Introduction.
Heterogeneity is a characteristic feature of malignant tumours. It challenges the treatment regimens as well as can impair the diagnostic accuracy. Glioblastoma multiforme (GBM), a high-grade malignant glial tumour, is known for the extreme morphological heterogeneity giving rise also to the term itself.

Aim of the study was to evaluate heterogeneity of pathogenetically and diagnostically important cardinal tumour features, namely, cellular proliferation and tumour suppressor protein expression in GBMs.

Material and methods. The study group comprised 101 GBMs, retrospectively identified by archive search. The inclusion criteria comprised validated diagnosis (by World Health Organisation criteria) and lack of prior treatment. Recurrent GBMs as well as other glial and non-glial tumours were excluded from the study. Insufficient tissue materials comprising stereotactic biopsies and tissues affected by widespread necrosis (exceeding 90%) were also excluded. Proliferation activity (by Ki-67) and expression of aberrant p53 protein was detected by immunohistochemical investigation (IHC) of formalin-fixed, paraplast-embedded tumour samples. Polymeric visualisation system was used to detect bound primary antibodies. The expression of each antigen was measured by computed morphometry in at least 200 cells of hot and cold spots in each tumour. The data were expressed as the relative value. Heterogeneity was estimated as the mathematical difference between the highest and lowest expression value in each tumour. Descriptive statistics was applied. The 95% confidence intervals (CI) were determined as well.

Results. The highest proliferation activity ranged 15 – 95%; mean 43.9% [95% CI = 40.3 – 47.6]. The lowest proliferation activity ranged 2 – 95%, mean 20.1% [16.8 – 23.4]. The mean proliferation heterogeneity was 23.8% [21.5 – 26.2]; range 0 – 67%. The mean heterogeneity of p53 protein expression was 11.7% [8.9 – 14.6], ranging 0 – 75%.

Conclusions. GBM is characterized by marked heterogeneity regarding proliferation rate and expression of p53 protein that may affect diagnostic accuracy and grading of gliomas in small samples of tissue material as well as survival in case of small residual tumour after surgical treatment.

Key words: glioblastoma, heterogeneity, immunohistochemistry, proliferation

INTRODUCTION
The issue of intratumoural heterogeneity (ITH) is a well-known feature of malignant tumours. The tumour clonal evolution model described by P. Nowell in 1976 explained the ITH on Darwinian selection over time, generated by genetic instability (Nowell, 1976). This selection results in origination of different cellular subpopulations with distinct mutations and protein expression profiles leading to various biological features (Nowell, 1976). Such heterogeneity provides the tumour with several advantages promoting tumour growth and progression as well as resistance to therapy, but it can also lead to erroneous diagnosis.

ITH is described in many neoplasms including glioblastoma (GBM), which is an extremely heterogeneous tumour in many aspects. ITH in glial tumours has also been described regarding cellular proliferation and p53 protein expression (Coons and Johnson, 1993; Dalrymple et al., 1994; Ren et al., 2007; Soussi and Lozano, 2005). However, some of the studies are small, comprising only 11 gliomas (Ren et al., 2007).

In addition, the ongoing technological progress both in diagnostic evaluation and treatment necessitates integrated evaluation, including high-affinity investigation and sensitive visualisation systems. The implementation of immunohistochemistry as a practical surrogate for molecular analysis also has to be evaluated in sufficiently large tumour groups as such substitution has showed high efficiency in certain epithelial tumours (Bhargava et al., 2010).

Amplification of oncogenes and inactivation of tumour suppressor genes are frequently found genetic abnormalities in human cancers including GBMs (Ohgaki and Kleihues, 2007; Ng et al., 2012). TP53 is a well-known tumour suppressor gene that has been widely studied over these past decades in many cancers. The gene encodes p53 protein that is involved in cell cycle regulation and prevents the proliferation of genetically damaged cells. TP53 is frequently mutated in GBMs, including 28% of primary and 65% of secondary GBM (Ohgaki and Kleihues, 2007). p53 overexpression is frequently seen in many TP53 mutations thus immunohistochemistry can be used as surrogate method for detection TP53 mutation (Milinkovic et al., 2012).

Uncontrolled cellular proliferation is the hallmark of the neoplastic process. It directly represents tumour behaviour. Thus, proliferation activity, expressed also...
as proliferation index can be used as an auxiliary tool in evaluation of grade in glial tumours together with morphological features (Louis et al., 2007). However, regional heterogeneity of proliferative activity and p53 protein expression is an important factor that influences accurate estimation of tumour and its malignancy grade.

AIM OF THE STUDY
The aim of this study was to evaluate the heterogeneity of p53 protein expression and proliferation activity by Ki-67 performing immunohistochemical investigation of glioblastoma tissue material.

MATERIAL AND METHODS

Patients and tissue specimens
In this study, formalin-fixed paraffin-embedded tissues from 101 glioblastoma patients were investigated. The consecutive cases were identified by retrospective archive search in a single university hospital. Only primarily diagnosed, histologically proven cases of GBMs which unequivocally met the criteria of classification of tumours of the central nervous system, issued by World Health Organisation (Louis et al., 2007) were included in the study. The inclusion criteria comprised also the lack of prior treatment. Recurrent GBMs as well as other glial and non-glial tumours were excluded from the study. Insufficient tissue materials comprising stereotactic biopsies and tissues affected by widespread necrosis (exceeding 90%) were also excluded.

Immunohistochemical visualization
Immunohistochemical visualisation of p53 and Ki-67 proteins was performed. Briefly, 3 μm-thick tissue sections were cut on electrostatic slides, deparaffinized in xylene and hydrated in a series of graded ethanol. Heat induced antigen retrieval was performed in a microwave oven using basic TEG buffer (pH 9.0). After blocking of endogenous peroxidase, the sections were incubated with primary antibodies at room temperature for 60 min. The characteristics of primary antibodies were following: anti-Ki-67, monoclonal mouse antibody against human antigen, clone MIB-1; anti-p53, monoclonal mouse antibody against human antigen, clone DO-7. The bound primary antibodies were detected by high-sensitivity polymeric visualisation system EnVision linked with horseradish peroxidase. For the development of colour signal, 3,3’-diaminobenzidine was used as chromogen. All immunohistochemistry reagents were produced by Dako, Glostrup, Denmark.

Evaluation of immunohistochemical staining and data analysis
Computed morphometry was performed using specialised software Kappa Image Base (KAPPA optoelectronics Inc., United States of America). The optical system was represented by Axiolab (Zeiss, Oberkochen, Germany) microscope, and Kappa CF 11 DSP camera was used for image capture. Hot and cold spots were identified at scanning magnification (100x). The scoring was performed in at least 200 tumour cells using high-power magnification of 400x. Only nuclear expression of markers was scored. The expression of Ki-67 and p53 was evaluated quantitatively as the relative value: percentage of positive tumour cells over total tumour cells (%). An evaluation of highest and lowest relative values of expression (%) for Ki-67 and p53 were measured in GBM specimens. The heterogeneity was calculated as a difference between highest and lowest finding in the particular tumour. Descriptive statistical analysis was performed including calculation of 95% confidence interval (CI) by CIA software as described by Altman et al., 2000.

RESULTS
The study group comprised 101 GBM patients (52 males, 49 females) with the mean age of 61.2 years [95% CI = 58.8 – 63.5].

Expression of p53 and Ki-67 was confined to the nuclei of neoplastic cells (Figure 1). The staining was heterogeneous in distribution (Table 1) and varied in different fields. Thus, the highest proliferation activity ranged 15 – 95%, resulting in mean value 43.9% [40.3 – 47.6]. The lowest proliferation activity ranged 2 – 95%, mean 20.1% [16.8 – 23.4]. The mean proliferation heterogeneity by the difference between the highest and lowest proliferation activity was 23.8% [21.5 – 26.2]; range 0 – 67%.

Fig. 1. Diverse p53 protein expression and proliferation activity in glioblastoma. A. Intense p53 expression in tumour cells. Note the characteristic nuclear reactivity. Immunoperoxidase (IP), anti-p53, original magnification (OM) 100x. B. Lack of p53 expression in tumour cells. IP, anti-p53, OM 100x. C. High proliferation activity in tumour cells. IP, anti-Ki-67, original magnification (OM) 100x.
Table 1. Proliferation activity by Ki-67 and p53 expression values in glioblastoma

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Highest value, %</th>
<th>Lowest value, %</th>
<th>Heterogeneity, %</th>
</tr>
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<tbody>
<tr>
<td>Ki-67</td>
<td>Range 15 – 95</td>
<td>2 – 95</td>
<td>0 – 67</td>
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<tr>
<td></td>
<td>Mean 43.9</td>
<td>20.1</td>
<td>23.8</td>
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<td></td>
<td>95% CI 40.3 – 47.6</td>
<td>16.8 – 23.4</td>
<td>21.5 – 26.2</td>
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<td>p53</td>
<td>Range 0 – 100</td>
<td>0 – 100</td>
<td>0 – 75</td>
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<tr>
<td></td>
<td>Mean 34.1</td>
<td>22.3</td>
<td>11.7</td>
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<tr>
<td></td>
<td>95% CI 26.4 – 41.2</td>
<td>15.2 – 29.5</td>
<td>8.9 – 14.6</td>
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Abbreviations in the Table: CI, confidence interval for the mean

Both highest and lowest expression of p53 protein reached extreme values: 0 – 100%. Aberrant p53 protein expression was absent (0%) in 23 GBMs. Only rare tumour cell nuclei (1 – 5%) expressed p53 in 8 GBMs. Moderate p53 expression (6 – 50%) was found in 40 GBMs. However, 30 GBMs showed strong expression of p53 in more than 50% of nuclei. The mean heterogeneity by the difference between the highest and lowest relative expression was 11.7% [8.9 – 14.6], ranging 0 – 75%.

Fig. 2. Distribution of the highest and lowest Ki-67 proliferation indices and p53 expression values in glioblastoma by box-plot (2A) and point scatter (2B) analysis.

Abbreviations in the Figure: Ki67Max, the highest proliferation activity; Ki67Min, the lowest proliferation activity; p53Max, the highest relative expression of p53 protein; p53Min, the lowest relative expression of p53 protein

DISCUSSION

This study demonstrated that GBMs show significant ITH of cell proliferation and p53 protein expression. Such heterogeneity may pose a problem in the examination of small tissue material.

There are very few studies regarding p53 expression heterogeneity in GBMs. We found a single one by Ren et al., 2007. They performed microdissection in different areas of 11 gliomas, including 8 GBMs, followed by TP53 gene mutation analysis and p53 protein immunohistochemistry in the dissected neoplastic tissues. Seven gliomas had p53 gene mutations and five from these were positive for p53 protein. The authors noticed the presence of different TP53 gene mutations in distinct areas within the same tumour. Coexistence of wild-type and mutated TP53 was also found in different areas from the same GBM (Ren et al., 2007). Such findings indicate high rate of p53 gene heterogeneity in GBMs. p53 protein expression by immunohistochemistry seems to be more influenced from heterogeneity because not all mutations result in p53 protein over-expression. The present study confirms also that immunohistochemistry can be successfully applied as surrogate method in GBM research. Thus, larger tumour group comprising more than hundred cases could be investigated. The examination can also be included in clinical diagnostic pathway to characterise the individual tumour heterogeneity.

Proliferation activity, known also as proliferation fraction or Ki-67 proliferation index is a powerful prognostic indicator in a variety of neoplasms. There is strong correlation with the grade of malignancy and proliferation index in glial neoplasms (Louis et al., 2007). Proliferation index in diffuse astrocytomas (grade 2) is usually less than 4%, with the mean 2.5 %, anaplastic astrocytomas (grade 3) show indices in the range of 5 – 10%. For GBMs (grade 4) mean values of 15 – 20% have been reported (Louis et al., 2007). However, considerable overlap of values among different tumour grades have been described (Torp, 2002). Thus, Ki-67 cannot be used alone as a diagnostic tool. Despite this limitation, evaluation of Ki-67 is a useful adjunct in morphological grading of astrocytomas in combination with histopathological features as well as clinical data and radiologic findings. It can also help in some problematic cases, for example, evaluating neoplasm with low-grade histology in the available tissue material if other factors indicate high-grade tumour. On the other hand, regional heterogeneity has been demonstrated in cell proliferation in different areas within the same tumour (Coons and Johnson, 1993). The highest proliferation in GBMs has been shown in those cells located at the solid tumour-infiltrated parenchyma interface (Dalrymple et al., 1994).
The heterogeneity can unpredictably influence the survival after surgical treatment leading to small residual tumour volume. Regarding the diagnostic accuracy, the highest impact of heterogeneity can be expected in small tissue samples such as stereotactic biopsy specimens because it may not include areas with the highest proliferation activity or sufficient histopathological features of the neoplasm we are dealing with. Thus, we should be aware of diagnostic accuracy limitations in small tissue samples.

**CONCLUSIONS**

GBM is characterized by extreme heterogeneity regarding proliferation rate and expression of tumour suppressor proteins. The identified heterogeneity can affect diagnostic accuracy and grading of gliomas in small samples of tissue material as well as the outcome after surgical treatment.

**Conflict of interest:** None

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