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Analysis of the prognostic significance of selected morphological and immunohistochemical markers in ependymomas, with literature review

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Abstract

Aim: *Ependymal tumours are relatively uncommon primary neoplasms of the central nervous system. Histological criteria distinguishing ependymoma and anaplastic ependymoma are not clear-cut and other parameters are required to allow more precise prognostication in these tumours. We analysed the histological and immunohistochemical features of these tumours (Ki-67, cyclin D1, EGFR, hTERT, Olig2) and correlated them with the clinical outcome.*

Material and methods: *We analysed 39 patients with grade II ependymoma (30) and anaplastic ependymoma (9). Twenty-eight tumours developed in children and the remaining 11 patients were adults with intracranial and intraspinal tumours. Eighteen patients died during the follow-up period.*

Results and conclusions: *Overall survival was reduced significantly for paediatric patients and patients with intracranial tumour. High-grade tumours, increased mitotic index and increased cellularity had an unfavourable influence on survival. Other histological parameters such as nuclear atypia, necrosis and microvascular proliferation did not alter the survival rate. Increased Ki-67 and cyclin D1 indices correlated with worse prognosis. Furthermore, any level of cyclin D1 expression in WHO grade II ependymomas was strongly associated with higher risk of death. No correlation was identified between Olig2, EGFR and hTERT expression and the outcome of the patients.*

Key words: *ependymoma, immunohistochemistry, mitotic index, Ki-67, Olig2, cyclin D1, prognosis.*

Introduction

Ependymomas are neoplasms that arise from the ependymal lining of the cerebral ventricles and/or the central canal of the spinal cord. They constitute approximately 3-9% of all primary tumours of the central nervous system (CNS). Ependymomas occur more commonly in children and in this age group their frequency ranges from 10.1 to 21.4% of primary CNS neoplasms [18]. There is a significant

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correlation between the age of a patient and tumour location: about 90% of paediatric ependymomas develop intracranially, whereas intraspinal location is most common in adults [3,7,9].

The most recent WHO classification (2007) divides ependymal tumours into four entities: subependymoma, myxopapillary ependymoma, ependymoma, and anaplastic ependymoma. They fall into three histological grades (WHO grade I-III). The diagnostic criteria of the classical (grade II) and anaplastic (grade III) ependymomas are defined in the current WHO classification, but they are not very clear and this makes their application rather troublesome. These criteria include nuclear atypia, increased cellularity and mitotic activity [18]. The value of other markers has not been established unequivocally. The Ki-67 labelling index (LI) was shown as a significant prognostic factor in several publications [2,6,10,16,36,45]. However, the threshold value of Ki-67 LI that distinguishes less malignant ependymomas from anaplastic ones is variable. Olig2 is a transcription protein active in embryonic cells of CNS, progenitors of oligodendroglial cells and oligodendroglial tumours [20]. However, expression of Olig2 is not limited to oligodendrogliomas, as it was identified in other CNS tumours [17,24] and may play a role in pathogenesis of ependymoma. The well-known regulatory protein cyclin D1 is involved in the control of the cell cycle at the G1/S checkpoint. Its impact on progression-free survival was determined in ependymomas by one publication only [45]. A high level of hTERT expression implies immortalization of neoplastic cells as the cell with telomerase expression obviates apoptosis in case of cycle-dependent shortening of chromosomal telomeres. A significant impact of hTERT expression on patients' survival with ependymomas was reported in a few reports; however, all of them analysed intracranial tumours [22,40].

Therefore, we attempted to verify the significance of the histopathological features discriminating classical from anaplastic ependymoma, including the mitotic index, cellularity, vascular proliferation, and nuclear atypia and some immunohistochemical markers.

Material and methods

A group of 39 ependymomas was retrieved from the files of the Department of Neurosurgery of the Medical University of Gdańsk, the Department of Neurosurgery of the Pomeranian Traumatology Center in Gdańsk, the Department of Neurosurgery of the Medical University of Łódź and the Department of Neurosurgery, Polish Mother Memorial Hospital Research Institute, Łódź, over the period of 1991-2008. The only criterion was availability of the tissue material and the clinical data. Twenty-eight patients were children and the remaining 11 patients were adults. The average age for these respective groups was 4.7 and 38.7 years (age range for children 0.5 to 12.9 years, adults 18.5 to 60.7).

Males slightly predominated in the whole group (22 males and 17 females). Twenty-four tumours were intracranial and nine were intraspinal. Location of the tumour was unknown in 6 cases. Nineteen patients died during the follow-up period (mean time of their clinical follow-up 37.2 months). The mean time of follow-up was 67.4 months for the whole group. Four patients were excluded from the survival analysis as they died in the postoperative period.

For each patient, formalin-fixed, paraffin-embedded archival paraffin blocks were available. Routinely (H&E) stained sections were obtained from the block containing the most abundant and representative tumour tissue. Re-evaluation of all morphological features was performed by two pathologists (AZ, WB). The diagnosis was verified based on the WHO classification of CNS tumours [18].

The following histological features were analysed:

- Cellularity was defined as the most crowded cellular area in the tumour that was further analysed. Cellularity was calculated in 10 HPFs (high power field = 400× magnification) that were then scanned and analysed by means of the computer program MultiScanBase 14.02. Since the area of one HPF for the Olympus BX40 microscope is about 0.237 mm^2, we recalculated the obtained results for 1 mm^2.
- Mitotic index. Mitotic figures were counted in the whole area of the tumour present in the slide. The approximate total tumour surface analysed was also calculated and the corrected mitotic index (CMI, number of mitoses per mm^2 of the tumour) was defined.
- Nuclear atypia was evaluated as present or absent, based on presence of nuclear hyperchromasia, irregularity of the shape and variety of size of the nucleus.

- Vascular proliferation was defined as hyperplasia of endothelial cells forming multilayered small vessels or glomeruloid structures.
- Necrosis. Presence or absence of necrosis in the tumour tissue was recorded.

Immunohistochemical analysis

Immunohistochemical staining was performed on deparaffinized 4 μm thick tissue sections on SuperFrost Plus slides using the Envision (Dako) DAB-based system for visualization. For antigen retrieval tissue cooker and buffer pH 6.1 (Dako) was used. The following primary antibodies were used: anti-Ki-67 monoclonal antibody (MIB1, Dako, 1 : 100), anti-hTERT (NCL-hTERT, Novocastra, 1 : 100), anti-epidermal growth factor receptor (EGFR) (EGFR.25, Novocastra, RTU), anti-Olig2 (polyclonal, IBL, 1 : 800), and anti-cyclin D1 (DCS-6, Novocastra, RTU).

The evaluation of antigen expression was determined by two methods: quantitative and semiquantitative:

Quantitative methods. Percentage of positive cells was evaluated by one pathologist (AZ) without knowledge of the clinical data. For each slide, a minimum of the 10 most immunoreactive microscopic fields were examined at high power magnification (400×), counting at least 1000 tumour cells in the areas devoid of necrosis. We counted the cells using the image analysis program MultiScanBase 14.02, on images acquired by an Olympus 5060 digital camera. This quantitative method was used for evaluation of Ki67, Olig2, and cyclin D1 expression.

Semiquantitative method. Hscore (HS) method was used for semiquantitative evaluation of protein expression. The score was obtained with the following formula: 3 × percentage of strongly stained cells + 2 × percentage of moderately stained cells + percentage of weakly stained cells, giving a range of 0 to 3. This method was applied for scoring of all markers examined.

We statistically analysed the clinical and histological features of the tumours, including expression of the selected immunohistochemical markers (Ki-67, cyclin D1, hTERT, EGFR). For categorical data, the χ^2 test was used. Survival curves were calculated and compared by means of the Kaplan-Meier method and the F Cox test. All p values were considered to be statistically significant if less than 0.05. For statistical analysis the program STATISTICA v 8.0 was used.

Results

There were 30 cases of WHO grade II ependymoma and 9 cases of anaplastic ependymoma. The mitotic index was in the range 0-4/mm^2. Nuclear atypia was identified in 5 cases (13%). Cellularity ranged from 2495 to 17 027 cells/mm^2. Microvascular proliferation and necrosis were identified in 12 (31%) and 17 (44%) cases, respectively.

For the entire cohort of patients, overall survival was reduced significantly for paediatric patients (p = 0.008) and patients with intracranial tumour (p = 0.002). High-grade tumours (p = 0.007, Fig. 1A), increased corrected mitotic index (CMI > 0.17/mm^2, p = 0.002, Fig. 1B) and cellularity (> 8800 cells/mm^2, p = 0.04, Fig. 1C) exerted an unfavourable influence on survival. Other histological parameters such as nuclear atypia (p = 0.532), necrosis (p = 0.187) and microvascular proliferation (p = 0.403) did not influence the survival rates. The mitotic index (p = 0.0002) and necrosis (0.026) were important histological features discriminating between WHO grade II and anaplastic ependymoma.

The expression of Ki-67 protein (Fig. 2A) was identified in all ependymomas. Increased Ki-67 LI (> 3%) was associated with worse prognosis (p = 0.002, Fig. 1D). The anaplastic ependymomas presented higher Ki-67 LI than classical ependymomas (p = 0.002).

Cyclin D1 expression (Fig. 2B) was revealed in 18 tumours (46%). Increased expression of cyclin D1 (HS ≥ 0.2) correlated with shorter survival (p = 0.045, Fig. 1E). Furthermore, any level of cyclin D1 expression (HS > 0) in WHO grade II ependymomas was strongly associated with higher risk of death (p = 0.015, Fig. 1F).

The expression of Olig2 (Fig. 2C) was identified in 20/39 (51%) ependymomas. There was no correlation between Olig2 expression and the clinical outcome (p = 0.194).

The expression of telomerase (Fig. 2D) was revealed in 28/39 (72%) ependymomas and it did not influence patient survival (p = 0.41). However, its expression was more common in adults (p = 0.034) and intraspinal tumours (p = 0.001).

Overexpression (Fig. 2E) of epidermal growth factor receptor (EGFR) was identified in 79% (31/39) of ependymomas and its expression correlated with longer survival, but it did not achieve a level of statistical significance (p = 0.060).

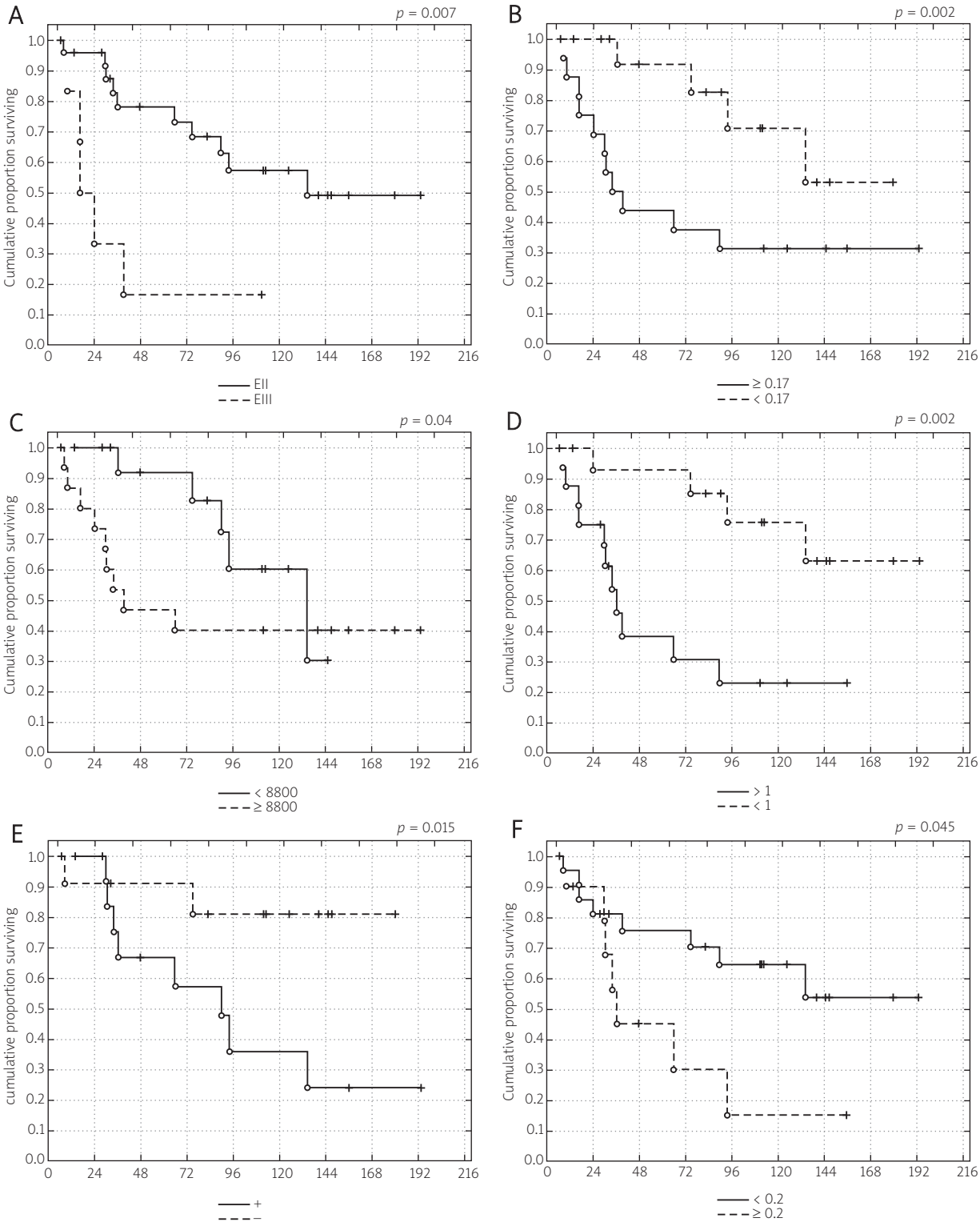

Fig. 1. The impact of morphological and immunohistochemical status on overall survival in patients with ependymoma: tumour grade (**A**), mitotic index (**B**), cellularity (**C**), Ki67HS (**D**), cyclin D1 (**E**), cyclin D1 in ependymomas grade II (**F**).

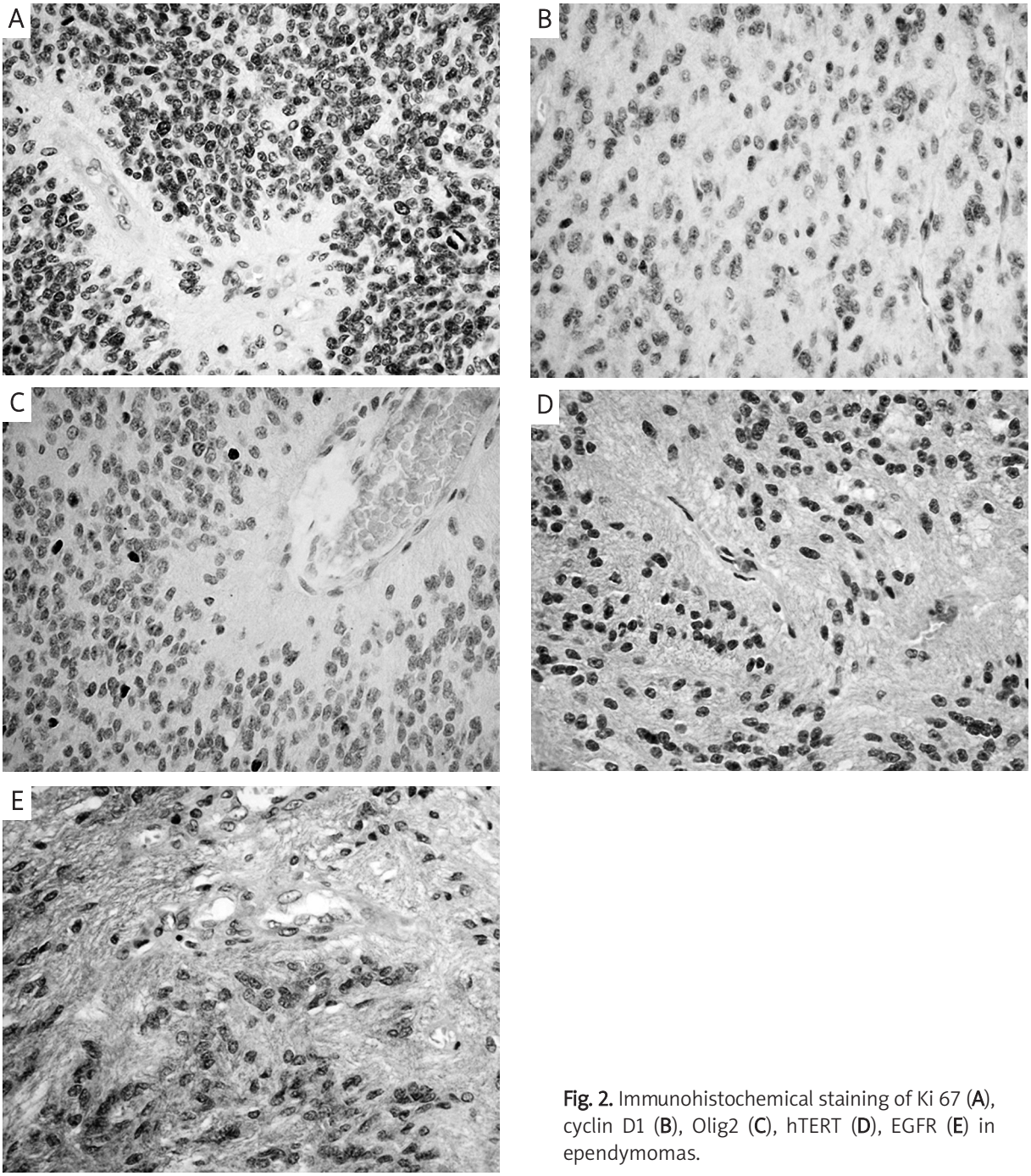

Fig. 2. Immunohistochemical staining of Ki 67 (**A**), cyclin D1 (**B**), Olig2 (**C**), hTERT (**D**), EGFR (**E**) in ependymomas.

The results of the univariate analysis indicated that the tumour grade (HR = 4.59, CI 95% 1.5-14.03, p = 0.015) and expression of Ki-67HS (HR 3.79, CI95% 1.23-4.03, p = 0.007) influenced survival significantly in all ependymomas and Ki67HS (HR = 3.79, CI 95% 1.61-8.97, p = 0.001) and cyclin D1 HS (HR = 3.13, CI95% 1.24-7.93, p = 0.037) in WHO grade II ependymomas.

Discussion

The clinical course in patients with ependymomas depends on a variety of factors. The patient's age and tumour location are closely related to each other as most paediatric ependymomas develop intracranially, whereas adults usually suffer from spinal ependymomas. The better clinical outcome observed in adults may result from this preferential

anatomical location of the tumour rather than from the patient's age only. Intraspinal ependymomas, which are more frequent in adults, are more amenable to total resection, and this may be a critical factor explaining better prognosis in this group of patients [38]. We also confirmed worse survival of paediatric patients than in adults.

Our results also support the previous data concerning better outcome in patients with lower grade tumours [23, 41]. Morphological differentiation between WHO grade II and grade III ependymomas relies on factors that remain subjective. In some reports, the interpersonal discrepancy of grading in the same series of ependymomas amounted to one third of cases [45]. This stems from the various proposals of ependymoma grading. According to Mansur *et al*. the diagnosis of anaplastic ependymoma should be based on the presence of one of two histopathological features: either vascular proliferation or more than 5 mitotic figures per 10 HPF [19]. Concerning the diagnosis of this tumour, Korshunov *et al*. [13] suggested the presence of one of three criteria – cellularity, atypia or vascular proliferation – whereas Ho *et al*. [10] proposed analysis of four parameters: cellularity, mitotic index exceeding 4 figures per 10 HPF, necrotic areas, and endothelial proliferations. Presence of each parameter was assigned a numerical value of one point. A score of two or more points was proposed as diagnostic of anaplastic ependymoma. These features were mentioned in earlier publications, but were not present in such a systemic manner [15,21,37]. In a few publications, the criteria for grading ependymomas were not well specified, precluding comparison of their results [12,25,42].

We undertook verification of several morphological criteria analysed quantitatively. Analysis of the most cell-populated area and its precise calculation showed that cellularity exceeding 8800 cells/mm^2 correlated with less favourable survival. Likewise, total mitotic activity scored in the whole tumour area by means of CMI conferred worse survival if CMI exceeded 0.17/mm^2. Both these results support the rationale for precise determination of the threshold levels that may appear suitable in differentiation between more and less clinically favourable cases of ependymomas that may be grouped into respective histological grades.

Increased cellularity is one of the important diagnostic criteria for anaplastic ependymoma. However, the definition of this feature varies in the different reports. Ho *et al*. reported that mere presence of hypercellular areas, irrespective of degree of hypercellularity, may worsen prognosis in patients with ependymoma [10]. On the other hand, Schwartz *et al*. divided all ependymomas into two groups, with high and low cellularity, and observed a significant decrease of survival in patients with highly cellular tumours [39]. In the view of other authors, more diffuse increase in cellularity may confer more clinical aggressiveness to the tumour than cases with discrete foci of hypercellularity [29].

The results of morphometric methods estimating ependymoma cellularity were not unequivocal. Zamecnik *et al*. [45] suggested a cut-off point for highly cellular tumours at the level of 300 cells per 1 HPF. Likewise, Figarella *et al*. [4] used a threshold level at 15 000 cells per 5 HPFs. However, their results are difficult to compare with ours, as the actual size of 1 HPF was not given in their reports. To overcome this problem, we used a value corrected index of cellularity, evaluating number of cells per 1 mm^2. In our view, further analyses are indispensable to develop, verify and standardize the methods evaluating the tumour cellularity.

The proposed threshold levels of mitotic index discriminating clinically relevant cases varied from 1 to 7 mitotic figures per 10 HPFs [4,10,15,16,45]. The discrepancy between various reports may stem from several determinants, as reviewed by Gal *et al*. [5]. Some problems are methodological, while others may result from different characteristics of tumours in studied patient populations. To avoid some of the methodological problems, and to standardize the mitotic surveying, we used the volume corrected mitotic index, which relies on the same principle as the value corrected index of cellularity. Using this approach in the present study, we determined a relatively low cut-off point CMI equal to 0.17 per 1 mm^2. This is equivalent to identifying a single mitotic figure in 6 mm^2, or about 25 HPFs. In a small biopsy of a 3rd or 4th ventricle tumour, one unequivocal mitotic figure identified in a small biopsy may indicate worse prognosis in a patient with ependymoma.

The other morphological factors (nuclear atypia, vascular proliferations, necrosis) did not correlate with the survival of patients with ependymoma. Nuclear pleomorphism was uncommonly seen in our series, occurring in 12.8% of cases in contrast to the range of 18.5 to 45% reported in the literature. In

other reports this feature does not appear to be a significant factor influencing survival [10,39,45]. Kurt *et al.* suggested it might be of value; however, they identified this correlation in intracranial ependymomas [16].

Vascular proliferation is a well-known phenomenon in glioblastoma and oligodendroglioma. It is infrequently seen in ependymomas [4,32,36]. We observed vascular proliferations in 31% of tumours and this falls in the range reported in other publications (33-62%) [10,16,32,45]. Similarly to most of these reports, we found no correlation with survival of patients with ependymoma.

Necrosis is one of the prominent morphological features in malignant gliomas, including ependymomas [18]. We identified it in 44% of ependymomas. This frequency was similar to other publications, which showed necrosis occurring in 45-58% of ependymomas. Although necrosis was more frequently seen in anaplastic ependymomas (78%), it was also identified in about 33% of classical ependymomas [10,16,45]. Seemingly, necrosis has less impact on prognosis in ependymomas as a single parameter compared with other diffuse gliomas, and this observation is in agreement with previous reports [10,16,45]. Overall, our results showed that the mitotic index and cellularity influenced the survival of patients with ependymomas, while other microscopic features may be of secondary prognostic significance only.

Expression of Ki67, a proliferation marker, is present in ependymomas, irrespective of type and grade [4,16,43,45] but the mean Ki67 labelling index (LI) ranged from 1 to 25% [4,28,36,43,45]. The mean value of Ki67 LI (3%) in our study fell in the lower values of this range. Anaplastic ependymomas usually have higher Ki67 LI than grade II ependymomas [35,36,45]. However, there is not an accepted threshold of that value separating these two groups of tumours and patients with favourable and unfavourable outcome. Various cut-off points of Ki67 LI in relation to the clinical course are reported by investigators [2,6,10,16,36,45]. This variety of opinions makes application of Ki67 LI difficult as a prognostic factor. However, some authors suggest that it plays an independent prognostic role in ependymomas [31,43].

There are several causes of the discrepancies in values of Ki67 LI in various reports. Inter- and intraobserver differences of Ki67 evaluation and differences in the immunohistochemical procedures may contribute to this issue [8,30]. Preusser *et al.* identified a wider range of Ki67 LI values in the same series of cases if analysed by inexperienced pathologists (14-25.4%) in contrast to experienced ones (17.4-22.1%) [30]. Some contribution may derive from the number of cells counted while assessing the LI. This number ranged from 500 to 2000 cells, which is in accordance with methods used in the current study [36,43], while others did not precisely state the methodology of counting. Furthermore, we also determined Ki67 expression using the HS method. This method evaluates Ki67 expression in the entire tumour area. It is easy to apply and, in our view, is as accurate as Ki67 LI.

Cyclin D1 expression was found in 46% of our cases. This value is in agreement with other reports (48-64%). The cyclin D1 index did not differ between classical and anaplastic ependymomas, without an impact on overall survival [28,45]. We found a correlation between cyclin D1 HS index and patient survival. Furthermore, we discovered that any expression of cyclin D1 in classical ependymomas seemed to confer worse prognosis for the patient. Although Zamecnik *et al.* [45] and Prayson [28] evaluated cyclin D1 expression in ependymomas, their studies concerned entirely different patient populations. The former study concerned children with intracranial ependymomas, while the latter included patients at any age with various locations of the tumour.

Similar to the results of Ishizawa *et al.* [11], we found Olig2 expression in about 50% of ependymomas. There was no evidence of a dependency between Olig2 expression and patient survival.

Results of evaluation of expression of EGFR in ependymomas are controversial. It has been a topic of a few recent investigations which demonstrated its presence in about 40-60% of intracranial tumours [14,22]. On the other hand, Ridley *et al.* found EGFR in one of 63 cases (2%); however, they analysed childhood ependymomas only [35]. Our results support the former results, with EGFR expression detected in 79% of cases. In agreement with some of the published reports [33,35], we did not find any impact of EGFR expression on survival. Only Korshunov *et al.* [14] suggest a significant impact of EGFR on progression-free survival. They also identified differences in EGFR expression in respect to the tumour grade. We share the observations of Mendrzyk *et al.* [22], who

did not find differences in EGFR expression between WHO grade II and III ependymomas.

We detected expression of hTERT in 72% of ependymomas without any impact on survival, as found by few authors [14,15]. The study by Tabori [40] concerned a paediatric population only, while the demographic characteristics of patients with ependymomas were not provided by Mendrzyk *et al*. [22]. Both of these publications used a microarray tissue technique to examine expression of hTERT. With the evident advantages of this method, we must remember its limitations, especially in the case of non-uniform distribution of an antigen in the tumour tissue. On the other hand, according to Wu *et al*. [44] the antibody NCL-hTERT (Novocastra) may recognize not only hTert but nucleolin too, making this immunohistochemical expression non-specific.

In conclusion, we confirmed the prognostic significance of two ependymoma subtypes: intracranial, preferentially occurring in children, and intraspinal, predominantly in adults. The other prognostic factors influencing the clinical course of ependymoma were tumour cellularity, mitotic figures, and expression of Ki67 and of cyclin D1. All of them had a significant impact in univariate analysis. The value and reproducibility of the HS method for evaluation of Ki67 and cyclin D1 expression should be verified in a larger group of tumours.

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