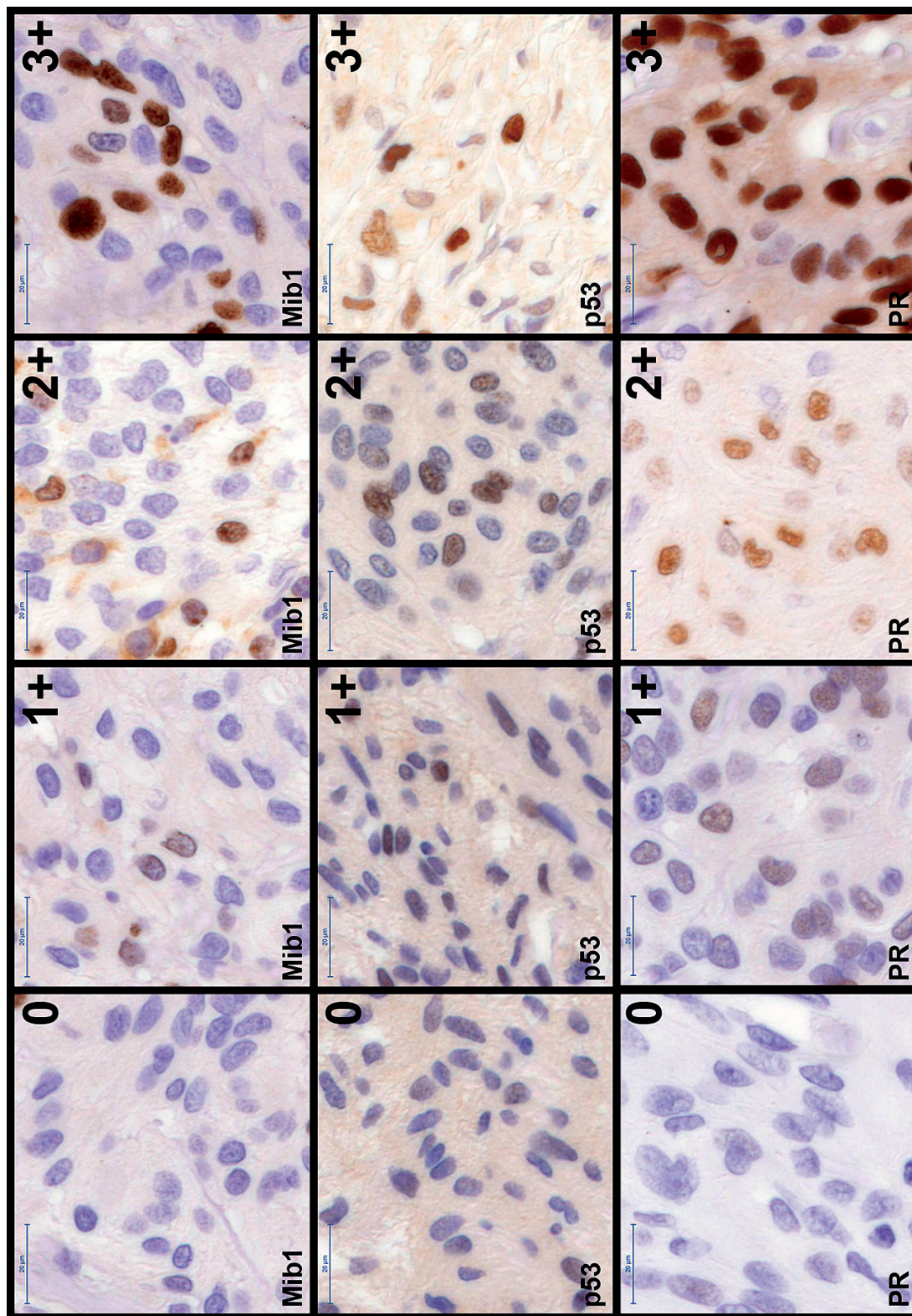
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Fig. 2. Ki-67 (clone Mib1), p53, and progesterone receptor (PR) immunostain with representative images of the different immunolabeling intensities: negative (0), minimal positivity (1+), moderate positivity (2+), strong positivity (3+). Scale bar 20 µm.
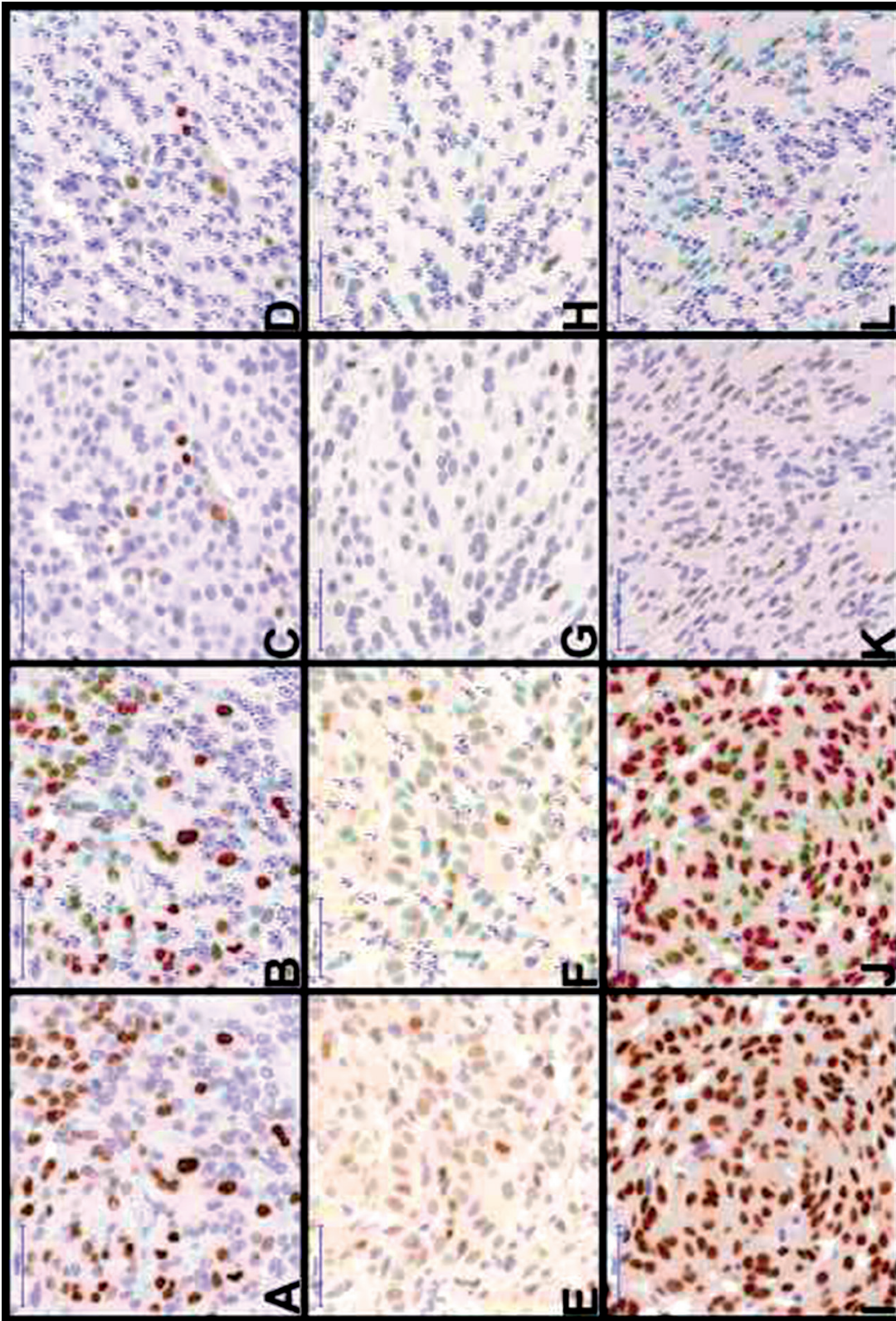
Fig. 3. Ki-67 (clone Mib1), p53 and progesterone receptor (PR) immunopositive cells counted using ImageJ software. A, C, E, G, I, K pictures are the originals, whereas B, D, F, H, J, L show cells numbered with ImageJ Cell Counter plug-in. The numbers from 1-4 stand for the negative, 1+, 2+ and 3+ cells, respectively. A-B pictures are immunostained for Mib1 (43,5% positivity). C-D pictures are immunostained for Mib1 (6.9% positive). E-F pictures are stained for p53 (58.9% positive). G-H pictures immunostained for p53 (9.8% positive). I-J pictures stained for PR (93.9% positive). K-L pictures immunostained for PR (31.3% positive).

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mark) according to the manufacturer's protocol with 1 : 100, 1 : 100 and 1 : 200 dilution for p53, PR and Ki-67, respectively. Sections were incubated with the primary antibody for 6 hours at room temperature; the visualization was performed with SuperSensitive™ One-step Polymer-HRP Detection System on Leica Bond Max™ fully automated IHC stainer with negative controls (omitting the primary antibody).

All of the H&E and immunostained TMA sections were scanned with a Panoramic Scanner (3DHistech, Budapest, Hungary). Two digital images were taken at 400x magnification from each tissue samples, in total 6 from each case. According to the intensity of nuclear staining of cells, 4 semi quantitative scores were applied: 0 (none), 1+ (weak), 2+ (moderate) and 3+ (strong) (Fig. 2).

Images in 10 reference cases were analysed quantitatively with ImageJ (NIH, Bethesda, USA) software Cell Counter function, to determine the exact percentage of immunopositive cells (Fig. 3). These images were used as reference cases to aid accurate semi-quantitative assessment in all cases. This is a method easily and reliably applicable in the routine pathological diagnostic practice, similarly to the assessment of percentage of immunopositive cells in other tumours.

Not only the percentage value of immunopositive cells but also the average labelling intensity score (0-3+; for reference images see Fig. 2) of the staining were calculated in each picture. Similarly to the histoscore of breast carcinoma i.e. the multiple of the percentage of the positive cells and the average intensity of the positive cell nuclei [11] were calculated.

Data were analysed with SPSS 22.0 for Windows (IBM, Armonk, NY, USA) statistical programme, using Kruskal-Wallis H-test, Mann-Whitney U-test and Wilcoxon signed ranks test. The patients were grouped by the grades (65 WHO grade I; 33 WHO grade II and 16 WHO grade III) and also based on the recurrence or relapse (R/R group) showed up at least 5 years after resection: patients without recurrence or relapse (non-R/R group), patients with definitive relapse or recurrence (R/R group).

The non-R/R cases were WHO grade I, while the R/R group have 18 WHO grade I, 9 WHO grade II and 2 WHO grade III tumour in the 1st histological sample.

Ethical approval has been obtained from the Institutional Research Ethics Committee (Number: DEOEC RKEB: 2437-2005).

Results

The 70 patients' average age was 56 years at the time of the first pathological examination. There were no significant differences between the R/R group and the non-R/R group. There were 16 patients (3 male and 13 female; average age 54 years) without recurrence or relapse (non-R/R group) with at least 5 years' survival after surgery and 31 patients (8 male and 23 female; average age 53 years, overall time of recurrence 19.6 months) with definitive relapse or recurrence (R/R group). Further 23 patients (5 male and 18 female, average age 59 years) were operated within 5 years without recurrence/relapse; however the time window was too short to include them in the non-R/R group. There were 65 WHO grade I cases, 33 WHO grade II cases and 16 WHO grade III cases. All of the non-R/R cases were WHO grade I. The R/R group contained 19 WHO grade I, 9 WHO grade II and tree WHO grade III cases according to the 1st neuropathological diagnosis of the first surgical specimen. There were 8 patients whose subsequent surgical specimens had higher WHO grade than the first; 15 patients whose first and last cases both showed the same grade; and 6 patients who only had 1 histological sample and the recurrence/relapse was diagnosed by imaging techniques.

The histological subtypes were not statistically different between the R/R group and non-R/R group: there were 6 meningothelial, 5 transitional, 3 fibrous and 2 psammomatous in the non-R/R group, while 9 meningothelial, 6 transitional, 3 fibrous, one psammomatous, one clear cell, 8 atypical and 3 anaplastic in the R/R group. There was no increased tendency for recurrence for any WHO grade 1 subtype. Among grade 2-3 meningiomas, there was no specific subtype which had higher frequency of recurrence than the respective grade in general.

There is a significant correlation between WHO tumour grade and Mib1 LI (%) ($p < 0.001$), Mib1 staining intensity ($p = 0.001$), Mib1 histoscore ($p < 0.001$), p53 staining intensity ($p < 0.001$), p53 histoscore ($p = 0.031$), PR LI (%) ($p < 0.001$), PR intensity ($p < 0.001$) and PR histoscore ($p < 0.001$), respectively (Kruskal-Wallis test). Comparing only grade I and grade II tumours there is a significant correlation with Mib1 LI (%) ($p < 0.001$), Mib1 intensity ($p < 0.001$), Mib1 histoscore ($p < 0.001$), p53 intensity ($p = 0.001$), PR LI (%) ($p = 0.014$), PR intensity ($p = 0.029$) and PR histoscore ($p = 0.013$). Comparing grade II and

Table I. Comparison of grade I, grade II and grade III cases with Kruskal-Wallis test, and the WHO grade pairs with Mann-Whitney test. Immunostain percentage, intensity (average intensity of cells: 0, 1, 2 or 3) and histoscore (intensity × percentage) for Mib1, p53 and pogesterone receptor (PR)

	Mib1 Percentage	Mib1 Intensity	Mib1 Histoscore	p53 Percentage	p53 Intensity	p53 Histoscore	PR Percentage	PR Intensity	PR Histoscore
Kruskal-Wallis	0.000	0.001	0.000	0.316	0.000	0.031	0.000	0.000	0.000
Mann-Whitney grade I-II	0.000	0.000	0.000	0.272	0.001	0.065	0.014	0.029	0.013
Mann-Whitney grade II-III	0.449	0.320	0.831	0.654	0.049	0.376	0.008	0.008	0.009
Mann-Whitney grade I-III	0.000	0.086	0.000	0.198	0.000	0.023	0.000	0.000	0.000

Table II. Comparison of the non-recurrence/relapse (non-R/R) cases and recurrence/relapse (R/R) cases' first surgical specimens without regarding the grade (first row) and only in WHO grade I cases (second row)

	Grade	Mib1 Percentage	Mib1 Intensity	Mib1 Histoscore	p53 Percentage	p53 Intensity	p53 Histoscore	PR Percentage	PR Intensity	PR Histoscore
Mann-Whitney any grades	0.003	0.000	0.004	0.000	0.027	0.955	0.069	0.207	0.497	0.215
Mann-Whitney grade I	1.000b	0.009b	0.126b	0.029b	0.032b	0.195b	0.038b	0.708b	0.708b	0.858b

Table III. Comparison of the first and last surgical specimens of the recurrence/relapsed (R/R) cases with Mann-Whitney test (first row) and Wilcoxon signed rank test (second row). Immunostain percentage, intensity (average intensity of cells: 0, 1, 2 or 3) and histoscore (intensity x percentage) for Mib1, p53 and progesterone receptor (PR)

	Grade	Mib1 Percentage	Mib1 Intensity	Mib1 Histoscore	p53 Percentage	p53 Intensity	p53 Histoscore	PR Percentage	PR Intensity	PR Histoscore
Mann-Whitney progression	0.001	0.002	0.098	0.001	0.861	0.006	0.553	0.154	0.154	0.159
Wilcoxon progression	0.007	0.042	0.237	0.050	0.042	0.484	0.559	1.000	0.545	0.876

grade III tumours there is a significant correlation with p53 intensity ($p = 0.049$), PR LI (%) ($p = 0.008$), PR intensity ($p = 0.008$) and PR histoscore ($p = 0.009$). When comparing grade I and grade III tumours there is a significant correlation of the higher grade with increased Mib1 LI (%) ($p < 0.001$), Mib1 histoscore ($p < 0.001$), p53 intensity ($p < 0.001$), p53 histoscore ($p = 0.023$), PR LI (%) ($p < 0.001$), PR intensity ($p < 0.001$), PR histoscore ($p < 0.001$) (Mann-Whitney test) (Table I, Fig. 4).

Irrespective of the grades of the R/R group, comparing the non-R/R and R/R groups there is a significant correlation with the Mib1 LI (%) ($p < 0.001$), Mib1 intensity ($p = 0.004$), Mib1 histoscore ($p < 0.001$), p53 LI (%) ($p = 0.027$), and the WHO grade ($p = 0.003$) (Fig. 5). In WHO grade I tumours in the R/R group there is a significant correlation with the Mib1 LI (%) ($p = 0.009$), Mib1 histoscore ($p = 0.029$), p53 LI (%) ($p = 0.032$), p53 histoscore ($p = 0.038$) (Table II, Fig. 6).
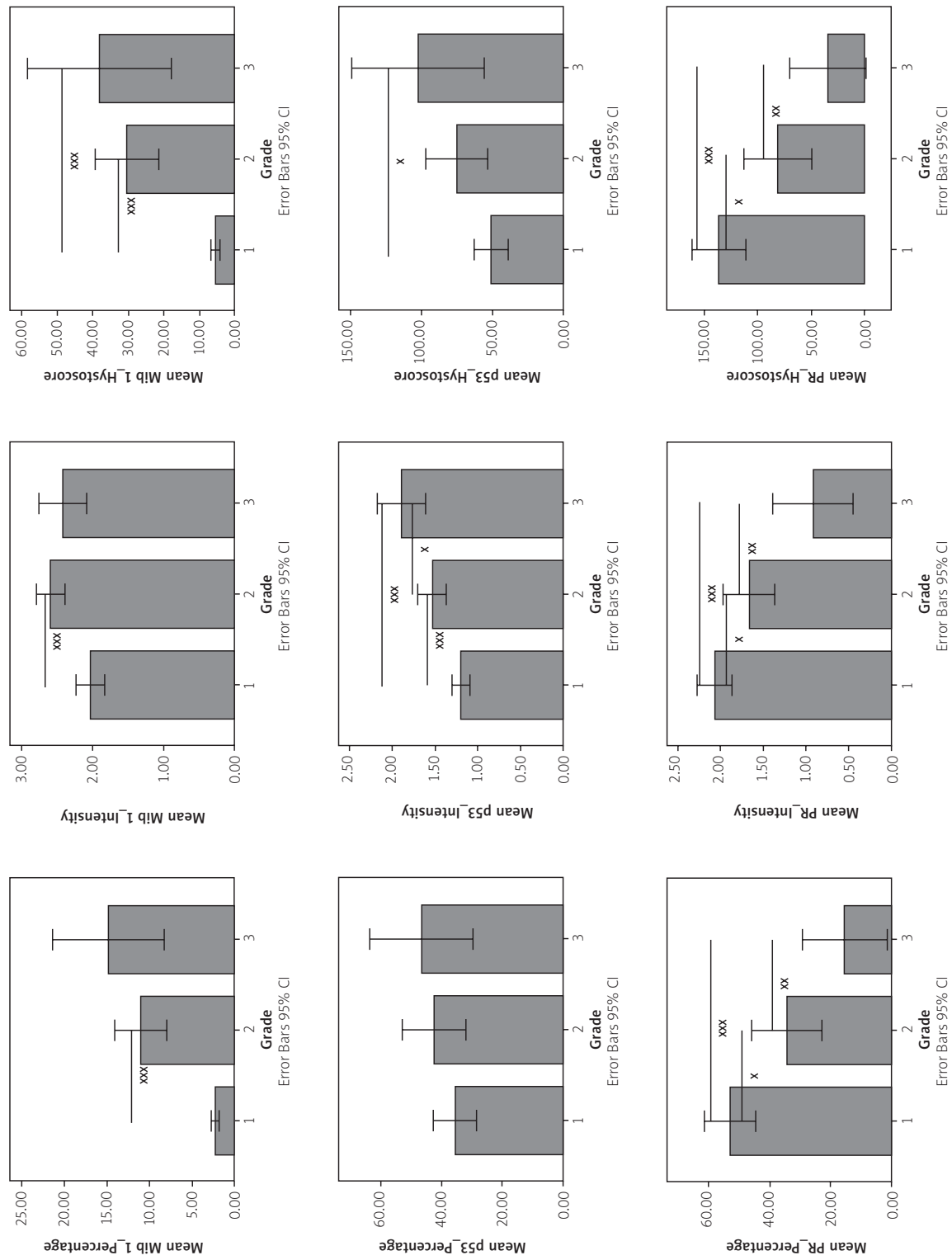
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Fig. 4. Percentage (%), intensity (0, 1+, 2+, 3+) and histoscore (intensity × percentage) of immunostain with Ki-67 (clone Mib1), p53 and progesteron receptor (PR) for WHO grade I, II and III.
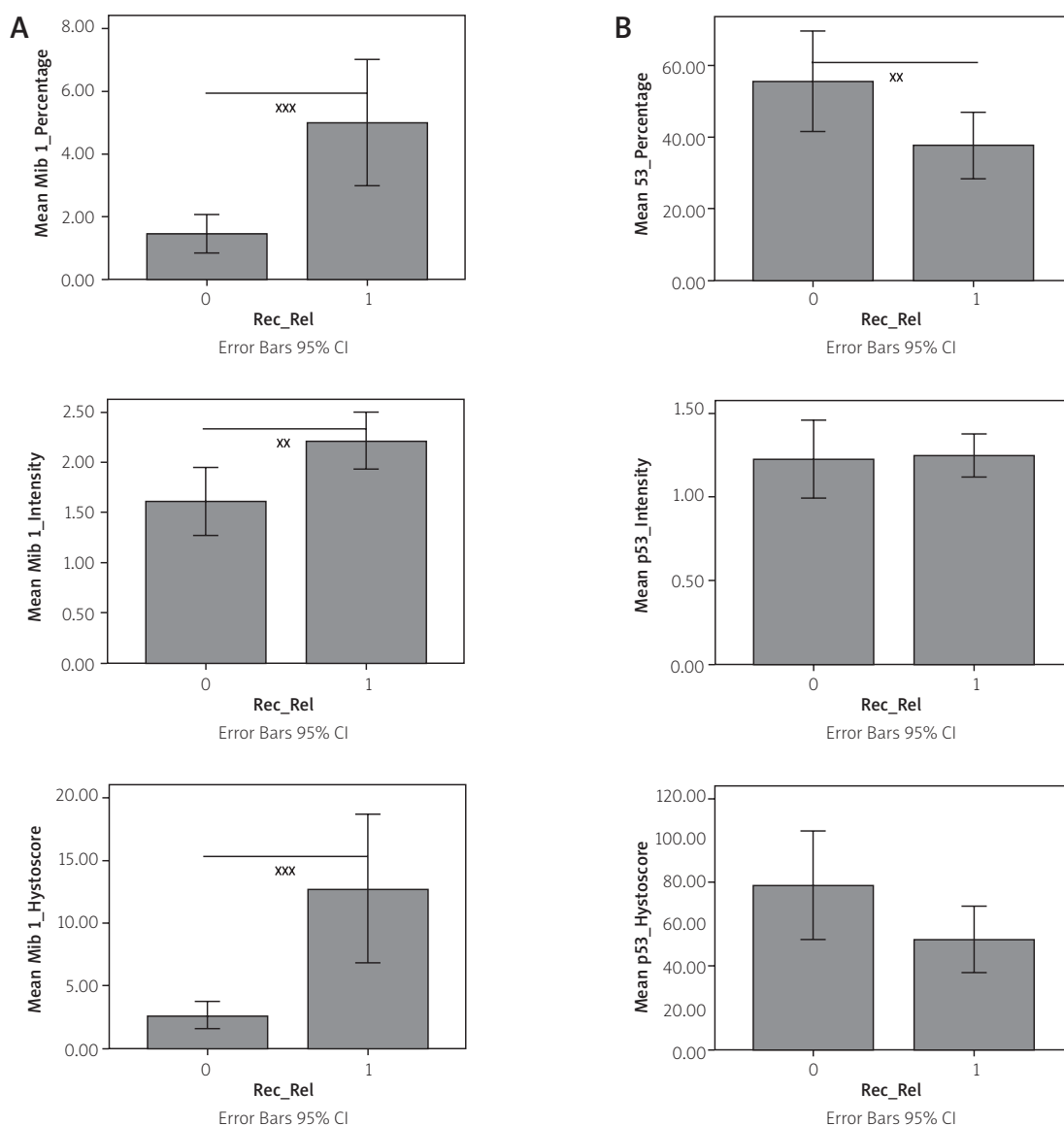
Fig. 5. Percentage (%), intensity (0, 1+, 2+, 3+) and histoscore (intensity x percentage) of immunostain with Ki-67 (clone Mib1) **(A)**, and p53 **(B)** for non-reccurence/relapse (non-R/R) and reccurence/relapse (R/R) cases, without regarding the WHO grades.

In the R/R groups when comparing the first case with the recurrent/relapsed cases there is a significant difference between the Mib1 LI (%) (p = 002), Mib1 histoscore (p = 0.001), p53 intensity (p = 0.006) and the grade (p = 0.001); and with Wilcoxon signed rank test when compared the first and last case of the same patient, there is a significant difference in the grade (p = 0.007), Mib1 LI (%) (p = 0.042), Mib1 histoscore (p = 0.050), and p53 LI (%) (p = 0.042) (Table III, Fig. 7).

According to our data, the WHO grade has strong forward proportion to Mib1 and p53 and an inverse proportion to the PR immunostain (as shown in several previous papers). As a quantitative marker the Mib1 has a better correlation with percentage, whereas p53 with intensity and histoscore. Therefore, the panel of PR, p53, Mib1 is sufficient to characterize meningioma immunohistochemically regarding the risk of recurrence as an integral part of the routine diagnostic histopathological practice.
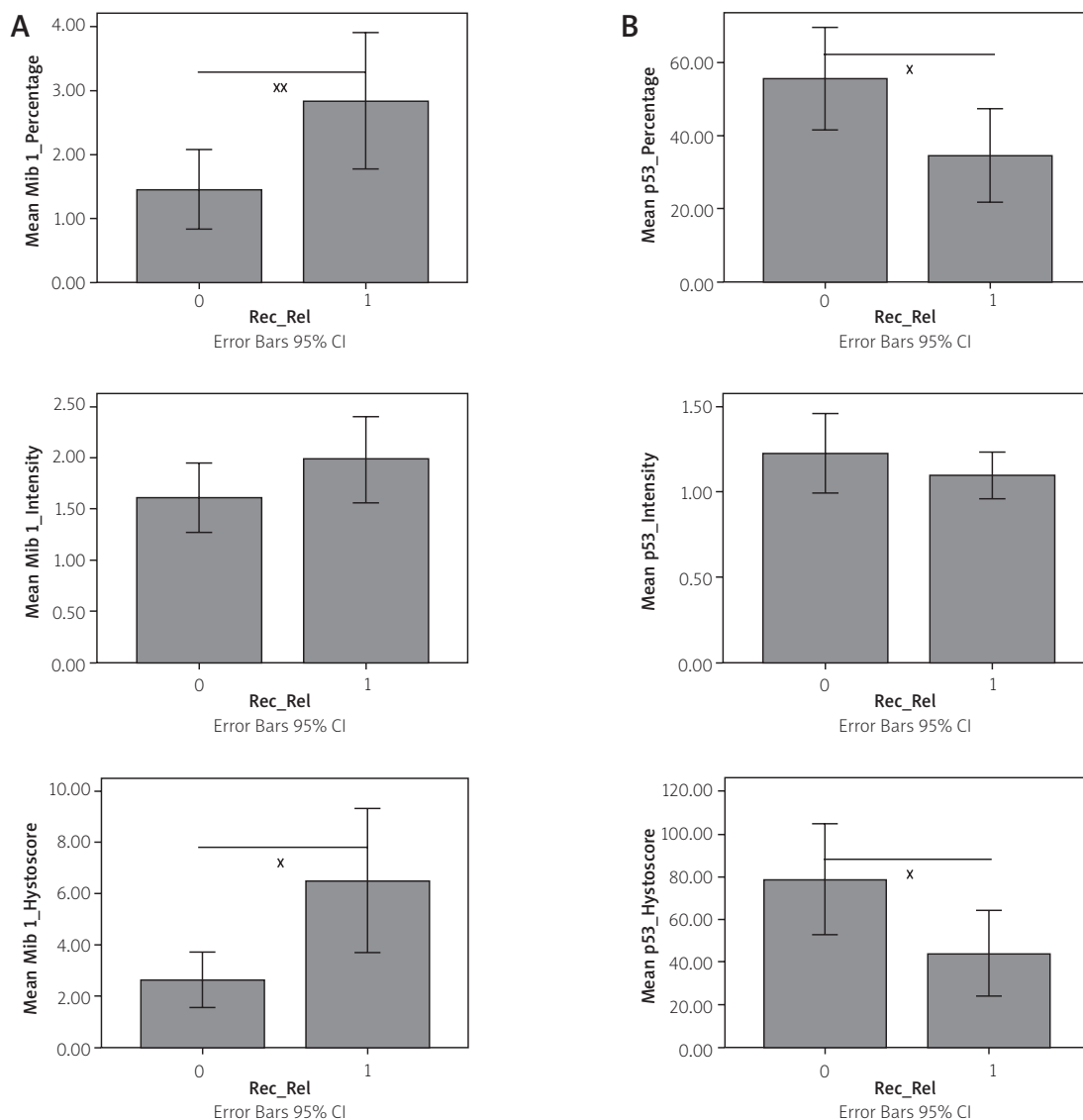
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Fig. 6. Percentage (%), intensity (0, 1+, 2+, 3+) and histoscore (intensity x percentage) of immunostain with Ki-67 (clone Mib1) **(A)**, and p53 **(B)** for non-reccurence/relapse (non-R/R) and WHO grade I of the reccurence/relapse (R/R) cases.

Discussion

Meningioma is one of the most common intracranial tumours with high incidence in the neurosurgical practice. The histological subtypes are well characterised by the WHO, and the grading is based on these histological characteristics, morphological findings and the mitotic ratio. The Simpson Grading System also can provide further information of the probability of the recurrence [30].

The aim of this study was to establish an easy-to-use immunohistochemical panel for the routine neuropathological use, which can predict meningioma relapse/recurrence. This is particularly relevant for tumours in problematic localization (e.g. falx meningiomas).

For validation we analysed the changes in immunohistochemical characteristics and expression patterns during relapse/recurrence and their relation to tumour grade.

Meningiomas usually are non-infiltrative neoplasms therefore complete surgical resection is curative. However, the tumour may spread laterally in small
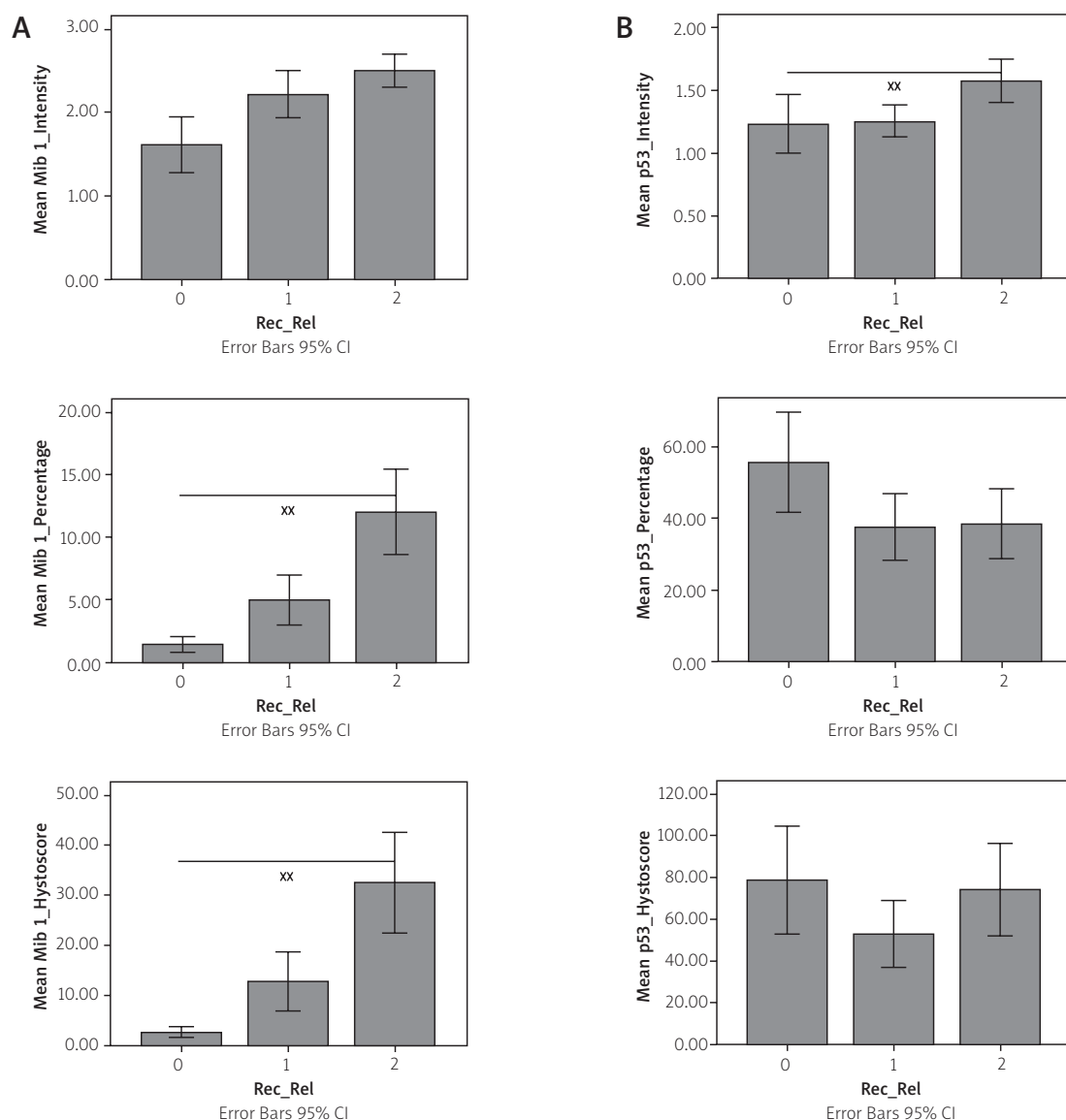
Fig. 7. Percentage (%), intensity (0, 1+, 2+, 3+) and histoscore (intensity x percentage) of immunostain with Ki-67 (clone Mib1) **(A)**, and p53 **(B)** for the 1st, 2nd and last surgical specimens of the recurrence/replased (R/R) cases.

nests in the dura mater which could be a source of recurrence. Hence no chemotherapy is effective even in high grade meningiomas – radiotherapy increases malignant transformation [33] – another argument for discovery of relatively simple predictive markers of tumour progression and recurrence.

Although the Mib1 labelling index can be different according to which laboratory-performed reaction [8], standardized method can help the data interpretation and comparison both for routine and experimental practice. In accordance with previous studies the higher initial Mib1 LI has a predictive value

regarding increased probability of recurrence. In R/R cases during evolution in time (i.e. time between 1st and last surgical procedure) there was an increase in Mib1 LI consistent with the known fact that tumour progression may occur over time which is reflected by increased proliferative potential and higher WHO grade.

The routinely used p53 antibody does not differentiate between the wild type and the mutant protein. Interestingly, the p53 LI and histoscore (but not the labelling intensity) has an inverse correlation with the chance of recurrence in the WHO grade I tumours

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in our study, but if we examine all the WHO grades, the increased staining in the higher grades, changes to forward proportion, similarly to prior studies [7,14,15]. This may be explained by the fact that the p53 immunoreactivity does not distinguish between the wild type (WT) and mutant protein; in non-recurrent cases increased normal protein may have a beneficial effect as p53 is involved in DNA damage repair. In contrast, in recurrent cases p53 is more likely to be mutant and ineffective thereby contributing to tumour growth and recurrence. Mutation analysis could answer this problem, however, the focus of our study is on immunohistochemical markers, and therefore it is beyond the scope of the current project. Today the antibodies specific to mutated p53 are not routinely used therefore not applied in this study. The p53 LI and histoscore decreased during time to recurrence which may indicate decreased levels of WT protein.

PR has an inverse relation with tumour grade in concert with previous reports [10,18,20,25] with no predictive value regarding recurrence.

Using p53 and Ki-67 molecular markers and the relatively simple and quick assessment method the increased risk of recurrence can be reliably predicted. However, it is foreseeable that the presented method has the potential for further improvement with the use of digitalized histological specimens, because this enables automated quantitative image analysis as an integral component of the diagnostic process.

In summary, we have demonstrated a rather simple immunohistochemistry-based method with routinely used molecular markers to identify patients with increased risk of recurrence. Further work is needed to validate our work in more patients, multiple centres and in a prospective manner with long follow-up. The combination of histological, surgical and imaging markers may be a more sensitive tool to predict recurrence and this can also be tested in future studies.

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Disclosure

Authors report no conflict of interest.

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