Association of loss of heterozygosity with shorter survival in primary glioblastoma patients

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Loss of heterozygosity (LOH) co-deletion 1p/19q, *MGMT* promoter methylation and/or *IDH1* mutation generally signify a better prognosis for patients with glioma. However, the influence of 1p/19q co-deletion and the LOH on other chromosomes in primary glioblastoma on survival is still debatable. The aim of our study was to identify LOH on chromosomes 1p, 19q, 9p, 10q, 13q, and 17p, and evaluate their impact either alone or 1p/19q co-deletion or by groups of LOH on the overall survival of 42 primary glioblastoma patients without an oligodendroglial component. These patients were additionally molecularly characterized for *EGFR* amplification, *IDH1* mutations and *TP53* mutations. We assessed their influence on the overall survival of glioblastoma patients. LOH in at least one of the loci on all examined chromosomes was detected in 65% of cases and was significantly associated with shorter overall survival (hazard ratio 3.07; 95% CI: 1.29-7.31, p = 0.006). 1p/19q co-deletion was infrequent (7.14%) and had no impact on overall survival. Our results indicate that in primary glioblastoma a specific LOH group analysis may be important for the prognosis. LOH 1p/19q co-deletion is rare in glioblastoma without an oligodendroglial component and has no impact on patient survival.

Key words: glioblastoma, LOH 1p, 19q, 9p, 10q, 13q and 17p, survival.

Introduction

Glioblastoma (GB) carries complex genetic alterations resulting in a different molecular and epidemiological profile. In primary glioblastoma, the most common molecular alterations are LOH 10q (over 70%), *EGFR* amplification (about 40%), *MDM2* amplification, LOH 10p and 10q, $p16^{INK4a}$ and *PTEN* mutation. In secondary glioblastoma, the first common molecular event in multistep carcinogenesis is the mutation of *IDH1*, *TP53*, and LOH on 17p, 10q and 19q [1-3].

There are three molecular markers related to better outcome of gliomas: *MGMT* promoter methylation is associated with a stronger benefit of radiochemotherapy in glioblastomas; *IDH1* mutations are a strong and independent predictor of survival in both low-grade and high-grade gliomas; while 1p/19q co-deletion strongly predicts prolonged response to treatment and longer

survival in oligodendroglial tumors [3-6]. However, recent data showed that the prognostic significance of these markers should be used with caution [7].

MGMT promoter methylation has been associated with better survival in glioblastoma treated with radiotherapy and alkylating agents [6]. In our previous study, *MGMT* promoter methylation had no prognostic value in glioblastoma patients who were treated only with surgery and radiotherapy [8]. With regards to *MGMT* methylation status and the occurrence of *EGFR* amplification, the group of glioblastoma that shows no hypermethylation with amplification constituted about 19% of cases and the same percentage was a group with hypermethylation and amplification. These patients could potentially benefit from bimodal chemotherapy with an alkylating agent and/or *EGFR* blocker additionaly [9].

Mutations of the *IDH1/IDH2* gene inversely correlated with glioma grade [3, 10]. *IDH1/IDH2* mutations occurred mainly in secondary glioblastomas (> 70%), associated with young age of patients and with an increase in overall survival, but were very rare in primary glioblastomas (< 5%) [3]. *IDH2* mutations are less frequent and prevail in anaplastic oligodendrogliomas (5%) and oligoastrocytomas (6%) [3]. 100% of patients with 1p/19q co-deletion had *IDH1/IDH2* gene mutations and indicated occurrence of an oligodendroglial component in glioblastoma [11].

LOH 1p was detected in up to 24% of glioblastomas [12-14] and the frequency of LOH 1p is similar in primary and secondary glioblastoma (12% and 15%, respectively) [15], but its prognostic significance is not well established. It has been suggested that the isolated deletion of 1p may be associated with longer survival in high-grade gliomas composed of either pure astrocytic or mixed astrocytic-oligodendroglial phenotypes [16]. In one study, LOH 1p was found to be associated with longer survival in glioblastoma patients [17]; however, another study reported no influence of isolated LOH 1p on survival [18]. LOH 19q (a common deletion at 19q13.3) frequency varies from 5% to 33% [12, 14, 18] and it is more frequent in secondary glioblastoma (54%) than in primary glioblastoma (6%) [15]. However, its impact on the prognosis in GB is not clear and remains controversial [14, 17, 19, 20]. Although the co-deletion LOH 1p/19q is an important predictive marker of chemosensitivity in oligodendrogliomas [5], its occurrence is not frequent in primary GB [14, 18, 21]. The clinical significance of 1p/19q in glioblastoma is reported by some [4, 18] but another study showed no impact on the prognosis of GB [21].

It was reported that LOH 10q is associated with shorter survival and poor clinical outcome [1, 2], whereas other studies have found no such association [18, 22]. The frequency of LOH on chromosome 10 in primary and secondary GB is similar (47% in primary vs. 54% in secondary GB) but the differences concern the pattern of chromosomal loss [23]. The loss of an entire copy of chromosome 10 was usually observed in primary glioblastoma, while LOH is restricted to 10q in secondary glioblastoma [1, 23].

Despite intensive investigations the molecular prognostic factors for primary glioblastoma are still debatable. The aim of our study was to evaluate the impact of LOH on chromosomes 1p, 19q, 9p, 10q, 13q, 17p either alone or by groups of LOH and *TP53* mutations, and *EGFR* amplification on the survival of primary glioblastoma patients. We also focused on the occurrence and clinical significance of LOH 1p/19q in GB without an oligodendroglial component.

Material and methods

Tumor samples and DNA extractions

The material consisted of tumor tissue with primary GB and paired peripheral blood from 42 patients (agreement of Bioethical Committee of Medical University of Lodz, Poland RNN/192/03/KE). Tumor samples were obtained from 42 patients with primary glioblastoma (21 males and 21 females) who were treated in the Departments of Neurosurgery of "Copernicus" Memory Hospital in Lodz, Barlicki Clinical Hospital of Medical University of Lodz, Regional Specialist Hospital in Olsztyn, and Perzyna Memorial Hospital in Kalisz, Poland, from 2002 to 2005. Informed consent from patients was obtained in every case.

The mean age of the patients was 59.1 ±11.8. All tumors were histopathologically examined and classified according to the World Health Organization (WHO) classification of tumors of the CNS [24].

Patients with primary glioblastoma were selected on the basis of a short clinical history and the presence of histopathological features of glioblastoma without evidence of precursor low-grade astrocytomas at the first biopsy [25]. No oligodendroglial components were found in the histopathological study. According to the classical model of the molecular GB pathway, primary glioblastoma is characterized by high incidence of EGFR amplification (about 40%) and low frequency of *IDH1* mutations (< 5%); additionally, *TP53* mutations are less frequent in primary GB [26, 27]. To ascertain that the tumors studied were primary glioblastomas we used the following molecular criteria: frequencies of *EGFR* amplification (37.5%), *IDH1* mutation (2.4%), and *TP53* mutation (26.2%); the results were partly published before (*EGFR* amplification and *TP53* mutations) [8]. Moreover, the 1p/19q co-deletion was infrequent (3 cases), and in 2 of these cases there was no co-presentation with *IDH1* mutation (one of these cases was not determined for *IDH1* mutation), which is typical for primary glioblastoma without an oligodendroglial component [11] (Table I). It means that these cases showed molecular markers typical for primary GB.

Table I. Clinical data and results of molecular alterations analysis

No.	AGE/SEX	OS (MS)	LOH10p	LOH9p	LOH17p	LOH13q	LOH1p	LOH19q	*EGFR* AMPL	*TP53* MUTATION – EXON (E), CODON, TYPE OF MUTATION AND EFFECT	*IDH1* MUTATION
1.	69/M	3	yes	yes	yes	NI	no	no	yes	E 8, 273, CGT>CAT, Arg-His	no
2.	57/M	11	yes	no	yes	no	no	yes	yes	E 8, 282, CGG>TGG, Arg-Trp	no
3.	67/F	2	no	no	no	yes	no	yes	no	E 5, 175, CGC>CAC, Arg-His E 8, 282, CGG>TGG, Arg-Trp	no
4.	76/F	5	yes	yes	NI	no	yes	no	yes	no	no
5.	43/F	12	no	yes	no	no	no	no	no	E 5, 173, GTG>TTG, Val-Leu	no
6.	70/F	11	no	no	no	no	no	no	no	E 5,179, CAT> GAT, His-Asp	no
7.	62/F	20	no	no	no	no	no	no	no	no	no
8.	72/F	5	no	no	no	no	**yes**	**yes**	no	no	ND
9.	64/M	14	yes	NI	NI	NI	no	yes	yes	no	no
10.	71/F	19	yes	yes	no	no	yes	no	yes	no	no
11.	54/M	12	yes	no	no	no	yes	no	yes	no	no
12.	68/F	9	no	no	no	no	no	yes	no	no	no
13.	66/F	2	no	NI	NI	no	NI	yes	yes	no	no
14.	51/M	10	no	NI	NI	no	no	no	yes	no	no
15.	60/F	9	NI	yes	NI	no	no	NI	yes	no	no
16.	69/F	11	no	yes	no	no	no	no	no	no	no
17.	50/M	15	yes	no	no	no	no	yes	no	no	no
18.	69/F	12	yes	NI	no	no	no	no	yes	no	no
19.	67/M	13	yes	no	yes	yes	no	no	no	E 6, 190, CCT>TCT, Prol-Ser	no
20.	52/F	3	no	no	no	no	no	yes	no	no	no
21.	65/F	7	no	no	no	no	no	no	no	no	no
22.	74/M	8	no	no	no	no	no	no	no	no	no
23.	34/M	28	no	no	no	no	no	no	no	E 8, 273, CGT>TGT, Arg-Cys	no
24.	40/M	26	no	no	no	no	no	no	no	no	no
25.	75/F	48	no	no	no	no	no	no	no	E 7, 234, TAC>CAC, Trp-His	yes
26.	44/M	20	no	no	no	yes	no	no	no	no	no
27.	60/M	3	yes	no	no	no	no	no	yes	no	no
28.	64/M	14	ND	ND	ND	ND	ND	ND	ND	no	no
29.	62/M	12	no	no	no	no	no	no	yes	no	no
30.	66/M	26	ND	ND	ND	ND	ND	ND	ND	no	no
31.	55/F	22	no	no	no	no	no	no	no	no	no
32.	51/M	8	no	no	no	no	no	no	no	E 7, 237, ATG>ATA Met-Ile	no
33.	75/F	11	no	no	yes	no	no	no	no	E 8, 267, CGG>TGG, Arg-Trp	no
34.	68/F	5	yes	yes	no	no	yes	no	no	no	no
35.	47/F	17	yes	no	no	no	**yes**	**yes**	yes	no	no
36.	50/F	11	yes	no	no	no	no	no	no	no	no

Table I. Cont.

No.	AGE/SEX	OS (MS)	LOH10p	LOH9p	LOH17p	LOH13q	LOH1p	LOH19q	*EGFR* AMPL	*TP53* MUTATION – EXON (E), CODON, TYPE OF MUTATION AND EFFECT	*IDH1* MUTATION
37.	23/M	6	no	no	no	no	no	no	no	no	no
38.	52/M	16	no	yes	no	no	no	no	yes	no	no
39.	58/F	18	no	no	yes	no	no	no	no	E 6, 215, AGT>AAT, Ser-Asn	no
40.	52/M	11	yes	no	no	no	no	no	no	no	no
41.	51/M	12	no	no	no	yes	yes	yes	yes	no	no
42.	59/M	13	no	no	no	no	no	no	no	no	no

OS – overall survival, ms – months, ampl – amplification, ND – not done, NI – non-informative

All patients underwent total or partial surgery and radiotherapy and 7 underwent chemotherapy (temozolomide). The patients who received chemotherapy were not treated routinely with temozolomide according to the previous standard protocol therapy in the years 2002-2005 in Poland.

DNA was isolated by standard proteinase K digestion and phenol/chloroform extraction from frozen tumor tissue samples taken before radio- and/or chemotherapy, and from paired samples of peripheral blood leukocytes (WBC). Histological assessment of tissue fragments chosen for this study confirmed that all specimens consisted of at least 80% tumor cells.

Loss of heterozygosity analysis

Loss of heterozygosity analysis was performed using paired tumor specimens and corresponding peripheral blood samples. The LOH on chromosomes 1p, 9p, 10q, 13q, 17p, and 19q were examined using PCR with the markers D1S508 (approximate chromosomal localization 1p36.23), D9S156 (9p22), D9S162 (9p21-9p22), D10S587 (10q25-10q26), D10S536 (10q23), D13S256 (13q21-13q14), D17S675 (17p13.2) D19S206 (19q13.3), and D19S219 (19q13.3). Forward primers were fluorescence-labeled at the 5' end. The PCR was performed in thermocycling conditions established individually for each pair of primers. The PCR products were denatured and separated by gel electrophoresis in a LiCor automatic sequencer system for the analysis of PCR-generated alleles.

TP53 sequencing analysis

Four genomic regions of the *TP53* gene (exons 5-8) were amplified by PCR, as described previously [8]. Sequence analysis was performed by the dideoxy termination method using the SequiTherm Excel DNA Sequencing Kit (Epicentre Technologies, Madison, WI) and commercially available fluorescent-labeled primers as described previously [8]. Products of the sequencing reaction were visualized and analyzed using a LiCor automated laser fluorescence sequencer.

IDH1 sequencing analysis

Exon 4, including codon 132 of the *IDH1* gene, was amplified by PCR and sequenced using the dideoxy termination method and SequiTherm Excel DNA Sequencing Kit (Epicentre Technologies). The commercially available primers used for PCR amplification of the DNA sequences were: IDH1 – 5'-GGCACCCATCTTCTGTGTTT-3' (sense) and 5'-ATATATGCATTTCTCAATTTCA-3' (antisense). The sequencing primers used were: IDH1 exon 4 – 5'-CGGTCTTCAGAGAAGCCATT-3' (sense) and IDH1 exon 4 – 5'-CACATTATTGCCAACATGAC-3' (antisense). A Li-Cor automatic sequencer system was applied for the separation and analysis of PCR sequencing products.

EGFR amplification analysis

Multiplex PCR was performed for evaluation of *EGFR* amplification with superoxide dismutase 1 (*SOD1*) used as a reference gene as described previously [28].

Statistical analysis

The Kaplan-Meier method was used to estimate overall survival, defined as the time from the date of the first surgery to the last observation. Differences in survival distributions were evaluated using a log-rank test. Univariate and multivariate survival analyses were performed using the Cox proportional hazard regression model. All results were considered statistically significant when two-sided p was < 0.05.

Results

Loss of heterozygosity

Loss of heterozygosity in at least one of the loci on all examined chromosomes was detected in 65% (26/40) of the informative cases. The results of LOH

Table II. Frequency of LOH on examined chromosomes

	LOH 1p	LOH 19q	LOH 9p	LOH 10q	LOH 13q	LOH 17p	LOH 1p/19q	TOTAL LOH
Percent of informative cases	17.9% (7/39)	25.6% (10/39)	22.6% (8/36)	35.8% (14/39)	10.5% (4/38)	14.3% (5/35)	7.14% (3/35)	65% (26/40)

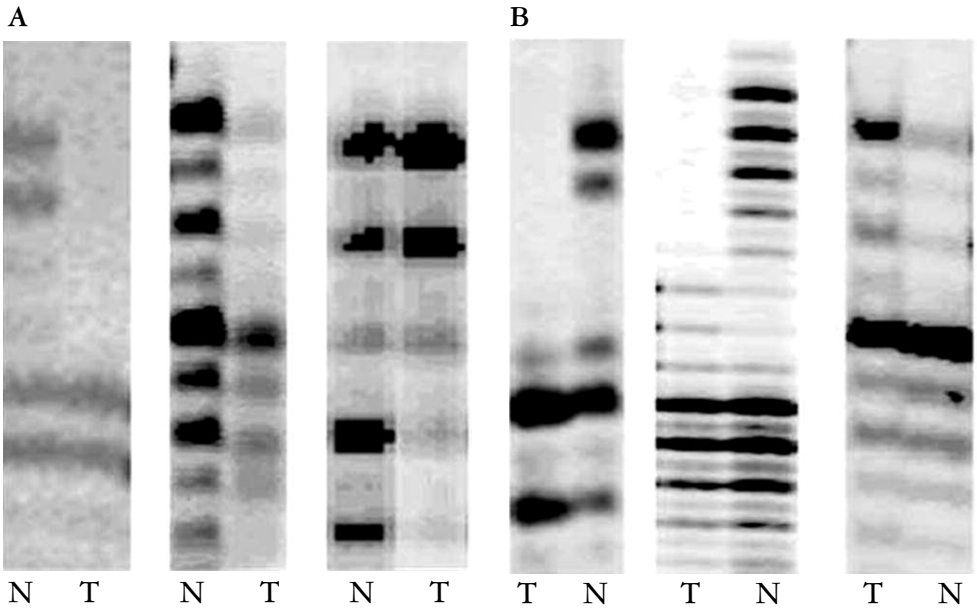

Fig. 1. The representative results of LOH analysis: A) LOH 1p and B) LOH 19q. T – tumor sample; N – a corresponding normal tissue (blood)

are shown in Table I and II. Representative results for LOH 1p and 19q analysis are shown in Fig. 1.

Loss of heterozygosity and the influence on prognosis and age

The mean survival for the whole group of 42 patients was 12.9 ±8.5 months; median was 11.5 months (range 2-48). The correlations between LOH and survival are shown in Table III.

The clinical outcome of patients with LOH in at least one of the loci examined on all chromosomes, except LOH 13q (any LOH; n = 26), was significantly worse than that of patients without LOH (no LOH; n = 14) (log-rank, p = 0.007) (Fig. 2). LOH 13q was excluded from this calculation because it was associated with a hazard ratio (HR) below 1.00, while all other LOHs were associated with HR > 1.00. The median overall survival of the patients with any LOH was 11 months (range 2-19 months), while median survival time was 13.0 months in the group of patients without LOH (range 6-48 months). Cox univariate analysis confirmed that patients with LOH at any of the loci examined was related to a significantly increased risk of death (hazard ratio 3.07; 95% CI: 1.29-7.31; p = 0.006). There was no statistically significant association between LOH 1p/19q and survival (hazard ratio 1.17; 95% CI: 0.36-3.8; p = 0.79). There was no statistically significant association between other LOH, as a single parameter, and overall survival (Table III). The correlation between LOH (as a single parameter or a group of LOH) and age also was not statistically significant.

EGFR, *TP53* and *IDH1* alterations and the influence on prognosis and age

EGFR amplification was identified in 37.5% (15/40). *IDH1* mutation was found only in one case (2.4%) (case 25). In this case there was no 1p/19q codeletion. One case was not determined for *IDH1* mutation. The *TP53* mutations were identified in 26.2% (11/42) of cases within exons 5, 6, 7 and 8. All the mutations of TP53 were missense and are equally distributed through the exons (Table I). There was no difference in survival between patients with these molecular alterations and patient age.

Discussion

The clinical outcome of glioblastoma patients with loss of heterozygosity on chromosomes 1p, 19q, 9p, 10q,

Table III. Correlations between LOH and survival

	Median survival time	Hazard ratio (HR) p-value
LOH 1p (+) (n = 7)	12 months (range 5-19)	HR 1.31; 95% CI: 0.57-3.03; p = 0.537
LOH 1p (–) (n = 32)	11 months (range 2-48)	
LOH 19q (+) (n = 10)	10 months (range 2-17)	HR 1.87; 95% CI: 0.88-3.98; p = 0.121
LOH 19q (–) (n = 29)	12 months (range 3-48)	
LOH 9p (+) (n = 8)	10.0 months (range 3-19)	HR 1.62; 95% CI: 0.72-3.66; p = 0.261
LOH 9p (–) (n = 28)	11.5 months (range 2-48)	
LOH 10q (+) (n = 14)	11.5 months (range 3-19)	HR 1.43; 95% CI: 0.71-2.89; p = 0.318
LOH 10q (–) (n = 25)	11 months (range 2-48)	
LOH 13q (+) (n = 4)	12.5 months (range 2-20)	HR 0.9; 95% CI: 0.33-2.71; p = 0.924
LOH 13q (–) (n = 34)	11 months (range 2-48)	
LOH 17p (+) (n = 5)	11.0 months (range 3-18)	HR 1.36; 95% CI: 0.51-3.58; p = 0.549
LOH 17p (–) (n = 30)	12 months (range 2-48)	
LOH 1p/19q (+) (n = 3)	12 months (range 5-17)	HR 1.17; 95% CI: 0.36-3,8; p = 0.79
LOH 1p/19q (–) (n = 32)	11 months (range 2-48)	
Any LOH (+) (n = 26)	11 months (range 2-19)	HR 3.07; 95% CI: 1.29-7.3; **p = 0.006**
Any LOH (–) (n = 14)	13.0 months (range 6-48)	

13q and 17p has been reported previously, but their prognostic influence in glioblastoma is still controversial [14, 18, 20-22, 29]. In this study LOH in at least one of the loci in all examined chromosomes was detected in 65% of cases. The presence of LOH at any of the loci examined (except LOH 13q) was related to a significant unfavorable impact on the overall survival of the patients (hazard ratio 3.07; 95% CI: 1.29-7.31; p = 0.006) (Table III). In contrast, in a large GB study no correlation was found with survival for LOH 1p, 19q, LOH 9p or LOH 10q or other molecular alterations (*EGFR* amplification, *CDK4* amplification, INK4A/ARF deletion) analyzed either alone or by groups of alterations [19]. We did not find any prognostic significance of LOH as a single parameter (Table III).

A shorter median survival in patients with LOH 10q had been reported previously [1] especially at the *PTEN* locus [22]. On the other hand, Houillier *et al*. found no correlation between LOH 10q and survival [19], which was consistent with our study.

In our study the frequency of LOH10q was 38.5%. This frequency has been reported in approximately 36-76% of GB cases depending on the microsatellite markers used [1, 19, 22]. The 10q25, 10q23-24, 10p14-p15 and 10q23 regions were reported as most frequently deleted on chromosome 10 [1, 22]. The loci of several tumor suppressor genes have been identified on chromosome 10, i.e. *PTEN/MMAC1* on 10q23.3 (satellite marker D10S1765) and suppressor gene *DMBT1* (10q25.3-q26) [30, 31]. *PTEN* is a regulator of cell cycle, progression and apoptosis via the PI3K-AKT pathway, and is frequently lost in GB, mainly involved in late sequences of genetic alteration [32]. The LOH of the *DMBT1* gene locus occurs in 21% to 79% of GB cases [22, 23]. Homozygous deletion of the *DMBT1* gene was detected at a similar frequency (about 20%) in primary glioblastomas and secondary glioblastomas, suggesting that loss of *DMBT1* is involved in the pathogenesis of both subtypes of glioblastoma and was significantly associated with shorter overall survival [33].

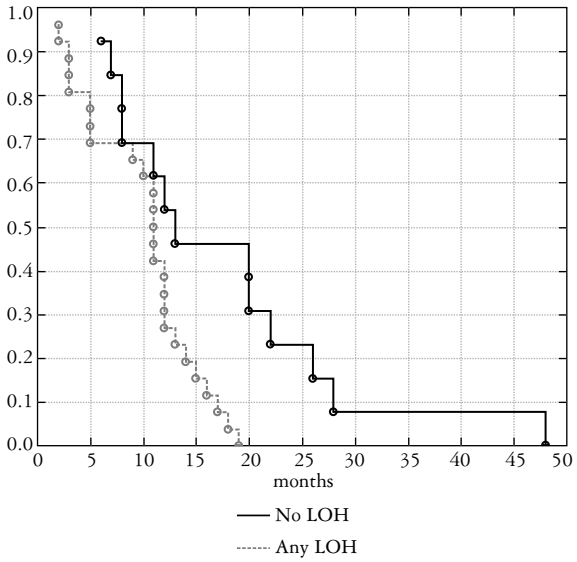

Fig. 2. Kaplan-Meier estimates for overall survival in all patients based on LOH status. Median overall survival of any LOH (+) patients was 11 months (range from 2-19 months), while median survival time in the LOH (–) group was 13.0 months (range from 6-48 months) (p = 0.007)

In this study, LOH 9p (locus on chromosome 9p21-22) was detected in 22.2% of cases. The *CDKN2A* (*INK4A/ARF*) genetic locus on chromosome 9p21 encodes two tumor suppressors: the *p16INK4A* cell cycle suppressor and $p14^{ARF}$ [34, 35], a regulator of *TP53* stability. LOH 17p was detected in 14.3% and like *TP53* mutation it has no impact on prognosis. In a previous study, poorer survival was associated significantly with the occurrence of either deletion of p16 ($p = 0.031$), LOH on 9p ($p = 0.016$), or LOH on 10q ($p = 0.0007$) in high-grade gliomas, but LOH 17p and *TP53* mutation had no statistically significant effect on survival after adjustment for age [36].

Loss of heterozygosity at 13q, which includes the *RB1* gene, has been detected in 12% of primary glioblastomas [37], which is similar to our results (10%). LOH 13q was excluded from the analysis because it was associated with a hazard ratio (HR) below 1.00, while all other LOHs were associated with HR > 1.00. We have not found in the literature data about the influence of LOH 13q on patient survival in GB.

In the present study, the frequencies of LOH 1p, LOH 19q and co-deletion of LOH 1p/19q were 17.9%, 25.6% and 7.14%, respectively. These results are consistent with other studies; the ranges of frequency of LOH 1p, 19q and LOH 1p/19q co-deletion were as follows: 0-24%, 5.3-33% and 0-13.3%, respectively [12, 18].

Although the co-deletion LOH 1p/19q is an important diagnostic and prognostic marker of chemosensitivity in oligodendrogliomas [5], its occurrence is not frequent in primary GB [12, 14, 18] and is mainly associated with the oligodendroglial component (GB-O). The frequency of GB-O in the whole group of glioblastomas has been observed in the range from 4.2% up to 27.2% [17, 21, 29, 38]. In GB-O cases, the frequency of LOH 1p was from 12 to 24%, 19q was from 32 to 43%, and combined 1p/19q was from 22 to 28% [39, 40]. In the more recent EORTC/NCIC trial study concerning a large group of 360 GB cases, an oligodendroglial component was found in 15% of all cases but co-deletion of 1p/19q was found in only one case [41]. Intriguingly, a more detailed study in GB-O using microdissection of tumor tissues with astrocytic and oligodendroglial components revealed no difference in the pattern of genetic alterations on chromosomes 1p and 19q in the same tumors [21]. In our study LOH 1p/19q co-deletion was infrequent and there was no co-presentation with *IDH1* mutation, which is consistent with the histopathological lack of oligodendroglial component in these cases. Although the occurrence of 1p/19q loss has been associated with better prognosis in glioblastoma [4, 18], another study showed no impact on the prognosis of GB, even within the oligodendroglial component (GB-O) [21]. In our study there was no influence of LOH 1p/19q co-deletion on overall patient survival.

Conclusions

Although the present study did not show any prognostic significance of LOH as a single parameter, the presence of LOH in at least one of the loci on all examined chromosomes (except LOH 13q) was related to a significant unfavorable impact on the overall survival of the patients.

We concluded that in primary glioblastoma a specific LOH group analysis may be important for the prognosis. In contrast to oligodendroglioma and secondary glioblastoma, 1p/19q co-deletion is rare in glioblastoma without an oligodendroglial component and does not influence glioblastoma patients' survival.

The authors declare no conflicts of interest.

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