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CLINICAL STUDY

TP53 and p53 statuses and their clinical impact in diffuse low grade gliomas

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Abstract *TP53* is a pivotal gene frequently mutated in diffuse gliomas and particularly in astrocytic tumors. The majority of studies dedicated to *TP53* in gliomas were focused on mutational hotspots located in exons 5–8. Recent studies have suggested that *TP53* is also mutated outside the classic mutational hotspots reported in gliomas. Therefore, we have sequenced all *TP53* coding exons in a retrospective series of 61 low grade gliomas (LGG) using high throughput sequencing technology. In addition, *TP53* mutational status was correlated with: (i) p53 expression, (ii) tumor type, (iii) chromosome arms 1p/19q status and (iv) clinical features of patients. The cohort included 32 oligodendrogliomas (O), 21 oligoastrocytomas (M) and 8 astrocytomas (A). *TP53* mutation was detected in 52.4 % (32/61) of tumors (34 % of O, 71.4 % of M and 75 % of A). All mutations (38 mutations in 32 samples) were detected in exons 4, 5, 6, 7, 8 and 10. Missense and non-missense mutations, including seven novel mutations, were detected in 42.6 and 9.8 % of tumors respectively. *TP53* mutations were almost mutually exclusive with 1p/19q co-deletion and were associated with: (i) astrocytic phenotype, (ii) younger age, (iii) p53 expression. Using a threshold of 10 % p53-positive tumor cells, p53 expression is an interesting surrogate marker for missense *TP53* mutations (Se = 92 %; Sp = 79.4 %) but not for non-missense mutation (18.4 % of mutations). *TP53* and p53 statuses were not prognostic in LGG. In conclusion, we have

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identified novel *TP53* mutations in LGG. *TP53* mutations outside exons 4–8 are rare. Although it remains imperfect, p53 expression with a threshold of 10 % is a good surrogate marker for missense *TP53* mutations and appears helpful in the setting of LGG phenotype diagnosis.

Keywords Low grade glioma · TP53 · P53 · Mutation · Expression · Prognosis

Introduction

TP53 is a well-known tumor suppressor gene that has been extensively studied over these past decades in cancers. *TP53* encodes p53, a transcription factor regulating cell cycle to prevent proliferation of genetically damaged cells with oncogenic properties. In addition, *TP53* is involved in modulation of multiple cell functions that are pivotal in cancer biology. Indeed, mutant p53 participates to tumor cells invasion, proliferation and survival [1]. Somatic *TP53* alterations are frequent in most human cancers, ranging from 5–80 % depending on type and stage of tumors. Most of these alterations are missense mutations (~75 %) leading to complete or partial loss of p53 functions (http://p53.iarc.fr/).

TP53 mutation is an early event in gliomagenesis [2–4]. In diffuse gliomas, and particularly in astrocytomas, *TP53* is frequently mutated (~50 %). *TP53* is also found mutated in oligoastrocytomas and oligodendrogliomas, albeit at a lower rate (~40 % and ~10 % of cases, respectively) [5]. Most studies investigating *TP53* in diffuse gliomas were focused on mutational hotspots (exons 5–8 sparing exons 1, 2, 3, 4, 9, 10 and 11) (Supplementary Data 1). However, it has been shown that in primary glioblastoma that frequent *TP53* mutations are also located outside these classic mutational hotspots within the transactivation domain, the prolin-rich domain and the splice donor sites [6].

Although the threshold of p53-positive tumor cells predicting *TP53* mutation is not consensual, p53 expression determined by immunohistochemistry (IHC) is used as a surrogate marker of *TP53* mutations in tumors. Indeed, many *TP53* mutations result in p53 stabilization and an increased percentage of stained cells using IHC. However, this parallel is not appropriate for null mutation since there is no protein expression and for other gene alterations not leading to p53 accumulation [7].

The prognostic value of *TP53* mutations remains uncertain, especially in diffuse low grade gliomas (WHO grade II, LGG). Heterogeneity of cohorts and techniques used to assess *TP53* or p53 statuses may explain, at least partly, the conflicting results reported in the literature [3, 5, 7–11].

These data prompt us to sequence all the *TP53* coding-exons using high-throughput sequencing method and to assess in a large retrospective series of LGG: (i) the prevalence of *TP53* mutations, (ii) the correlation between *TP53* mutation and p53 expression and (iii) the pathological and clinical values of *TP53* mutation and p53 expression.

Materials and methods

Material

Sixty-one ($n = 61$) patients and tumors operated between July 1987 and December 2011 in the groupe hospitalier Pitié Salpêtrière were included in the present study. They were selected in our database based on the following inclusion criteria: (i) age at diagnosis of 18 or above (ii) diagnosis of grade II astrocytoma, oligodendroglioma or oligoastrocytoma, (iii) available tumor DNA or frozen tissue, (iv) previously determined chromosome arms 1p/19q statuses, (v) documented clinical outcome and (vi) no contrast enhancement on brain MRI. The patients have signed a consent form for molecular analysis.

TP53 sequencing

Tumor DNA was extracted from frozen samples using a standard protocol (Qiagen, QIAmp DNA minikit). A first amplification of all exons (1–11) was performed using high-fidelity Fast-start polymerase (Roche®). Primers' sequences and the conditions of the touchdown polymerase chain reaction (PCR) are reported in the Supplementary Data 2 and 3 respectively. After amplification, primary amplicons were normalized to equimolar concentrations and pooled for groups of 11 amplicons (1 group = 1 patient). Each group was then labeled using a combination of two different Multiplex IDentifiers (MIDs), according to the manufacturer's specifications (Roche/454 Life Sciences®).

Sequencing, emulsion PCR and pyrosequencing steps were conducted using the GS Junior instrument (Roche/454 Life Sciences®) according to the manufacturer's instructions. Data analysis was conducted using Genomic Workbench (CLCBio) and Amplicon Variant Analysis (Roche®) softwares. The sequence used as *TP53* reference was NM_000546. The mean coverage depth for all exons was 99X. Every mutation found was then validated using Sanger's method (direct sequencing).

P53 and Ki67 expression using immunohistochemistry

Paraffin-embedded tissue slices, 3 µm in thickness, were immunostained. Monoclonal antibody anti-p53 (clone DO-

7) from DAKO (Trappes, France) was used at a titer of 1:100 using the BenchMark XT automate (Ventana®). The number of tumor cells with strong p53 expression (out of at least 500 nuclei) was quantified through manual counting by two operators. p53 immunostaining was performed in 59/61 samples. Ki67 expression was investigated using Ki67 antibody (clone Mib1, 1/100, Dako®) as previously reported [12].

Chromosome arms 1p/19q deletion assessment

Loss of heterozygosity (LOH) on chromosome arms 1p and 19q was studied using microsatellite analysis as described before [13].

IDH1/2 mutation statuses

IDH1/2 mutation statuses were assessed as described elsewhere [14].

Statistical analysis

Overall survival (OS) was evaluated by the time from radiological or histopathological diagnosis to death. Survival curves were drawn using Kaplan–Meier method and compared using log-rank test. A p value < 0.05 was considered as significant. The correlation between *TP53* mutation or p53 expression and other parameters (gender, tumor type, chromosome arms 1p/19q status, *TP53* mutation or p53 expression status) were performed using χ^2 test. Means' ages between two groups were compared using Student test. Comparison of the distribution of Ki67 index across groups was performed using Mann–Whitney test.

Results

Characteristics of tumors and patients

The current study included 61 LGG: (i) 32 WHO grade II oligodendrogliomas, (ii) 8 WHO grade II astrocytomas and (iii) 21 WHO grade II oligoastrocytomas. Chromosome 1p/19q co-deletion was present in 34.4 % (21/61) of cases including 16 oligodendrogliomas.

Median age at diagnosis was 39.3 years (Interquartile Range—IR−, 19.8 years). Gender ratio of the cohort was 1.2 (33 males/28 females). Median follow-up and median overall survival of the cohort was 8.3 years [CI (5.4–11.3)] and 3.6 years [CI (2.5–4.7)] respectively (Table 1).

Most of the patients underwent partial or total resection of the tumor (85.7 %); the others underwent diagnostic biopsy (14.3 %).

TP53 sequencing

In our series of 61 LGG, 38 mutations were detected in 32 tumors (52.4 %). Six tumors exhibited two mutations within *TP53*. There were 30 transitions and 8 transversions, leading to 31 missense mutations, 5 nonsense mutations, and 2 aberrant splicing mutations, as shown in Table 2.

TP53 mutations were detected in exon 4 ($n = 4$), exon 5 ($n = 8$), exon 6 ($n = 5$), exon 7 ($n = 4$), exon 8 ($n = 14$) and exon 10 ($n = 1$). No mutation was detected in exons 1, 2, 3, 9 and 11. The location and the frequency of *TP53* mutations in our series are reported in Fig. 1.

The demographic and molecular data according to *TP53* mutational status are reported in Table 3. Patients with *TP53*-mutated tumor are younger than patients with *TP53* wild-type LGG ($p = 0.01$), but there is no difference

Table 1 Characteristics of the patients and the tumors

Variable		
Age [1st percentile–3rd percentile](years)		30.3–50.1
Gender M/F (sex ratio)		33/28 (1.2)
Median follow-up (years, $CI_{95\%}$)		8.3 (5.4–11.3)
Surgery (n, %)	Biopsy	8 (14.3 %)
	Partial resection	24 (42.9 %)
	Total resection	24 (42.9 %)
Tumor type (n, %)	Oligodendroglioma	32 (52.5 %)
	Astrocytoma	8 (13.1 %)
	Oligoastrocytoma	21 (34.4 %)
Median overall survival (years, $CI_{95\%}$)[a]		3.6 (2.5–4.7)
Chromosome arms 1p/19q co-deletion (n, %)		21 (34.4 %)

n number, *SD* standard deviation

[a] 13 deceased patients during the study

Table 2 *TP53* mutations detected in our series of diffuse low grade gliomas

Tumor ID	*N*	Exon	Codon	Base change	AA change
1237	1	4	90	c. 269C>T	Ser90Phe
1945	1	4	100	c. 298C>T	Gln100X
1289, 2289	2	4	110	c. 329G>T	Arg110Leu
371	1	5	158	c. 473G>A	Arg158His
2324	1	5	175	c. 523C > G	Arg175Gly
1237	1	5	175	c. 524G>A	Arg175His
1223	1	5	135	c. 405C>G	Cys135Trp
833	1	5	135	c. 404G>A	Cys135Tyr
2064	1	5	168	c. 503A>G	His168Arg
575	1	5	179	c. 536A>G	His179Arg
1945	1	5	143	c. 427G>A	Val143Met
2363	1	6	197	c. 589G>C	Val197Leu
1811, 461, 1967	3	6	213	c. 637C>T	Arg213X
371	1	6	220	c. 659A>G	Tyr220Cys
1207	1	7	238	c. 713G>T	Cys238Phe
2468	1	7	239	c. 716A>G	Asn239Ser
2160	1	7	245	c. 733G>A	Gly245Ser
2289	1	7	245	c. 734G>A	Gly245Asp
31, 551, 1171, 198, 475, 2255, 1598, 2292, 2306, 2380, 2412	11	8	273	c. 818G>A c. 817C>T	Arg273Cys
2243	1	8	273	c. 818G>C	Arg273Pro
1527	1	8	280	c. 838A>G	Arg280Gly
1131	1	8	292	c. 874A>T	Lys292X
575	1	10	359	c. 1076C>T	Pro359Leu
1811	1	intron 6 SD	NA	c. 672 + 1G>C	672 + 1G>C
2382	1	intron 9 SD	NA	c. 993 + 1G>A	993 + 1G>A

N number, *SD* splice-donor, *NA* not available, *AA* aminoacid

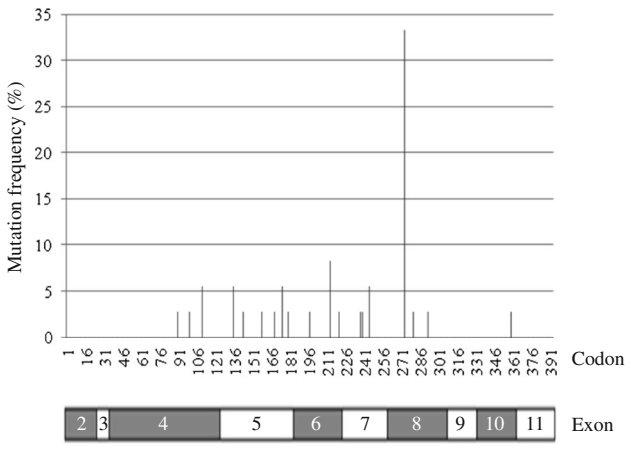

Fig. 1 Distribution of *TP53* mutation in our series of low grade gliomas. Although sequenced, exon 1 is not represented because it is a non-coding exon

Table 3 Clinical, pathological and molecular features of tumor with or without *TP53* mutation

	TP53 not mutated ($n = 29$)	*TP53* mutated ($n = 32$)	*p* value
Age mean (SD)	44.4 (13.2)	36.5 (10)	0.01
Gender			
Male	12 (36.3 %)	21 (63.7 %)	0.08
Female	17 (60.7 %)	11 (39.3 %)	
Tumor type			
Oligodendroglioma	21 (65.6 %)	11 (34.4 %)	0.01
Astrocytoma	2 (25 %)	6 (75 %)	
Oligoastrocytoma	6 (28.6 %)	15 (71.4 %)	
Chromosome arms 1p/19q co-deletion	18 (85.7 %)	3 (14.3 %)	2.7×10^{-5}
p53 overexpression	7 (22.6 %)	24 (77.4 %)	2.3×10^{-5}

considering gender. *TP53* mutations are significantly less frequent in oligodendrogliomas (34.4 %) compared to oligoastrocytomas (71.4 %) and astrocytomas (75 %) ($p = 0.01$). Chromosome 1p/19q co-deletion is rare in *TP53* mutated LGG compared to *TP53* wild-type LGG ($p = 2.7.10^{-5}$).

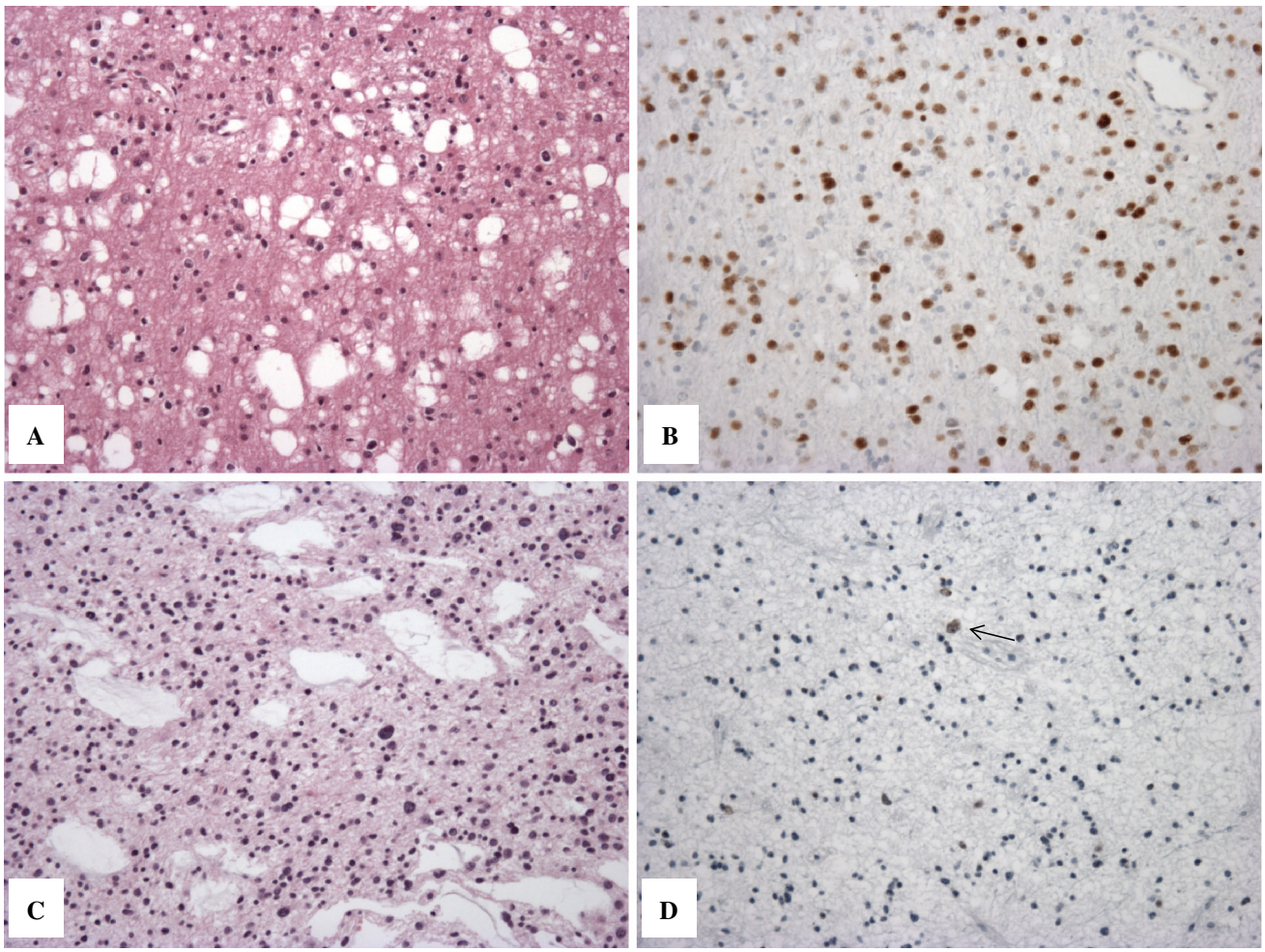

Fig. 2 p53 expression and *TP53* mutation. **a** and **b** a diffuse low grade glioma with missense *TP53* mutation and p53 overexpression. **c** and **d**, a diffuse low grade glioma with a non missense (stop) *TP53* mutation and low p53 expression (*arrow* indicates a positive cells). **a**, **c** hematoxylin eosin staining; **b**, **d** p53 staining. ×100 magnification

p53 expression using immunohistochemistry

Strong positive p53 immunostaining (Fig. 2) was observed in 62.7 % of LGG (37/59). A ROC curve correlating the percentage of p53-positive tumor cells and *TP53* mutational status of the tumor was established (Fig. 3). The best couple sensitivity/specificity was found for a threshold of 10 % [respectively 77.4 and 78.6 %; Predictive Positive Value (PPV) = 80.0 % and Predictive Negative Value (PNV) = 75.8 %]. Using 10 % of p53 positive tumor cells as threshold, a consistent correlation between p53 overexpression and *TP53* mutation was detected ($p = 2.3 \times 10^{-5}$).

The characteristics of the patients and tumors according to p53 expression status are reported in Table 4. No association was found between p53 expression and age or gender. However, similarly to *TP53* mutation, p53 overexpression is less frequent in oligodendrogliomas compared to astrocytomas and oligoastrocytomas ($p = 0.002$). Chromosome 1p/19q co-deletion and p53 overexpression were mutually exclusive ($p = 9.2 \times 10^{-4}$). However, in 4 samples both alterations were detected. Interestingly, in these cases the Ki67 index was significantly higher compared to tumors without this molecular pattern ($p = 0.03$).

Prognostic value of TP53 mutation and p53 expression

Considering OS, *TP53* mutation and p53 expression are not associated with patients' outcome ($p = 0.49$ in Fig. 4a and $p = 0.77$ in Fig. 4b respectively). Similarly, *TP53* mutations located in exons 5–8 have no prognostic value in the present series. Finally when considering both 1p/19q and *TP53* statuses, no prognostic significance was detected (i.e. 1p/19q intact and *TP53* wild-type LGG versus 1p/19q co-deleted and *TP53* wild-type LGG versus 1p/19q intact and *TP53* mutated LGG) (Supplementary Figure).

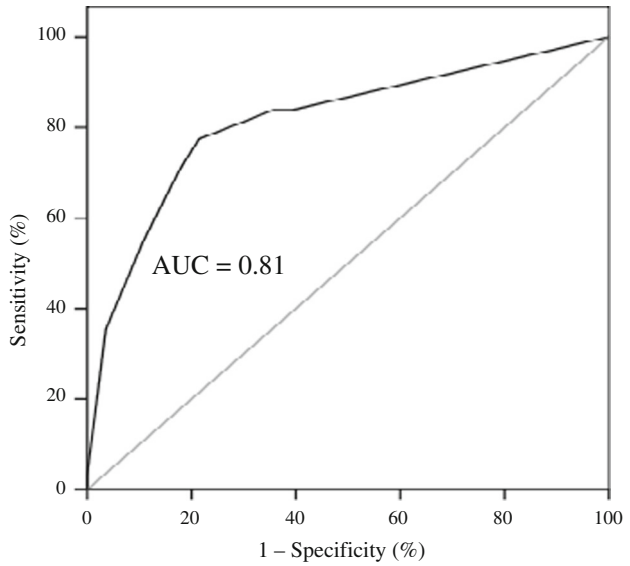

Fig. 3 Receiver operating characteristic (ROC) curve of p53 immunopositive cells percentage predicting *TP53* mutation. Area under ROC curve (AUC) was 0.81 ($*p = 3.5 \times 10^{-5}$). The best combination of sensitivity (77.4 %) and specificity (78.6 %) was provided for a threshold of 10 % p53-positive tumor cells

Discussion

TP53 is a critical gene in cancer including glial tumors. Indeed, *TP53* mutations have been detected in approximately 50, 40 and 10 % of astrocytomas, oligoastrocytomas and oligodendrogliomas respectively [5, 14]. The vast majority of published works focused on classic mutational hotspot exons and did not investigated the exons 1, 2, 3, 9, 10 and 11 [3, 5, 8, 10, 11, 15–46]. Only eight studies have explored non hotspot exons but in limited cohorts of LGG (Supplementary data 4). Therefore we have analyzed all *TP53* exons in a retrospective cohort of 61 LGG.

To our knowledge, our study investigated the largest cohort of LGG for *TP53* and p53 statuses. In agreement with previous studies, 52.4 % of the tumors exhibited *TP53* mutation, and most of them (31/38) have been already described in LGG. Interestingly, six *TP53* mutations have been detected in other tumors types (CNS and non-CNS) but never in LGG (Ser90Phe, Cys135Trp, Val143Met, Arg273Pro, Lys292X, 993+1G>A). Finally, one mutation has never been observed in cancer (according to IARCTP53 database). This mutation is a missense mutation located in exon 10, Pro359Leu. To assess the functional impact of this mutation we used Polyphen tool predicting impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations [47]. To our concern, a Polyphen score 0 suggested a benign effect of this substitution on p53 functions. Overall, 86.8 % of the *TP53* detected in our series are located in classic mutational hotspots supporting the proposed strategy to focus on these exons.

Our study confirmed already known data that *TP53* mutation and p53 are associated with astrocytic phenotype and are mutually exclusive with 1p/19q co-deletion [9, 12, 15, 48], which is related to oligodendroglial features. In our cohort of low grade oligodendrogliomas, 1p/19q co-deletion was detected in 50 % which is within the boundaries reported in the literature (i.e. 39–70 %) [9]. In contrast, *TP53* mutations are more frequent in our series of low grade oligodendrogliomas compared to data reported in the literature. A sampling bias might at least partially explain our results.

TP53 sequencing is time consuming and p53 expression is used as a surrogate marker in the setting of glioma diagnosis. Our study supports that immunohistochemistry, which is a straightforward technique that can be applied in routine histopathological assessment, may be used instead of molecular biology.

However, interpretation of p53 expression is heterogeneous across labs. The threshold of 10 % p53-positive tumor cells is debated [10–13, 43, 46, 48–51]. Indeed, p53 immunostaining is scored as positive or negative using

Table 4 Clinical, pathological and molecular features of tumors with and without p53 overexpression

	p53 not overexpressed ($n = 29$)	p53 overexpressed ($n = 30$)	p value
Age mean (SD)	41.8 (12.4)	39.0 (12.0)	0.39
Gender			
Male	15 (45.5 %)	18 (54.5 %)	0.60
Female	14 (53.8 %)	12 (46.2 %)	
Tumor type			
Oligodendroglioma	22 (71 %)	9 (29 %)	0.002
Astrocytoma	2 (28.6 %)	5 (71.4 %)	
Oligoastrocytoma	5 (23.8 %)	16 (76.2 %)	
Chromosome arms 1p/19q co-deletion	16 (80 %)	4 (20 %)	9.2×10^{-4}
TP53 mutation	7 (22.6 %)	24 (77.4 %)	2.3×10^{-5}

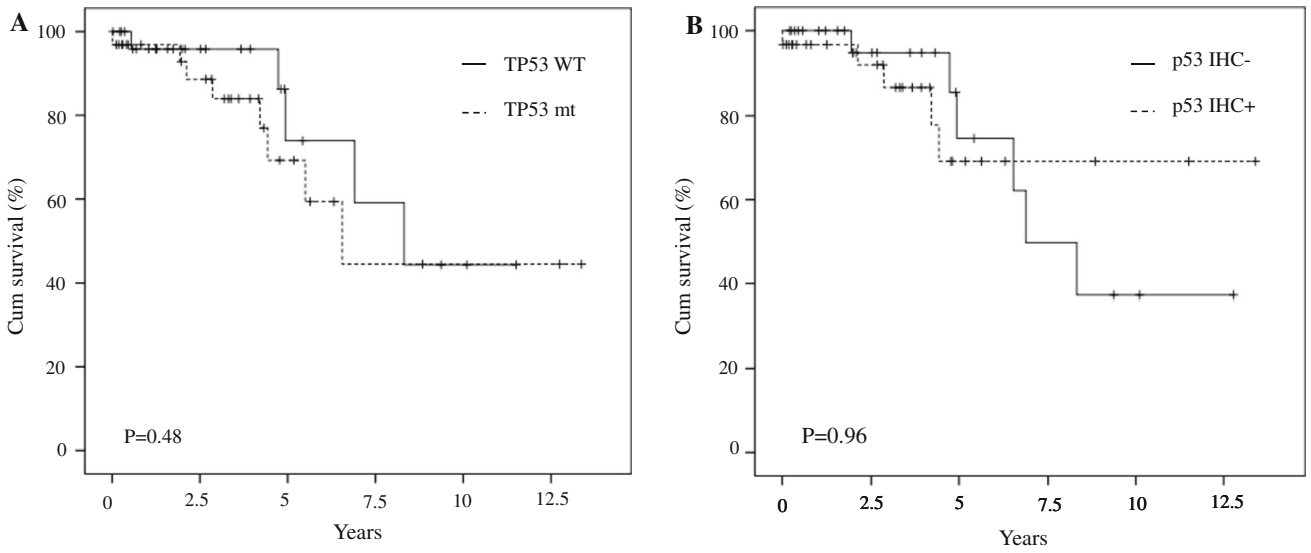

Fig. 4 Prognostic significance, in terms of overall survival, of *TP53* mutation (**a**) and p53 expression (**b**) in diffuse low grade gliomas. *WT* wild type, *mt* mutated, *IHC*− <10 % p53-positive tumor cells, *IHC*+ ≥10 % p53-positive tumor cells

varying thresholds [13, 46, 49–51] or using a "labeling index" [3]. In our study, a ROC curve demonstrated that the best cut-off to predict the mutational status of *TP53* was 10 % of p53-positive tumor cells with a PPV of 80.0 %, and a PNV of 75.8 %.

Among the seven *TP53*-mutated tumors without p53 expression, five of them carry a nonsense mutation (codon stop or aberrant splicing). Indeed, this is a known limitation for the use of p53 expression as *TP53* mutation sensor [7]. If we consider only *TP53* missense mutations (81.6 % of all mutations), p53 immunolabeling detects 92 % of mutated tumors.

One of the largest *TP53* study ($n = 124$) was conducted by Stander et al. [11]. Here, *TP53* mutations were associated with a shorter survival, but not p53 overexpression. However, previous work conducted in the same research group failed to demonstrate any link between *TP53* status and prognosis [10]. Similarly, Okamoto et al. [5]. showed a negative impact of *TP53* mutation on OS in univariate, but not in multivariate analysis. These studies illustrate the conflicting reports on prognostic value of this marker. Based on our large cohort and comprehensive analysis, neither *TP53* mutation nor p53 accumulation were found to be prognostic factors in LGG.

Finally, a recent study has shown the interest of combining *IDH* mutational, 1p/19q and p53 statuses in prognostic stratification of LGG [52]. Indeed, "triple-negative" LGG (i.e. *IDH*−, p53− and 1p/19q intact) exhibit dismal prognosis. We were unable to identify this prognostic impact in our cohort probably due to the limited number of "triple-negative" LGG ($n = 7$).

Conclusion

Our study is the largest investigating *TP53* and p53 statuses in LGG. *TP53* is mutated in 52.4 % of cases. *TP53* mutations outside mutational hotspots (exons 4–8) are rare (2.6 %) supporting targeted *TP53* sequencing in LGG. Interestingly, seven novel *TP53* mutations have been discovered in LGG. *TP53* mutations are associated with astrocytic phenotype, younger age and p53 overexpression. In contrast, they are mutually exclusive with 1p/19q co-deletion. Using a threshold of 10 % of p53-positive tumor cells, p53 expression is a good surrogate marker of missense *TP53* mutation. However, it should be used with caution since it misses ∼20 % of *TP53*-mutated tumors. *TP53* and p53 statuses are not prognostic in our series of LGG. Further analyses of *TP53* statuses in prospective series of LGG may overcome potential sampling biases observed in retrospective studies including the present work.

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Conflict of interest The authors have no conflicts of interest to declare.

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