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Promoter hypermethylation of genes encoding for RASSF/Hippo pathway members reveals specific alteration pattern in diffuse gliomas

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ABSTRACT

RASSF/Hippo pathway alterations are poorly characterized in diffuse gliomas. We assayed promoter methylation of *LATS1/2*, *MST1(STK4)/MST2(STK3)*, *RASSF1*, *RASSF2*, *Nore1A/RASSF5*, *RASSF6*, and *RASSF10* genes in 133 diffuse Grade II-III-IV gliomas, using methylation-specific PCR or PCR coupled to Cobra. RASSF/Hippo pathway was highly silenced in gliomas, particularly *RASSF1A* (79.4%) and *LATS2* (35.9%). Most gliomas (75.2%) exhibited at least hypermethylation for two promoters of the RASSF/Hippo member's genes. The most frequent combination of promoter hypermethylation of one RASSF gene and one Hippo pathway member's gene was *RASSF1/LATS2*-coupled hypermethylation (n=44, 33.08%). Hypermethylated profiles were related to IDH mutation, yet not randomly in IDH-mutated gliomas, since *LATS2* promoter hypermethylation was more frequent in oligodendroglioma than in astrocytoma. *RASSF1* and *LATS2* promoter hypermethylation predicted a longer overall survival (OS). Considering hypermethylation of these two promoters, Cox regression analysis categorized the patients into three prognostic groups: i) high-risk (n=24, both *RASSF1* and *LATS2* unmethylated promoters, median OS=13 months); ii) intermediate-risk (n=65, *RASSF1* or *LATS2* hypermethylated promoter, median OS=50.5 months, HR=3.3, 95%CI [1.6 to 6.4], $P = 0.001$); iii) low-risk of death (n=44, both *RASSF1* and *LATS2* hypermethylated promoters, median OS=119 months, HR=75.1, 95%CI [3.3 to 15.1], $P = 0.001$). We have thus highlighted a simple two-gene (*RASSF1/LATS2*) methylation signature as a tool to stratify different prognostic groups of patients with diffuse glioma, adding further prognostic information within the IDH-mutated group.

INTRODUCTION

Diffuse gliomas, accounting for 80% of primary brain tumors in adults, are characterized by recurrent molecular alterations, and more particularly by mutations in the isocitrate dehydrogenase genes (IDH) 1 or 2 and co-deletion of 1p/19q [1]. IDH mutant gliomas manifest a CpG island methylator phenotype (G-CIMP), though its functional significance remains unclear [2, 3]. Among epigenetically silenced genes present in gliomas, genes encoding Ras association domain family (RASSF)/Hippo pathway proteins [2], a pathway required for cell homeostasis, are common [4].

The RASSF superfamily consists of 10 genes (named *RASSF1-10*), encoding proteins with several protein binding domains, enabling their interaction with a multitude of partners, and their subsequent participation to several cellular processes [5, 6]. The C-RASSF proteins (named RASSF1-6) are characterized by a C-terminal coiled-coil motif named SARAH (Salvador/RASSF/Hippo) domain. SARAH domain allows RASSF1-6 proteins to regulate the Hippo kinases, MST1/2 (mammalian STE20-like 1/2), namely orthologs of the "hippo" drosophila genes, which provided the name to this pathway composed of a kinase cascade [7]. Active (phosphorylated) MST1/2 kinases phosphorylate and activate large tumor suppressors 1/2 (LATS1/2) kinases, which in turn inactivate the transcriptional co-activators Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ). Following LATS1/2 down-regulation in gliomas [2], YAP1 and TAZ promote growth [8-10] and mesenchymal differentiation, respectively [11]. Although they do not express the SARAH domain, the N-terminal RASSF proteins (named RASSF7-10) were also shown to regulate YAP/TAZ, via their coiled-coil domain as shown for RASSF7 [12].

RASSF or hippo kinase inactivation triggering the YAP/TAZ activation mechanism, followed by proliferation and cell migration in many different tumor types [13], has not been systematically investigated in the glioma setting. From the sparse data available, *RASSF1* [14-16] and *RASSF10* [17] gene promoter are known to be frequently silenced in adult diffuse gliomas, whereas *RASSF2* [18] and *NORE1A/RASSF5* [19] gene promoters are

found hypermethylated in 10.6% and 4% of diffuse gliomas, respectively. Conversely, neither *RASSF3* [20], *RASSF7* [21], nor *RASSF8* [21] gene promoters appear silenced in gliomas. To our current knowledge, no data are available about the *RASSF4* and *RASSF6* gene promoter methylation frequency encountered in gliomas. Regarding Hippo pathway members, *MST1(STK4)/MST2(STK3)* methylation status in gliomas has not been documented, whereas common hypermethylation of *LATS* kinase genes has been reported in astrocytomas (63.66% and 71.5% for *LATS1* and *LATS2* kinases, respectively) [22].

Herein, we have shown that: i) *RASSF1A/Hippo* gene promoters are frequently hypermethylated in gliomas, with most gliomas studied (75.2%) exhibiting at least two *RASSF/Hippo* promoter gene hypermethylations; ii) *LATS2* promoter hypermethylation is a hallmark of oligodendroglial tumors; iii) combination of *RASSF1* or *LATS2* promoter hypermethylation allowed categorizing patients into three prognostic groups with high-, intermediate-, or low-risk of death. Both *RASSF1/LATS2* silencing predicted longer survival, possibly resulting from IDH mutant-associated CpG island methylator phenotype in gliomas.

MATERIALS AND METHODS

Tissue Samples

Between September 2001 and March 2012, the brain tumor registry of Caen University Hospital (Caen-UH) was searched to identify patients aged over 18 years with a diagnosis of WHO Grade II, III, and IV diffuse glioma. Overall, 133 patients were retrieved with sufficient tissue available for clinical, pathological, and radiological reviews, as well as additional biomarker studies, and with a minimum 1-month follow-up; they were included in on-going radiological PET and MRI observational studies at Caen-UH (<https://clinicaltrials.gov/>; identifier: NCT00850278, NCT01200134). As required by French laws, all patients provided informed consent, and the study was approved by the institutional ethics committee of Caen-UH (North-West Committee for Persons Protection III), France. All

tumor specimens were reviewed by an experienced neuropathologist (ELZ) who was in charge of confirming both diagnosis and tumor grade, according to the WHO 2016 classification system. In the absence of data, additional studies for molecular markers, including ATRX expression loss, mutation in IDH genes, *1p19q* co-deletion, and MGMT status, were performed at the Caen-UH [23]. The clinical data were retrieved from electronic medical charts, such as: i) date of initial surgery; ii) resection extent determined by the surgeon and corroborated by both the treating oncologist and interpreting neuroradiologist; iii) death or last follow-up date.

DNA Extraction and Methylation-Specific PCR assay

DNA samples were obtained using QIAmp DNA FFPE Tissue kit (Qiagen, Valencia, CA, Cat# 56404), with genomic DNA bisulfite modification performed by means of the EpiTect kit (Qiagen, Cat# 59104), according to the manufacturer's instruction. PCR was conducted with primers, as described in Table 1. *RASSF6* methylation status was determined using COBRA [28]. Water was substituted for DNA as a negative control, whereas cpGenome Universal methylation DNA (MPbiomedical, Santa Ana, CA, Cat# S7821) and DNA from lymphocytes of healthy volunteers were employed as a positive control for methylated and unmethylated alleles, respectively.

Identities of the PCR products were verified by sequencing, using the Abprism Byg-Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, Cat# 4314415) on AB model 310 or 377 DNA sequencers (Supplemental Figure S1).

Statistical Analyses

The comparison of proportions was based on either standard or exact Chi-square tests, depending on the sample size. Overall survival (OS) was calculated from the surgery date to death from any cause or censoring date, if alive. Survival curves were estimated with the Kaplan–Meier method. Univariate and multivariate Cox-proportional hazard models were used to assess the prognostic value of the promoter methylation status pertaining to

RASSF1/Hippo pathway genes. Hazard ratios (HR) were estimated with 95% confidence intervals (95%CI). Statistical significance was set at $P < 0.05$. The data were analyzed using IBM SPSS software, Version 22.

Betastasis online software (<http://www.betastasis.com/>, date of last access: 4.2.2012) was used for computing the *RASSF1A* and *LATS2* mRNA prognostic analyses in 329 glioma patients, with gene-expression data and OS information downloaded from the Rembrandt Glioma Dataset (Affymetrix HG U133 v2.0 Plus). OS analyses were dichotomized depending on the first quartile value.

RESULTS

Patient Characteristics and WHO 2016 Reclassification

Characteristics, treatment history, and pathologic data pertaining to 133 glioma samples from 133 patients studied, have been summarized in Table 2. The median age was 64.4 years [range: 18 to 80.22]. There were 56 females and 77 males. The median follow-up period was 28.35 months [range: 0.16 to 137 months]. According to the 2016 WHO classification [1], the 133 glioma samples were classified as follows: 14 Grade II, *isocitrate dehydrogenase 1/2 (IDH)*-mutant and *1p19q*-codeleted oligodendrogliomas (O); 26 Grade III, *IDH*-mutant and *1p19q*-codeleted anaplastic oligodendrogliomas (AO); 14 Grade II, diffuse and *IDH*-mutant astrocytomas (A-*IDH*^{MUT}); 19 Grade III, *IDH*-mutant anaplastic astrocytomas (AA-*IDH*^{MUT}); seven Grade IV, *IDH*-mutant glioblastomas (GB-*IDH*^{MUT}); 53 Grade IV, *IDH*-wildtype glioblastomas (GB-*IDH*^{WT}) (Table 2).

As expected, the 2016 WHO classification strongly impacted the patients' OS (Figure 1A, Tables 3 and 4): Patients with *IDH*-mutated glioma had a more favorable prognosis than those with *IDH*-wildtype glioma (Table 3); patients with oligodendroglial tumor (*IDH*-mt and *1p/19q*-codeleted) had the most favorable prognosis among *IDH*-mutated gliomas (Figure 1A,

Table 2); patients with *MGMT* promoter hypermethylated glial tumor had a more favorable prognosis than those with glial tumor and unmethylated *MGMT* (Table 3).

Characteristic Features of Promoter hypermethylation of genes that are members of the RASSF/Hippo pathway in Glioma samples

Promoter hypermethylation of RASSF/Hippo pathway member genes proved to be very common in diffuse gliomas: Only 7/133 gliomas (7 GB-IDH^{WT}) showed no hypermethylation of the studied gene promoters (Table 5, Supplemental Figure S2).

Among the RASSF family member genes, the most frequently hypermethylated promoters were *RASSF1* (n=104 [79.4%]), *RASSF6* (n=63 [55.3%]), and *RASSF10* (n=67 [55.4%]); among the Hippo pathway member genes, *LATS2* promoter hypermethylation was the most common event (n=47 (35.9%)) (Table 5).

Promoter hypermethylation of *RASSF1* and the Hippo pathway member genes was associated with integrated diagnosis according to the 2016 WHO classification (Table 5). The methylation frequency was higher in *IDH*-mutated gliomas (ranging from 7.6% to 88.6%, depending on the promoter studied) than in *IDH*-wildtype gliomas (ranging from 0% to 65.4%, depending on the promoter studied). This observation was significant for *RASSF1* gene ($P = 0.0013$), *RASSF5* ($P = 0.042$), *RASSF10* ($P < 0.001$), and *LATS2* promoter methylation ($P < 0.001$) (Supplemental Table S1). Among *IDH*-mutated gliomas, the promoter hypermethylation rates were not significantly increased when comparing Grade II to Grade III ($P > 0.5$, Table 5). However, interestingly, when comparing the silencing of RASSF/Hippo members by gene promoter hypermethylation in five primary *IDH*-muted gliomas and their *in situ* recurrence counterpart, for each case the presence of additional hyper-methylated RASSF/Hippo genes was noticed in the glioma recurrence tissues (data not shown). Finally, *LATS2* promoter hypermethylation was more common in oligodendroglial tumors (71.8%) than in astrocytomas (33.3%, Table 5). Using the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) Glioma Dataset [29], *LATS2* mRNA level was

confirmed to be lower in oligodendroglioma subtypes than in lower grade astrocytomas and glioblastomas (Supplemental Figure S3).

As hypermethylation of RASSF genes and one Hippo pathway member gene was by no means exclusive from the others, 75.2% of gliomas displayed hypermethylation of at least two promoters of genes encoding for RASSF/Hippo pathway members (Table 6). Co-occurrence of promoter methylation of RASSF genes was mainly *RASSF1/RASSF10* (n=63 ([47.3%]) and *RASSF1/RASSF6* (n=54, [40.6%]) (Table 6). Co-occurrence of promoter hypermethylation of RASSF and Hippo member genes consisted mostly of *RASSF1/LATS2* hypermethylation and *RASSF10/LATS2* hypermethylation in 33.08% (n=44) and 26.3% (n=35) (Table 6). By comparison, a combination of Hippo member promoter hypermethylation proved to be rare (Table 6).

RASSF and LATS2 Silencing Predicts Better OS in Glioma Patients

RASSF1 or *LATS2* promoter hypermethylation alone predicted a better OS in glioma patients (Table 7, Figure 1B-D). The median OS was 89.9 months for patients with a glial tumor with a hypermethylated *RASSF1* promoter versus 14.0 months for other patients, upon univariate analysis (HR=3, [95%CI: 1.8 to 5.1], $P < 0.001$, Table 7, Figure 1B). The median OS was 119 months for patients with a glial tumor with a hypermethylated *LATS2* promoter versus 26.4 months for the others, upon univariate analysis (HR=3.8, [95%CI: 2.0 to 7.2], $P < 0.001$, Table 7, Figure 1C). Next, the survival of resected glioma patients from the REMBRANDT Glioma Dataset was analyzed, demonstrating that low mRNA expression of *RASSF1A* or *LATS2* predicted better OS (logrank test, $P < 0.001$, [Supplemental Figure S4]).

Considering both *RASSF1* and *LATS2* promoter hypermethylation statuses, three patient risk groups, namely high-risk (n=24, both *RASSF1* and *LATS2* unmethylated promoters, median OS=13 months), intermediate-risk (n=65, *RASSF1* or *LATS2* hypermethylated promoter, median OS=50.5 months, HR=3.3, 95% CI [1.6 to 6.4], $P = 0.001$), and low-risk

(n=44, both *RASSF1* and *LATS2* hypermethylated promoters, median OS=119 months, HR=75.1, [95%CI 3.3 to 15.1], $P = 0.001$) (Figure 1D) could be distinguished.

After adjusting for standard risk factors (age, sex, 2016 WHO classification, and *MGMT* promoter hypermethylation), neither *LATS2* promoter hypermethylation alone (Supplemental Table S2) nor *RASSF1* promoter hypermethylation alone (Supplemental Table S3) independently influenced the survival of glioma patients ($P = 0.79$), ($P = 0.15$).

DISCUSSION

RASSF/Hippo pathway alterations are still poorly characterized in diffuse gliomas, although these alterations could significantly contribute to patient natural history by leading to YAP/TAZ dysregulation [8-11, 30]. We herein report on a first systematic epigenetic analysis of RASSF/Hippo pathway member genes pertaining to 133 patients with Grade II to IV gliomas re-evaluated according to the 2016 WHO classification [1].

First of all, these results confirm that *RASSF1* [14-16] and *RASSF10* [17] gene promoters are commonly found hypermethylated in diffuse gliomas, when compared to other tumor tissues [15], whereas *RASSF2* [18] and *RASSF5* [19] genes promoters are scarcely hypermethylated in gliomas. To our understanding, this is the first report on the common *RASSF6* inactivation detected in these tumors. The results show that MST kinases are not silenced by promoter hypermethylation, in contrast to LATS kinases that are actually silenced by hypermethylation in this setting [22]. Another striking result is that *LATS2* promoter hypermethylation occurs far more frequently in oligodendroglioma *IDH^{MUT}* and *1p19q* codeleted than in astrocytomas, *IDH^{MUT}*.

Additionally, most gliomas studied here were shown to carry multiple RASSF/Hippo pathway alterations. RASSF/Hippo pathway methylations were found to be more common in *IDH^{MUT}* glioma than in *IDH^{WT}* glioma. This finding could be related to an IDH mutation-associated constitutive CpG island methylator phenotype (G-CIMP). Currently, DNA

methylation profiling is emerging as a consistent tool enabling us to further dissect diffuse glioma classes. Interestingly, the “methylome” likely represents a combination of both somatically acquired DNA methylation changes and the cell of origin [31]. To our knowledge, multiple losses in RASSF/Hippo family members within a same tumor have not yet been investigated, even in non-central nervous system (CNS) tumors. Multiple losses in RASSF/Hippo family members could thus be a specific feature pertaining to diffuse gliomas, in contrast with circumscribed gliomas (notably, pilocytic astrocytoma) that do not show RASSF/Hippo hypermethylation [15]. This requirement to silence, in the diffuse glioma setting, several RASSF/Hippo isoforms via promotor hypermethylation sustains the concept that the lack of one isoform is not necessarily counterbalanced by the presence of another isoform [7, 32]. Though the six standard RASSF family members share some overlapping functions, they likewise exhibit specific properties and functions [7]. This likewise applies to the MST1/2 and LATS1/2 kinases [32]. Inactivating multiple RASSF/Hippo pathway members could definitively trigger the Hippo pathway’s switch off, resulting in oncogenic YAP [8-10] and TAZ [11] activation in diffuse gliomas, in addition to subsequent transformation of glial cells [30].

Genes encoding for RASSF/Hippo pathway members are considered to be tumor suppressor genes, with their promoter hypermethylation associated with poorer prognosis [33], except for LATS2 expression in nasopharyngeal carcinoma, which represents another CIMP tumor [34] predicting poor prognosis [35]. Among this whole set of genes encoding for RASSF/Hippo pathway members, only *LATS1* promoter methylation predicted a poorer prognosis, though without statistical significance, most likely due to the small sample size consisting of only 14 patients. *RASSF1* or *LATS2* promoter hypermethylation was shown to correlate with longer OS in our series, upon univariate analysis. Previous analyses focused on gliomas have brought up discordant results with respect to the RASSF pathway promoter methylation’s prognostic value [13-15]. Based on the scientific literature, it proves challenging to discuss our result pertaining to *RASSF1* or *LATS2* expression loss

observed in glioma patients, as well as their prognostic value, given that these findings must certainly be re-examined in the light of the changes made within the new 2016 CNS tumor classification [1]. In our series, IDH mutation and *1p19q* co-deletion were shown to be of high prognostic value. This, however, could have masked the prognostic value of *RASSF1* or *LATS2* methylation status upon multivariate analyses. However, the observation revealing that *RASSF1* or *LATS2* promoter hypermethylation actually correlates with longer OS proves to be in line with the report demonstrating that low *RASSF1A* or *LATS2* mRNA levels were able to predict superior OS in patients with IDH-mutated or IDH-Wild Type glioma (Supplemental Figure S4). In support of our published report demonstrating the impact of some hypermethylation on glioma patients' survival, it was recently reported that shifting of DNA pattern methylation from G-CIMP-high at initial diagnosis to G-CIMP-low at first recurrence was able to predict poor clinical outcome in glioma patients [3]. The observation that hypermethylation of Hippo kinase promoters could predict improved survival is additionally sustained by the now well-established concept that Hippo kinases, as Hippo pathway effectors, can exert either tumor oncogene or tumor suppressor functions, depending on the cellular context [36].

The mechanisms underlying the superior outcome of glioma patients with hypermethylated *RASSF1* or *LATS2* promoters are still unknown. In addition to acting as oncogene within a particular cellular context [35], as shown by the dual *LATS2* action as either apoptosis inducer or inhibitor depending on the cellular context [37], either *RASSF1A* or *LATS2*, or both may impact drug responsiveness. This is indeed the case for *RASSF1A* in non-small-cell lung cancer (NSCLC), given that NSCLC patients with *RASSF1A* loss exhibit superior OS when treated with paclitaxel/cisplatin doublet versus gemcitabine/cisplatin doublet, with *RASS1A* leading to nonresponse of tumor cells to gemcitabine treatment [33, 38]. Part of *RASSF1A* or *LATS2* oncogenic role may thus rely on drug resistance induction.

In conclusion, *RASSF1A*/Hippo signaling pathway alterations, frequently encountered in gliomagenesis, are associated with a more favorable prognosis as opposed to that reported

from other human cancers. A simple two-gene methylation signature enables us to both strikingly stratify different prognostic patient groups—notably by adding prognosis information to the *IDH*^{MUT} group and to designate YAP/TAZ—for which inhibitors are currently under development [40, 41], as a potential therapeutic target utility in glioma.

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G.L. and E.L.Z. conceived and designed the experiments and initiated and supervised the project. G.L. and S.L.C. performed experiments. E.P. assisted with REMBRANDT dataset exploration. All authors provided technical and scientific support. All authors wrote the manuscript and approved the final manuscript.

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Figure Legend

Figure1. Overall survival of glioma patients according to 2016 WHO glioma classification (**A**), and *RASSF1* (**B**), *LATS2* (**C**) or both *RASSF1* and *LATS2* (**D**) promoter hypermethylation. GII: Grade II, GIII: Grade III, GIV: Grade IV, A: diffuse astrocytoma, IDH-mutant, AA: anaplastic astrocytoma, IDH-mutant, AO: anaplastic oligodendroglioma, IDH-mutant and *1p19q*-codeleted, GB: glioblastoma (GB-*IDH*^{MT}: glioblastoma, IDH-wildtype and GB-*IDH*^{MUT}: glioblastoma, IDH-mutant), O: oligodendroglioma, *IDH*-mutant and *1p19q*-codeleted.

Table 1. Primer sequences.

	Sequence	T _M	Size (bp)	Reference
<i>LATS1</i> (Genbank access : NC_000006.12)				
U:	F: 5'-TGAATGATTAGAGTTGTGGGTGATGT-3' R: 5'-AAACATTTCCCAACATCACTTACACA-3'	60°C	128	[24]
M:	F: 5'-GAACGATTAGAGTTGCGGGCGAC-3' R: 5'-AACATTTCCCGACGTCGCTTACG-3'	62°C	126	
<i>LAST2</i> (Genbank access: NC_000013.11)				
U:	F: 5'-GGTGTGTTTGTGGATTGGTATGTGGTT-3' R: 5'-CATCTTCCCAAACACTCACACCACA-3'	60 °C	141	[24]
M:	F: 5'-TTCGTTCCGATTGGTATGCGGTC-3' R: 5'-CCATCTTCCCGAAACGCTCACG-3'		137	
<i>MST1/STK4</i> (Genbank access : NC_000020.11)				
U:	F: 5'-TTTGTGGGGTGGGTTTAGGAGGTTTGT-3' R: 5'-AACCAATAACCCCTCACCAACACAACAA-3'	63°C	125	[24]
M:	F: 5'-GCGGGGCGGGTTTAGGAGGTTTC-3' R: 5'-CCAATAACCCCTCACCGACGC-3'		120	
<i>MST1/STK3</i> (Genbank access : NC_000008.11)				
U:	F: 5'-TTTTAAGTGGGAGGGAGATTTGTTGTGG-3' R: 5'-AAAAACCAAAACACCAACCAACCAACC-3'	61°C	108	[24]
M:	F: 5'-CGGGAGGGAGATTCGTCGCG-3' R: 5'-AAACCGAAACACCGACCGACCG-3'	63°C	99	
<i>RASSF1</i> (Genbank access : NC_000003.12)				
U:	F: 5'-TTTGGTTGGAGTGTGTTAATGTG-3' R: 5'-CAAACCCACAACTAAAAACAA-3'	60°C	108	[25]
M:	F: 5'-GTGTTAACGCGTTGCGTATC-3' R: 5'-AACCCGCGAACTAAAAACGA-3'	62°C	96	
<i>RASSF2</i> (Genbank access : NC_000020.11)				
U:	F: 5'-AGTTTGTGTTGTTTTTTAGGTGG-3' R: 5'-AAAAACCAACAACCCCCACA-3'	63°C	108	[26]
M:	F: 5'-GTTTCGTCGTCGTTTTTTAGGCG-3' R: 5'-AAAAACCAACGACCCCGCG-3'			
<i>Nore1A/RASSF5</i> (Genbank access : NC_000001.11)				
U:	F: 5'-ATTTATATTTGTGTAGATGTTGTTTGGTAT-3' R: 5'-ACTTTAACAACAACAACCTTAACAACACTACA-3'	63°C	215	[27]
M:	F: 5'-CGTCGTTTGGTACGGATTTATTTTTTTCGGTTC-3' R: 5'-GACAACCTTAACAACGACGACTTTAACGACTACG-3'	62°C	202	

Sequences available in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>)

F, forward; R, reverse.

Table 2. Patient and tumor characteristics based on histology

	Oligodendro- glioma	Astrocytoma	Glioblastoma, <i>IDH</i>^{MUT}	Glioblastoma, <i>IDH</i>^{WT}
	n=40	n=33	n=7	n=53
Defining molecular alterations	<i>IDH1/2</i> mutation <i>1p19q</i> deletion	<i>IDH1/2</i> mutation ATRX loss	<i>IDH1/2</i> mutation ATRX loss	
Grading	14 Grade II 26 Grade III	14 Grade II 19 Grade III	7 Grade IV	53 Grade IV
Sex	18 (45%) men 22 (55%) women	18 (54.5%) men 15 (45.5%) women	3 (42.8%) men 4 (57.2%) women	38 (71.7%) men 15 (28.3%) women
Median age at diagnosis ([range])	48.9 years [27.5-80.2]	36.7 years [21.3-64.0]	44.6 years [35.6-73.5]	62.3 years [11.6-79.3]
Resection				
<i>Complete:</i>	15 (37.5%)	6 (18.2 %)	1 (14.3 %)	33 (43.4 %)
<i>Subtotal:</i>	3 (7.5%)	1 (3%)	1 (14.3%)	7 (13.2%)
<i>Partial:</i>	21 (52.5%)	25 (75.8%)	5 (71.4%)	16 (30.1%)
<i>Only biopsied:</i>	1 (2.5%)	1 (3%)	0	7 (13.2%)
Treatment following surgery				
<i>Stupp protocol</i> ^[39] :	3 (7.5%)	8 (24.3%)	4 (30%)	42 (79.2%)
<i>Radiotherapy:</i>	17 (42.5%)	6 (18.2%)	1 (14.3%)	4 (7.5%)
<i>Chemotherapy:</i>	5 (12.5%)	6 (18.2%)	1 (14.3%)	3 (5.6%)
<i>None:</i>	13 (32.5%)	13 (39.3%)	1 (57.1%)	3 (5.6%)
Median follow-up period ([range])	45.84 months [0.16-137.42]	50.54 months [2.5-121.48]	14.49 months [6.74-27.11]	33.1 months [0.16-54.55]
Recurrence (number / delay [range])	19 patients <i>median period:</i> 31.47 months [7.82-79.54]	17 patients <i>median period:</i> 35.42 months [7.59-61.77]	4 patients <i>median period:</i> 8.48 months [4.67-11.01]	34 patients <i>median period:</i> 9.17 months [3.55-37.52]
Median overall survival OS	119 months	92.2 months	19.7 months	14.1 months

OS: overall survival; IDH: isocitrate dehydrogenase; ATRX: alpha-thalassemia retardation syndrome.

Table 3. Known risk factors influencing the survival of glioma patients.

	Median OS	HR	CI95%	P-value
Age				
For each 5-year increase	Not applicable	1.2	(1.09 ; 1.32)	<0.001
Resection				
Total	34.9	1		
Subtotal	29.6	1.1	(0.45 ; 2.5)	0.99
Partial or biopsy	65.8	1.0	(0.61 ; 1.8)	
Histology				
oligodendroglioma	119.0	1		
astrocytoma	92.2	2.2	(0.77 ; 6.2)	<0.001
glioblastoma	14.1	88.4	(24.6 ; 317)	
Grading				
II	132.8	1		
III	89.9	2.5	(0.85 ; 7.4)	<0.001
IV	14.1	112.9	(27.9 ; 456)	
IDH1/2 mutation				
<i>IDH</i> ^{MUT}	119.0	1		
<i>IDH</i> ^{Wild type}	13.0	22.9	(10.5 ; 49.8)	<0.001
LOH 1p19q				
Yes	119.0	1		
No	25.0	8.2	(3.5 ; 19.2)	<0.001
MGMT promoter				
Hypermethylated	119.0	1		
Wildtype	21.0	3.7	(2.2 ; 6.2)	<0.001

Significant *P*-values are in bold.

OS: overall survival; 95% CI: 95% confidence interval; IDH: isocitrate dehydrogenase; LOH: loss of heterozygosity; MGMT: methylguanine-DNA methyltransferase.

Table 4. Overall Survival Median of Patient with glioma according to histology.

	OS median	HR	CI95%	<i>P</i> -value	Global <i>P</i>
O	132.8	1		0.40	<0.001
AO	107.7	2.0	(0.39-10.5)		
A	>137	1.7	(0.27-10.6)	0.036	
AA	64.4	8.2	(1.5-45.9)		
GB	14.1	193.5	(30.6-1222)		

A: diffuse astrocytoma, *IDH*-mutant, AA: anaplastic astrocytoma, *IDH*-mutant, AO: anaplastic oligodendroglioma, *IDH*-mutant and *1p19q*-codeleted, GB-*IDH*^{WT}: glioblastoma, *IDH*-wild type, GB-*IDH*^{MUT}: glioblastoma, *IDH*-mutant; O: oligodendroglioma, *IDH*-mutant and *1p19q*-codeleted; OS: overall survival; 95% CI: 95% confidence interval.

Table 5. RASSF/Hippo member promoter hypermethylation frequency according to 2016 WHO glioma classification.

Promotor methylation of gene	O		AO		A		AA		GB-IDHWT		GB-IDHMUT		P-value
	n	%	n	%	n	%	n	%	n	%	n	%	
<i>RASSF1</i>	11/13	84.6%	23/26	88.5%	13/14	92.9%	18/19	94.7%	34/52	65.4%	5/7	71.4%	0.029
<i>RASSF2</i>	1/13	7.7%	2/26	7.7%	1/14	7.1%	3/19	15.8%	3/53	5.7%	0/7	0%	0.77
<i>RASSF5</i>	3/13	23.1%	3/26	11.5%	0/14	0%	1/19	5.3%	0/51	0%	0/7	0%	0.015
<i>RASSF6</i>	6/9	66.7%	15/23	65.2%	7/12	58.3%	9/17	52.9%	21/47	44.7%	5/6	83.3%	0.37
<i>RASSF10</i>	6/11	54.5%	18/24	75.0%	8/13	61.5%	12/18	66.7%	17/48	35.4%	6/7	85.7%	0.0075
<i>LATS1</i>	2/13	15.4%	5/26	19.2%	1/14	7.1%	0/19	0%	9/52	17.3%	1/7	14.3%	0.45
<i>LATS2</i>	7/13	53.8%	21/26	80.8%	4/14	28.6%	7/19	36.8%	4/52	7.7%	4/7	57.1%	<0.001
<i>MST1/STK4</i>	1/13	7.7%	3/26	11.5%	2/14	14.3%	2/19	10.5%	5/53	9.4%	2/7	28.6%	0.80
<i>MST2/STK3</i>	2/13	15.4%	1/26	3.8%	2/14	14.3%	1/19	5.3%	1/53	1.9%	0/7	0%	0.22

The statistical analysis compares the frequencies of methylations between the different 2016 WHO glioma groups. Significant *P*-values are in bold.

A: diffuse astrocytoma, *IDH*-mutant, AA: anaplastic astrocytoma, *IDH*-mutant, AO: anaplastic oligodendroglioma, *IDH*-mutant and *1p19q*-codeleted, GB-*IDH*^{WT}: glioblastoma, *IDH*-wildtype, GB-*IDH*^{MUT}: glioblastoma, *IDH*-mutant; O: oligodendroglioma, *IDH*-mutant and *1p19q*-codeleted.

Table 6. Combination pattern of RASSF/Hippo pathway member methylation.

Hypermethylation coupled promotors (n)									
	<i>RASSF1</i> n =104	<i>RASSF2</i> n =10	<i>RASSF5</i> n =7	<i>RASSF6</i> n =63	<i>RASSF10</i> n =67	<i>LATS1</i> n =18	<i>LATS2</i> n =47	<i>MST1/ STK4</i> n =15	<i>MST1/ STK3</i> n =7
<i>RASSF1</i>	-	-	-	-	-	-	-	-	-
<i>RASSF2</i>	10 (7.5%)	-	-	-	-	-	-	-	-
<i>RASSF5</i>	6 (4.5%)	0	-	-	-	-	-	-	-
<i>RASSF6</i>	54 (40.6%)	5 (3.7%)	5 (3.7%)	-	-	-	-	-	-
<i>RASSF10</i>	63 (47.3%)	9 (6.7%)	3 (2.1%)	35 (26.3%)	-	-	-	-	-
<i>LATS1</i>	14 (10.5%)	4 (2.8%)	0	5 (3.7%)	8 (5.6%)	-	-	-	-
<i>LATS2</i>	44 (33%)	6 (4.5%)	5 (3.7%)	28 (18%)	35 (26.3%)	6 (4.5%)	-	-	-
<i>MST1/ STK4</i>	12 (9.0%)	2 (1.4%)	0	5 (3.7%)	7 (5.2%)	6 (4.5%)	7 (5.2%)	-	-
<i>MST1 /STK3</i>	7 (5.2%)	2 (1.4%)	0	3 (2.1%)	3 (2.1%)	3 (2.1%)	3 (2.1%)	2 (1.4%)	-

Table 7. RASSF/Hippo expression influencing glioma patient survival

	Median OS (months)	HR	CI95%	<i>P</i> -value
<i>RASSF1</i> promoter status				
Hypermethylated	89.9	1		<0.001
Wildtype	14.0	3.0	(1.8 ; 5.1)	
<i>RASSF2</i> promoter status				
Hypermethylated	28.8	1		0.91
Wildtype	64.4	1.1	(0.42; 2.6)	
<i>Nore1A/RASSF5</i> promoter status				
Hypermethylated	not reached	1		0.43
Wildtype	64.4	1.8	(0.43; 7.3)	
<i>RASSF6</i> promoter status				
Hypermethylated	64.4	1		0.28
Wildtype	50.5	1.3	(0.79; 2.3)	
<i>RASSF10</i> promoter status				
Hypermethylated	89.9	1		0.15
Wildtype	28.4	1.5	(0.88; 2.5)	
<i>LATS1</i> promoter status				
Hypermethylated	31.1	1		0.58
Wildtype	65.8	0.83	(0.44; 1.6)	
<i>LATS2</i> promoter status				
Hypermethylated	119.0	1		<0.001
Wildtype	26.4	3.8	(2.0; 7.2)	
<i>MST1/STK4</i> promoter status				
Hypermethylated	92.2	1		0.62
Wildtype	63.0	1.2	(0.57; 2.5)	
<i>MST1/STK3</i> promoter status				
Hypermethylated	not reached	1		0.10
Wildtype	63.0	5.3	(0.73; 38.3)	

Significant values are in bold.

Figure1

