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

Guénaëlle Levallet, Christian Creveuil, Lien Bekaert, Elodie A. Pérès, Gaëtane Planchard, Sylvie Lecot-Cotigny, Jean-Sébastien Guillamo, Evelyne Emery, Gérard Zalcman, Emmanuèle Lechapt-Zalcman

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

Guénaëlle Levallet,*† Christian Creveuil,‡ Lien Bekaert,§ Elodie Péres,* Gaëtane Planchard,† Sylvie Lecot-Cotigny,† Jean-Sébastien Guillamo,* Evelyne Emery,‡ Gérard Zalcman,¶‖ and Emmanuèle Lechapt-Zalcman*†**

From Normandie University,* UNICAEN, CEA, CNRS, ISTCT/CERVOxy group, Caen, France, GIP CYCERON ; the Service d'Anatomie et cytologie pathologique,† the Unité de recherche biomédicale,‡ and the Service de Neurochirurgie,§ CHU de Caen, Caen, France ; the Service d'Oncologie Thoracique,¶ Hôpital Bichat-Claude Bernard, AP-HP, Université Paris-Diderot, Paris, France ; CIC INSERM 1425-CLIP2 Paris-Nord,‖ Hôpital Bichat-Claude Bernard, AP-HP, Paris, France ; and the Service de Neuropathologie,** GHU Paris Psychiatrie et Neurosciences, Paris, France

Corresponding author (to whom reprints are to be requested):
Dr Guénaëlle Levallet

Unité ISTCT UMR6030 - CNRS-CEA-Normandie Université- Equipe CERVOxy - GIP CYCERON Campus Jules Horowitz - Bd Henri Becquerel - BP 5229 - 14074 CAEN Cedex 5.

E-mail: guenaelle.levallet@unicaen.fr

Running title: *RASSF1*/*LATS2* inactivation in gliomas

Disclosures: None declared.

Funding: Supported by Appel d'offre interne au CHU de Caen (APRI2007) (Internal call for tenders University Hospital Caen – 2007) to E.L.Z.

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* promotor 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-codeted) had the most favorable prognosis among *IDH*-mutated gliomas (Figure 1A,

Table 2); patients with *MGMT* promotor 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).

Promotor 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 *LAST2* 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.

Acknowledgements

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.

REFERENCES

1. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, Ohgaki H, Wiestler OD, Kleihues P, Ellison DW. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol., 2016, Vol 131 6(pg 803-20).
2. Turcan S, Rohle D, Goenka A, Walsh LA, Fang F, Yilmaz E, Campos C, Fabius AW, Lu C, Ward PS, Thompson CB, Kaufman A, Guryanova O, Levine R, Heguy A, Viale A, Morris LG, Huse JT, Mellinghoff IK, Chan TA. IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. Nature, 2012, Vol 483 7390(pg 479-83).
3. de Souza CF, Sabedot TS, Malta TM, Stetson L, Morozova O, Sokolov A, Laird PW, Wiznerowicz M, Iavarone A, Snyder J, deCarvalho A, Sanborn Z, McDonald KL, Friedman WA, Tirapelli D, Poisson L, Mikkelsen T, Carlotti CG Jr, Kalkanis S, Zenklusen J, Salama SR, Barnholtz-Sloan JS, Noushmehr H. A Distinct DNA Methylation Shift in a Subset of Glioma CpG Island Methylator Phenotypes during Tumor Recurrence. Cell Rep. 2018 Apr 10;23(2):637-651.
4. Fu V, Plouffe SW, Guan KL. The Hippo pathway in organ development, homeostasis, and regeneration. Curr Opin Cell Biol. 2017 Dec;49:99-107.
5. Donninger H., Vos M. D. and Clark G. J. The RASSF1A tumor suppressor. J Cell Sci, 2007, Vol 120 (pg 3163-3172).
6. Volodko N, Gordon M, Salla M, Ghazaleh HA, Baksh S. RASSF tumor suppressor gene family: biological functions and regulation. FEBS Lett. 2014 Aug 19;588(16):2671-84.
7. Iwasa H, Hossain S, Hata Y. Tumor suppressor C-RASSF proteins. Cell Mol Life Sci. 2018 May;75(10):1773-1787.
8. Mayrhofer M, Gourain V, Reischl M, Affaticati P, Jenett A, Joly JS, Benelli M, Demichelis F, Poliani PL, Sieger D, Mione M. A novel brain tumour model in zebrafish

reveals the role of YAP activation in MAPK- and PI3K-induced malignant growth. Dis Model Mech. 2017 Jan 1;10(1):15-28.

9. Wang Y, Pan P, Wang Z, Zhang Y, Xie P, Geng D, Jiang Y, Yu R, Zhou X. β-catenin-mediated YAP signaling promotes human glioma growth. J Exp Clin Cancer Res. 2017 Sep 29;36(1):136.
10. Zhang Y, Xie P, Wang X, Pan P, Wang Y, Zhang H, Dong Y, Shi Y, Jiang Y, Yu R, Zhou X. YAP Promotes Migration and Invasion of Human Glioma Cells. J Mol Neurosci. 2018 Feb;64(2):262-272
11. Li W, Dong S, Wei W, Wang G, Zhang A, Pu P, Jia Z. The role of transcriptional coactivator TAZ in gliomas. Oncotarget. 2016 Dec 13;7(50):82686-82699.
12. Zheng X, Dong Q, Zhang X, Han Q, Han X, Han Y, Wu J, Rong X, Wang E. The coiled-coil domain of oncogene RASSF 7 inhibits hippo signaling and promotes non-small cell lung cancer. Oncotarget. 2017 Aug 12;8(45):78734-78748.
13. Dubois F, Keller M, Calvayrac O, Soncin F, Hoa L, Hergovich A, Parrini MC, Mazières J, Vaisse-Lesteven M, Camonis J, Levallet G, Zalcman G. RASSF1A Suppresses the Invasion and Metastatic Potential of Human Non-Small Cell Lung Cancer Cells by Inhibiting YAP Activation through the GEF-H1/RhoB Pathway. Cancer Res., 2016, Vol 76 6(pg 1627-40).
14. Gao Y, Guan M, Su B, Liu W, Xu M, Lu Y. Hypermethylation of the RASSF1A gene in gliomas. Clin Chim Acta., 2004, Vol 349 1-2(pg 173-9).
15. Hesson LB, Cooper WN, Latif F. The role of RASSF1A methylation in cancer. Dis Markers., 2007, Vol 23 1-2(pg 73-87).
16. Kuo LT, Tsai SY, Chang CC, Kuo KT, Huang AH, Tsai JC, Tseng HM, Kuo MF, and Tu YK. Genetic and Epigenetic Alterations in Primary–Progressive Paired Oligodendroglial Tumors. PLoS One., 2013, Vol 8 6(pg e67139).
17. Hill VK, Underhill-Day N, Krex D, Robel K, Sangan CB, Summersgill HR, Morris M, Gentle D, Chalmers AD, Maher ER, Latif F. Epigenetic inactivation of the RASSF10

candidate tumour suppressor gene is a frequent and early event in gliomagenesis. Oncogene, 2011, Vol 30 (pg 978–989).

18. Perez-Janices N, Blanco-Luquin I, Tuñón MT, Barba-Ramos E, Ibáñez B, Zazpe-Cenoz I, Martinez-Aguillo M, Hernandez B, Martínez-Lopez E, Fernández AF, Mercado MR, Cabada T, Escors D, Megias D, Guerrero-Setas D. EPB41L3, TSP-1 and RASSF2 as new clinically relevant prognostic biomarkers in diffuse gliomas. Oncotarget., 2015, Vol 6 1(pg 368-80).
19. Lorente A, Mueller W, Urdangarín E, Lázcoz P, Lass U, von Deimling A, Castresana JS. RASSF1A, BLU, NORE1A, PTEN and MGMT expression and promoter methylation in gliomas and glioma cell lines and evidence of deregulated expression of de novo DNMTs. Brain Pathol., 2009, Vol 19 2(pg 279-92).
20. Hesson LB, Bièche I, Krex D, Criniere E, Hoang-Xuan K, Maher ER, Latif F. Frequent epigenetic inactivation of RASSF1A and BLU genes located within the critical 3p21.3 region in gliomas. Oncogene. 2004 Mar 25;23(13):2408-19.
21. Underhill-Day N, Hill V, Latif F. N-terminal RASSF family RASSF7-RASSF10. Epigenetics. 2011 Mar; 6(3): 284–292.
22. Jiang Z, Li X, Hu J, Zhou W, Jiang Y, Li G, Lu D. Promoter hypermethylation-mediated down-regulation of LATS1 and LATS2 in human astrocytoma. Neurosci Res. 2006 Dec;56(4):450-8.
23. Bekaert L, Valable S, Lechapt-Zalcman E, Ponte K, Collet S, Constans JM, Levallet G, Bordji K, Petit E, Branger P, Emery E, Manrique A, Barré L, Bernaudin M, Guillamo JS. [18F]-FMISO PET study of hypoxia in gliomas before surgery: correlation with molecular markers of hypoxia and angiogenesis. Eur J Nucl Med Mol Imaging., 2017, vol 44 8(pg 1383-1392).
24. Seidel C, Schagdarsurengin U, Blümke K, Würl P, Pfeifer GP, Hauptmann S, Taubert H, Dammann R. Frequent hypermethylation of MST1 and MST2 in soft tissue sarcoma. Mol Carcinog. 2007 Oct;46(10):865-71.

25. Schagdarsurengin U, Gimm O, Hoang-Vu C, Dralle H, Pfeifer GP, Dammann R. Frequent epigenetic silencing of the CpG island promoter of RASSF1A in thyroid carcinoma. Cancer Res. 2002 Jul 1;62(13):3698-701.
26. Hesson LB, Wilson R, Morton D, Adams C, Walker M, Maher ER, Latif F. CpG island promoter hypermethylation of a novel Ras-effector gene RASSF2A is an early event in colon carcinogenesis and correlates inversely with K-ras mutations. Oncogene. 2005 Jun 2;24(24):3987-94.
27. Hesson LB, Bièche I, Krex D, Criniere E, Hoang-Xuan K, Maher ER, Latif F. Frequent epigenetic inactivation of RASSF1A and BLU genes located within the critical 3p21.3 region in gliomas. Oncogene. 2004 Mar 25;23(13):2408-19.
28. Djos A, Martinsson T, Kogner P, Carén H. The RASSF gene family members RASSF5, RASSF6 and RASSF7 show frequent DNA methylation in neuroblastoma. Mol Cancer., 2012, Vol 13 (pg 11-40).
29. Gusev Y, Bhuvaneshwar K, Song L, Zenklusen JC, Fine H, Madhavan S. The REMBRANDT study, a large collection of genomic data from brain cancer patients. Sci Data. 2018 Aug 14;5:180158.
30. Wu LMN, Deng Y, Wang J, Zhao C, Wang J, Rao R, Xu L, Zhou W, Choi K, Rizvi TA, Remke M, Rubin JB, Johnson RL, Carroll TJ, Stemmer-Rachamimov AO, Wu J, Zheng Y, Xin M, Ratner N, Lu QR. Programming of Schwann Cells by Lats1/2-TAZ/YAP Signaling Drives Malignant Peripheral Nerve Sheath Tumorigenesis. Cancer Cell. 2018 Feb 12;33(2):292-308.e7.
31. Capper D, Stichel D, Sahm F, Jones DTW, Schrimpf D, Sill M, Schmid S, Hovestadt V, Reuss DE, Koelsche C, Reinhardt A, Wefers AK, Huang K, Sievers P, Ebrahimi A, Schöler A, Teichmann D, Koch A Hänggi D, Unterberg A, Platten M, Wick W, Witt O, Milde T Korshunov A, Pfister SM, von Deimling A. Practical implementation of DNA methylation and copy-number-based CNS tumor diagnostics: the Heidelberg experience. Acta Neuropathol. 2018 Aug;136(2):181-210.

32. Hergovich A. The Roles of NDR Protein Kinases in Hippo Signalling. Genes (Basel). 2016 May 18;7(5). pii: E21.
33. de Fraipont F1, Levallet G, Creveuil C, Bergot E, Beau-Faller M, Mounawar M, Richard N, Antoine M, Rouquette I, Favrot MC, Debieuvre D, Braun D, Westeel V, Quoix E, Brambilla E, Hainaut P, Moro-Sibilot D, Morin F, Milleron B, Zalcman G. An apoptosis methylation prognostic signature for early lung cancer in the IFCT-0002 trial. Clin Cancer Res., 2012, Vol 18 10(pg 2976-86).
34. Jiang W, Cai R, Chen QQ. DNA Methylation Biomarkers for Nasopharyngeal Carcinoma: Diagnostic and Prognostic Tools. Asian Pac J Cancer Prev. 2015;16(18):8059-65.
35. Zhang Y, Hu CF, Chen J, Yan LX, Zeng YX, Shao JY. LATS2 is de-methylated and overexpressed in nasopharyngeal carcinoma and predicts poor prognosis. BMC Cancer., 2010, Vol 10 (pg 538-50).
36. Cao J and Huang W. Two faces of Hippo: activate or suppress the Hippo pathway in cancer. Anticancer Drugs. 2017 Nov;28(10):1079-1085.
37. Furth N, Aylon Y. The LATS1 and LATS2 tumor suppressors: beyond the Hippo pathway. Cell Death Differ. 2017 Sep;24(9):1488-1501.
38. Grawenda AM, O'Neill E. Clinical utility of RASSF1A methylation in human malignancies. Br J Cancer., 2015, Vol 113 3(pg 372-81).
39. Stupp R1, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med., 2005, Vol 352 10(pg 987-96).
40. Elisi GM, Santucci M, D'Arca D, Lauriola A, Marverti G, Losi L, Scalvini L, Bolognesi ML, Mor M, Costi MP. Repurposing of Drugs Targeting YAP-TEAD Functions. Cancers (Basel). 2018 Sep 14;10(9). pii: E329. doi: 10.3390/cancers10090329.

41. Kim HB and Myung SJ. Clinical implications of the Hippo-YAP pathway in multiple cancer contexts. BMB Rep. 2018 Mar;51(3):119-125.

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*^{WT}: 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
LATS1 (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	
LAST2 (Genbank access: NC_000013.11)				
U:	F: 5'-GGTGTTTTGTTTGGATTGGTATGTGGTT-3' R: 5'-CATCTTCCCAAAACACTCACACCACA-3'	60 °C	141	[24]
M:	F: 5'-TTCGTTCGGATTGGTATGCGGTC-3' R: 5'-CCATCTTCCCGAAACGCTCACG-3'		137	
MST1/STK4 (Genbank access : NC_000020.11)				
U:	F: 5'-TTTGTGGGGTGGGTTTAGGAGGTTTGT-3' R: 5'-AACCAATAACCCCTCACCAACACAACAA-3'	63°C	125	[24]
M:	F: 5'-GCGGGGCGGGTTTAGGAGGTTC-3' R: 5'-CCAATAACCCCTCACCGACGC-3'		120	
MST1/STK3 (Genbank access : NC_000008.11)				
U:	F: 5'-TTTTAAGTGGGAGGGAGATTTGTTGTGG-3' R: 5'-AAAAACCAAAACACCAACCAACCAAACC-3'	61°C	108	[24]
M:	F: 5'-CGGGAGGGAGATTCGTCGCG-3' R: 5'-AAACCGAAACACCGACCGACCG-3'	63°C	99	
RASSF1 (Genbank access : NC_000003.12)				
U:	F: 5'-TTTGGTTGGAGTGTGTTAATGTG-3' R: 5'-CAAACCCCACAAACTAAAAACAA-3'	60°C	108	[25]
M:	F: 5'-GTGTTAACGCGTTGCGTATC-3' R: 5'-AACCCCGCGAACTAAAAACGA-3'	62°C	96	
RASSF2 (Genbank access : NC_000020.11)				
U:	F: 5'-AGTTTGTTGTTGTTTTTTAGGTGG-3' R: 5'-AAAAAACCAACAACCCCCACA-3'	63°C	108	[26]
M:	F: 5'-GTTCGTCGTCGTTTTTTAGGCG-3' R: 5'-AAAAACCAACGACCCCCGCG-3'			
Nore1A/RASSF5 (Genbank access : NC_000001.11)				
U:	F: 5'-ATTTATATTTGTGTAGATGTTGTTTGGTAT-3' R: 5'-ACTTTAACAACAACAACTTTAACAACTACA-3'	63°C	215	[27]
M:	F: 5'-CGTCGTTTGGTACGGATTTTATTTTTTTCGGTTC-3' R: 5'-GACAACTTTAACAACGACGACTTTAACGACTACG-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, *IDH*^{MUT}**	**Glioblastoma, *IDH*^{WT}**
	n=40	**n=33**	**n=7**	**n=53**
Defining molecular alterations	*IDH1/2* mutation *1p19q* deletion	*IDH1/2* mutation ATRX loss	*IDH1/2* 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				
Complete:	15 (37.5%)	6 (18.2 %)	1 (14.3 %)	33 (43.4 %)
Subtotal:	3 (7.5%)	1 (3%)	1 (14.3%)	7 (13.2%)
Partial:	21 (52.5%)	25 (75.8%)	5 (71.4%)	16 (30.1%)
Only biopsied:	1 (2.5%)	1 (3%)	0	7 (13.2%)
Treatment following surgery				
Stupp protocol [39]:	3 (7.5%)	8 (24.3%)	4 (30%)	42 (79.2%)
Radiotherapy:	17 (42.5%)	6 (18.2%)	1 (14.3%)	4 (7.5%)
Chemotherapy:	5 (12.5%)	6 (18.2%)	1 (14.3%)	3 (5.6%)
None:	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 *median period*: 31.47 months [7.82-79.54]	17 patients *median period*: 35.42 months [7.59-61.77]	4 patients *median period*: 8.48 months [4.67-11.01]	34 patients *median period*: 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					
IDH^{MUT}	119.0	1			
$IDH^{Wild\ type}$	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: isocytrate 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%	*P*-value	Global *P*
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-*IDH*WT		GB-*IDH*MUT		*P*-value
	n	%	n	%	n	%	n	%	n	%	n	%	
RASSF1	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**
RASSF2	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
RASSF5	3/13	23.1%	3/26	11.5%	0/14	0%	1/19	5.3%	0/51	0%	0/7	0%	**0.015**
RASSF6	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
RASSF10	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**
LATS1	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
LATS2	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**
MST1/STK4	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
MST2/STK3	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.

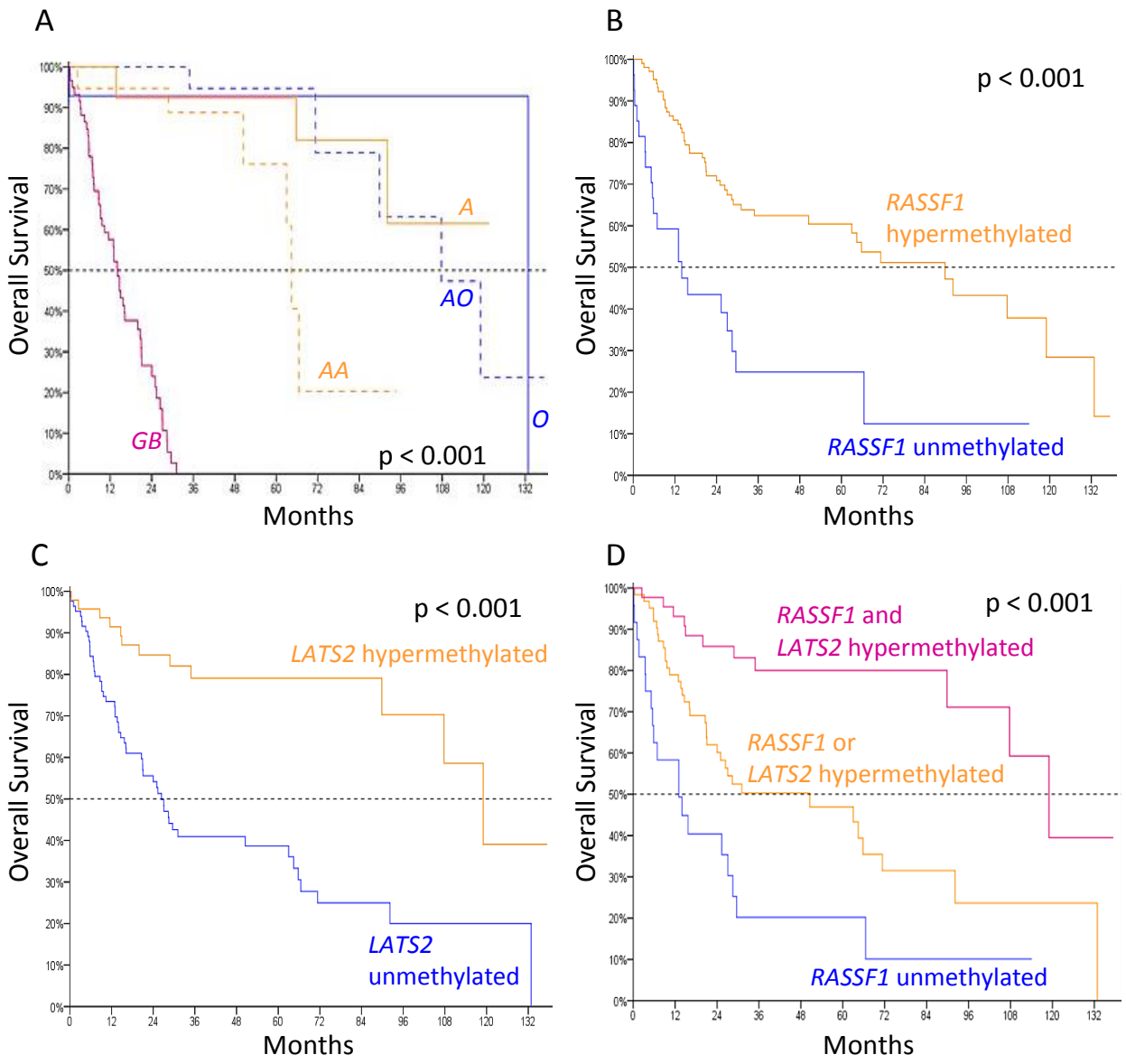
	Hypermethylation coupled promotors (n)								
	RASSF1 **n =104**	***RASSF2*** **n =10**	***RASSF5*** **n =7**	***RASSF6*** **n =63**	***RASSF10*** **n =67**	***LATS1*** **n =18**	***LATS2*** **n =47**	***MST1/ STK4*** **n =15**	***MST1/ STK3*** **n =7**
RASSF1	-	-	-	-	-	-	-	-	-
RASSF2	10 (7.5%)	-	-	-	-	-	-	-	-
RASSF5	6 (4.5%)	0	-	-	-	-	-	-	-
RASSF6	54 (40.6%)	5 (3.7%)	5 (3.7%)	-	-	-	-	-	-
RASSF10	63 (47.3%)	9 (6.7%)	3 (2.1%)	35 (26.3%)	-	-	-	-	-
LATS1	14 (10.5%)	4 (2.8%)	0	5 (3.7%)	8 (5.6%)	-	-	-	-
LATS2	44 (33%)	6 (4.5%)	5 (3.7%)	28 (18%)	35 (26.3%)	6 (4.5%)	-	-	-
MST1/ STK4	12 (9.0%)	2 (1.4%)	0	5 (3.7%)	7 (5.2%)	6 (4.5%)	7 (5.2%)	-	-
MST1 /STK3	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%		***P*-value**
RASSF1 promoter status					
Hypermethylated	89.9	1			**<0.001**
Wildtype	14.0	3.0	(1.8 ;	5.1)	
RASSF2 promotor status					
Hypermethylated	28.8	1			0.91
Wildtype	64.4	1.1	(0.42;	2.6)	
Nore1A/RASSF5 promoter status					
Hypermethylated	not reached	1			0.43
Wildtype	64.4	1.8	(0.43;	7.3)	
RASSF6 promoter status					
Hypermethylated	64.4	1			0.28
Wildtype	50.5	1.3	(0.79;	2.3)	
RASSF10 promoter status					
Hypermethylated	89.9	1			0.15
Wildtype	28.4	1.5	(0.88;	2.5)	
LATS1 promoter status					
Hypermethylated	31.1	1			0.58
Wildtype	65.8	0.83	(0.44;	1.6)	
LATS2 promoter status					
Hypermethylated	119.0	1			**<0.001**
Wildtype	26.4	3.8	(2.0;	7.2)	
MST1/STK4 promoter status					
Hypermethylated	92.2	1			0.62
Wildtype	63.0	1.2	(0.57;	2.5)	
MST1/STK3 promoter status					
Hypermethylated	not reached	1			0.10
Wildtype	63.0	5.3	(0.73;	38.3)	

Significant values are in bold.

Figure1


A
Overall Survival
Months
A
AO
AA
O
GB
p < 0.001
B
p < 0.001
RASSF1
hypermethylated
RASSF1 unmethylated
Overall Survival
Months
C
p < 0.001
LATS2 hypermethylated
LATS2
unmethylated
Overall Survival
Months
D
p < 0.001
RASSF1 and
LATS2 hypermethylated
RASSF1 or
LATS2 hypermethylated
RASSF1 unmethylated
Overall Survival
Months