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Rapid diagnosis of *IDH1*-mutated gliomas by 2-HG detection with gas chromatography mass spectrometry

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Abstract

The metabolic genes encoding isocitrate dehydrogenase (*IDH1, 2*) are frequently mutated in gliomas. Mutation of *IDH* defines a distinct subtype of glioma and predicts therapeutic response. *IDH* mutation has a remarkable neomorphic activity of converting α-ketoglutarate (α-KG) to 2-hydroxyglutarate (2-HG), which is now commonly referred to as an oncometabolite and biomarker for gliomas. PCR-sequencing ($n = 220$), immunohistochemistry staining (IHC, $n = 220$), and gas chromatography mass spectrometry (GC-MS, $n = 87$) were applied to identify *IDH* mutation in gliomas, and the sensitivity and specificity of these strategies were compared. PCR-sequencing and IHC staining are reliable for retrospective assessment of *IDH1* mutation in gliomas, but both methods usually take 1–2 days, which hinders their application for rapid diagnosis. GC-MS-based methods can detect 2-HG qualitatively and quantitatively, offering information on the *IDH1* mutation status in gliomas with the sensitivity and specificity being 100%. Further optimization of the GC-MS based methodology (so called as the mini-column method) enabled us to determine 2-HG within 40 min in glioma samples without complex or time-consuming preparation. Most importantly, the ratio of 2-HG/glutamic acid was shown to be a reliable parameter for determination of mutation status. The mini-column method enables rapid identification of 2-HG, providing a promising strategy for intraoperative diagnosis of *IDH1*-mutated gliomas in the future.

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Introduction

Comprehensive genomic studies have revealed that somatic mutations of isocitrate dehydrogenase (*IDH*) frequently occur in multiple types of human cancer,

including a subset of gliomas [1]. Subsequent studies have shown that *IDH* mutations may represent a powerful prognostic factor in gliomas [2]. *IDH1* mutation is also considered as a predictive biomarker in malignant astrocytoma for extensive resection and radio-chemotherapy to benefit patients [3]. Accordingly, *IDH* mutation has been introduced into the molecular subtyping and integrated diagnosis of gliomas [4, 5]. Nearly all *IDH* mutations target a single amino-acid residue in the enzymes' active sites, Arg132 in IDH1 and Arg172 and Arg140 in IDH2. Tumor-derived mutations in IDH1 and IDH2 abolish their normal catalytic activity in converting isocitrate to α-ketoglutarate (α-KG) [2, 6, 7]. The most remarkable feature of *IDH* mutation is the neomorphic gain-of-function: the NADPH-dependent reduction of α-KG to the normally trace metabolite 2-hydroxyglutarate (2-HG), which is now commonly referred to as an oncometabolite [6, 8].

As a direct metabolic consequence of *IDH* mutations, 2-HG can be accumulated to as high as 5–35 μmol/g in cases of glioma harboring *IDH* mutations [8]. Taking advantage of the abnormally high accumulation of 2-HG, magnetic resonance spectroscopy (MRS) [9, 10] as well as mass spectrometry [11, 12] have been reported to detect 2-HG and define the *IDH* mutation status in glioma tissues. As a non-invasive method to monitor 2-HG, the sensitivity of MRS will be sharply decreased especially in small-size tumors [13]. Although the accuracy could be improved in the high field MRS (7.0 Tesla), its clinical utility is not confirmed yet [14]. Because it is considerably more sensitive than MRS, gas chromatography mass spectrometry (GC-MS)-based methodology can detect metabolites in bio-samples with a good balance of sensitivity and reliability. GC-MS was first utilized for 2-HG detection in the urine samples of aciduria patients [15], and subsequently in serum samples of AML patients harboring *IDH1*-R132H or *IDH2*-R140Q mutation [16] as well as cultured cancer cell lines containing endogenous *IDH* mutations [17]. In present study, we used GC-MS to detect 2-HG qualitatively and quantitatively, and the results offered information on the *IDH1* mutation status in brain tumors with sensitivity and specificity of 100%. Further optimization of the GC-MS-based methodology enabled us to determine 2-HG within 40 min. Importantly, the ratio of 2-HG/glutamic acid was utilized to detect the mutation because there was very little 2-HG among wild-type neoplasm [18, 19]. And the ratio could accurately distinguish *IDH1*-mutated gliomas from wild-type, this may provide a new paradigm for intraoperative diagnosis and patient management in gliomas.

Materials and methods

Sample acquisition and statistical analysis

Details of patient enrollment, sample acquisition, and statistical analysis are shown in Supplementary Information. Consent forms were obtained from all patients after approval from local ethic committee in Huashan hospital. Clinical classification and grading of these tumor samples was performed by neuro-pathologists according to the 2016 WHO Classification of Tumors of the Central Nervous System [20]. Procedure for sequencing and IHC are provided in detail in Supplementary Information.

GC-MS-based 2-HG detection

Metabolite extraction was accomplished by adding 150 μL of −80 °C methanol: water mix (80%:20%; Cat#34860, Sigma-Aldrich) to the brain tissue (approximately 25 mg) followed by 20 s homogenization at 4 °C (OSE-Y10, Tiangen). These chilled, methanol extracted homogenized tissues were then centrifuged at 14,000 rpm for 6 min to sediment the cellular and tissue debris, and the 60 μL cleared tissue supernatants were transferred to a screw-cap V-type glass-bottom vial (VAAP-31509–1232–100, CNW) and dried in a vacuum-drying apparatus (Concentrator Plus, Eppendorf) at 30 °C for 10 min. For analysis, the residue was mixed with 20 μL N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA, Cat#394882, Sigma-Aldrich) and 35 μL of pyridine (Cat#270407, Sigma-Aldrich) at 70 °C for 20 min. One microliters aliquot of derivatized sample was injected into Aligent 7890 A gas chromatography coupled with Agilent 5975C mass spectrometer. A capillary column (Cat# 19091S-433, HP-5ms Intuvo, 30 m × 0.25 mm × 0.25 μm; Agilent Technologies) using helium as a carrier gas was utilized for separation. The parameters of GC-MS and data process are provided in Supplementary Information.

GC-MS-based 2-HG quantification

D-2-HG (Sigma-Aldrich, Cat#94577) as a standard sample was dissolved in ddH$_2$O at a concentration of 10 mM. Double-diluted samples were mixed with 100 μL of −80 °C methanol: water mix (80%:20%), and 40 μL of the mixture was transferred to a screw-cap V-type glass-bottom vial and dried in a vacuum-drying apparatus as described above, following the standard GC-MS method to detect 2-HG abundance. Accordingly, a 2-HG standard curve was made for its quantification.

For 2-HG quantification in glioma samples, metabolite extraction was accomplished by adding 200 μL of −80 °C
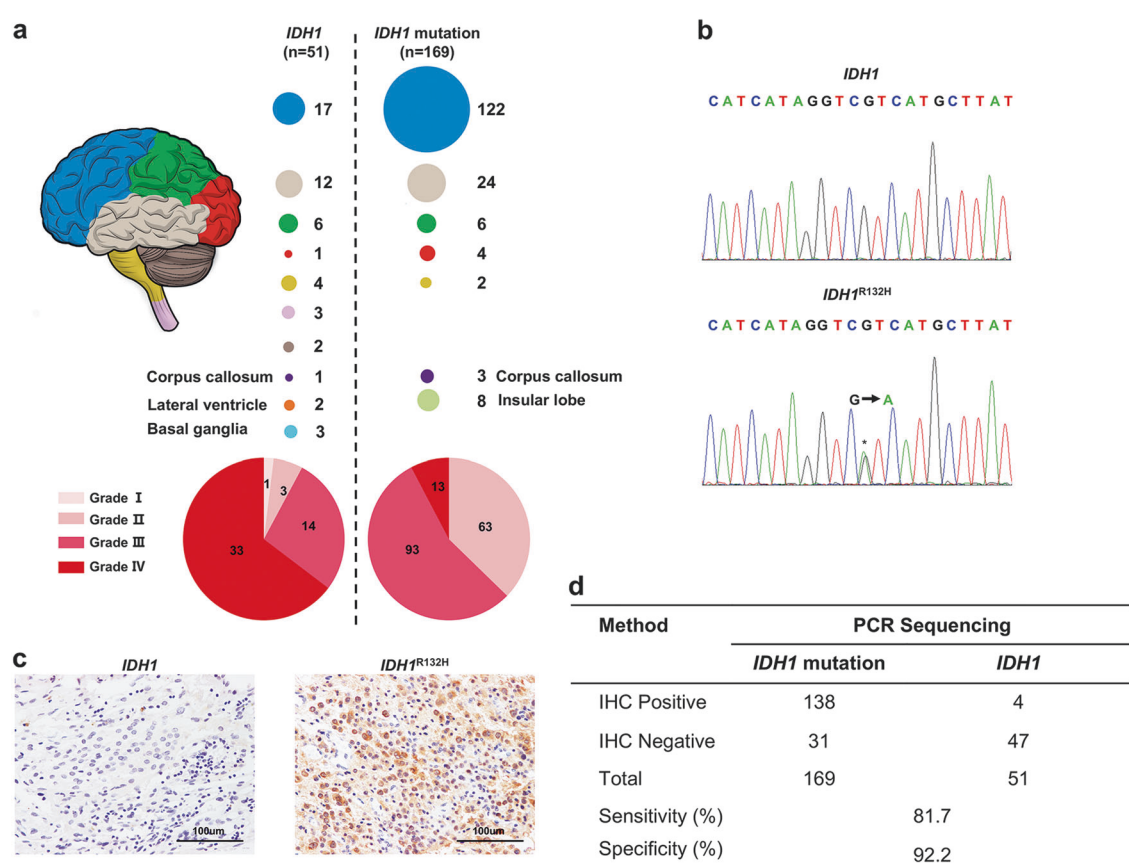
Fig. 1 Retrospective assessment of *IDH1* mutation. **a** The clinical characteristic of patient population. The circle represents the population size and the color corresponds to the location. The distribution of WHO grades (lower panel) is shown. The area of each part represents the percentage contributing to the total population. A total of 220 glioma samples were performed with **b** PCR-sequencing and **c** IHC staining to identify *IDH1*[R132H] mutation. Scale bars represent 100μm. According to the sequencing data, the sensitivity and specificity of the IHC staining method were shown (**d**)

methanol: water mix (80%:20%) to the brain tissue (approximately 10 mg) followed by 90 s homogenization (Tissuelyser-48, Shanghai Jingxin), and centrifugation at 14,000 rpm for 10 min at 4 °C. Two-hundred microliters of supernatant was transferred and dried at 30 °C for 1 h. The residue was mixed with 35 μL pyridine containing 20 mg/mL Methoxyamine hydrochloride (Sigma-Aldrich, Cat#226904) and then incubated at 70 °C for 0.5 h. Derivatization was performed at 70 °C for 40 min following addition of 20 μL of MTBSTFA. One-microliter aliquot of derivatized sample was injected into GC-MS for 2-HG detection and quantification.

Method validation

The mini-column method was validated according to the guidance set forth by the US-FDA for industry on bioanalytical method validation [21]. The selectivity, lower limit of quantitation (LLOQ), matrix effect, recovery, precision, accuracy, and stability were evaluated. Details and results was shown in Supplementary Information.

Results

Retrospective assessment of *IDH1* mutation by PCR-sequencing and IHC staining in gliomas

Among 220 glioma samples, 169 heterozygous *IDH1* R132H mutation were identified by PCR-sequencing (76.8%, The WHO grades and location were shown in Fig. 1a, b). We found a much higher ratio of *IDH1* mutation in lower grade gliomas (LGG, WHO Grades II & III, 92.3%) as compared to GBMs (7.7% of *IDH1* mutation glioma). Moreover, *IDH1*-mutated gliomas were mainly located in the frontal lobe (Fig. 1a), which is in line with previous studies (for characteristic of this population, Supplementary Table S1) [22].

Next, we assessed the *IDH1* mutation status of glioma samples by immunohistochemistry (IHC) using an antibody specifically recognizing *IDH1* R132H mutant (Fig. 1c). Of 169 *IDH1*-mutated samples, 138 (81.7%) cases were positive for the *IDH1* R132H mutation by IHC (Fig. 1d). Of 51 glioma samples with wild-type IDH1, 47 (92.2%) were confirmed by the negative staining with antibody against
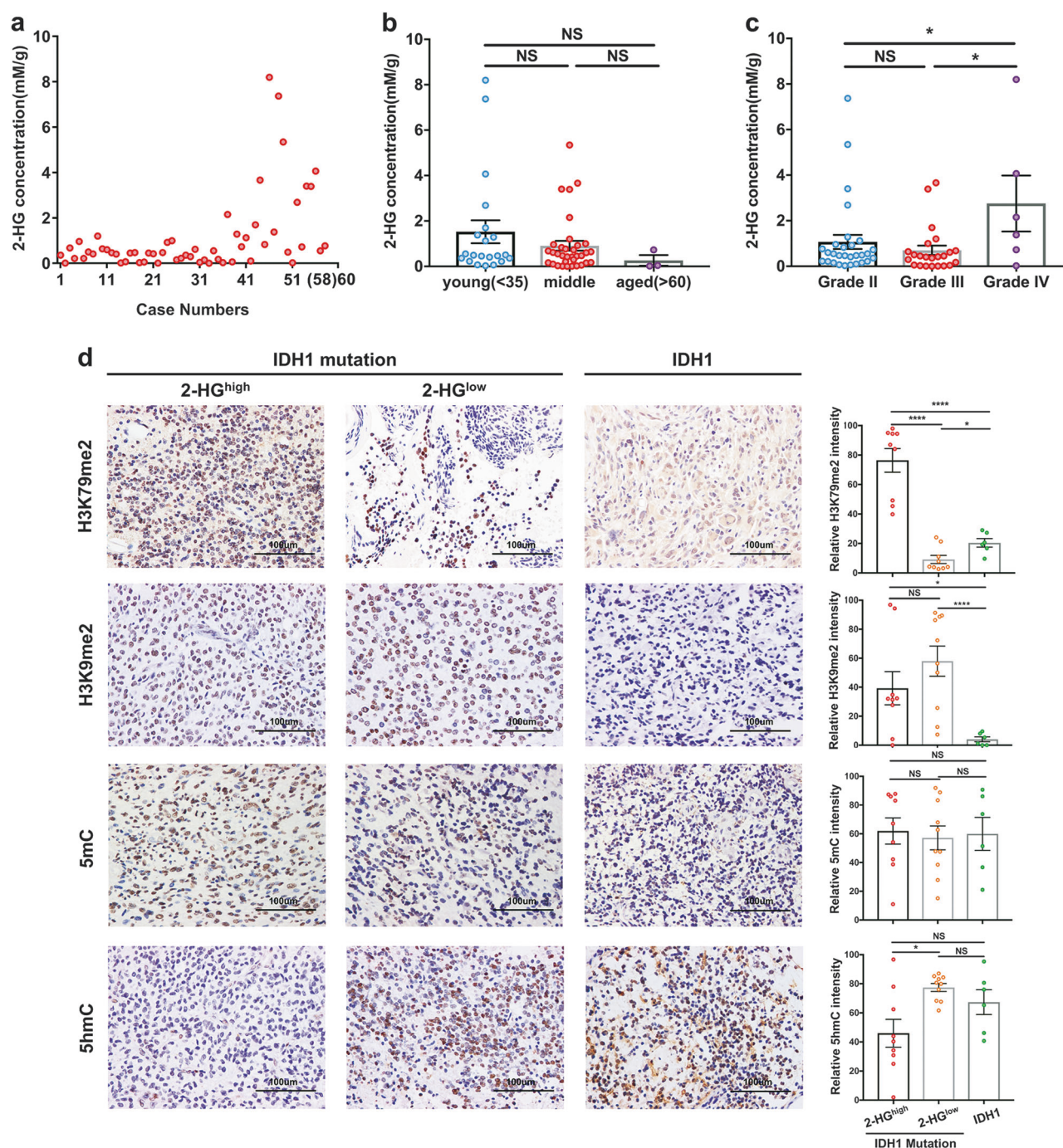
Fig. 2 Quantitative detection of 2-HG by GC-MS in *IDH1*-mutated gliomas. **a** The concrete 2-HG concentration. **b** High accumulation of 2-HG appears to be dominant in young patients (age < 35 years), middle-aged patients (35 years ≤ age ≤ 60 years), and **c** GBM samples. **d** Dose-dependent activity of 2-HG on epigenetic regulation by IHC. Representative IHC data in *IDH1* mutant glioma tissues (two left panels; the 2-HG^high and 2-HG^low groups) and WT tumors (right panel) are shown. Scale bars represent 100μm. Furthermore, quantification of these epigenetic modification intensity is shown. Mean ± SEM, 2-tailed *t*-test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

IDH1 R132H mutant, while four cases exhibited false positive IHC (Fig. 1d). With relatively high sensitivity and specificity, both PCR-sequencing and IHC staining can be used to assess the *IDH* mutation status postoperatively in gliomas. It has to be noted that both methods usually take 1–2 days, hindering their application for rapid diagnosis.

Quantitative detection of 2-HG by GC-MS in *IDH1*-mutated gliomas

The GC-MS technique offers a potentially sensitive and reliable assay not only for detecting 2-HG, but also for its quantification. During sample preparation of the GC-MS

based methodology, surgically dissected glioma samples (approximately 10 mg) were homogenized with pre-cold methanol following centrifugation, and the supernatant was then subjected to vacuum-drying, derivatization, and GC-MS detection and quantification. Standard curve was rebuilt adaptive to each detection in order to cover maximal response value within left 50% curve. In 58 cases of glioma samples harboring heterozygous *IDH1* R132H mutation identified by sequencing, we found that in 49/58 (84.5%) of tested samples the 2-HG level was below 2 mM/g tissue (Fig. 2a). In a few cases harboring *IDH1* mutation, 2-HG concentration could reach as high as 8.2 mM/g (Fig. 2a).

The levels of 2-HG appeared to be high in young *IDH1*-mutated patients (< 35 years, $n = 21$, 1.525 ± 2.30 mM/g) as well as middle-aged patients (35 years ≤ age ≤ 60 years, $n = 34$, 0.909 ± 1.22 mM/g), and tended to be reduced in elderly patients (> 60 years, $n = 3$, 0.263 ± 0.403 mM/g) (Fig. 2b). Due to the limited sample size of the elderly group, no statistical difference was detected (Fig. 2b). Additionally, we also observed that the 2-HG concentration was higher in GBM ($n = 6$, 2.757 ± 3.006 mM/g), compared with LGG including WHO Grade II ($n = 29$, 1.071 ± 1.663 mM/g) and Grade III ($n = 23$, 0.701 ± 0.988 mM/g, $P < 0.05$). (Fig. 2c).

Altogether, these results indicate that GC-MS could provide a reliable strategy to quantify 2-HG in gliomas harboring *IDH1* mutation using small amounts of tissue.

## Inhibition of histone demethylation by highly accumulated 2-HG in *IDH1*-mutated gliomas

IDH1 R132 mutations exclusively produce D-2-HG in primary tumor tissue [8], and D-2-HG acts as an antagonist of α-KG, competitively inhibiting multiple α-KG/Fe(II)-dependent dioxygenases, including both histone and DNA demethylases [23, 24]. In vitro studies have shown that D-2-HG has lowest half-maximal inhibitory concentration (IC_{50}) toward members of histone demethylases, ranging from 24 μM to 106 μM [23]. As shown in Fig. 2a, the concentration of 2-HG (i.e., D-2-HG) ranged from 5.4 μM/g to 8.2 mM/g in examined *IDH1*-mutated glioma samples, suggesting the possibility that histone demethylases may be inhibited in these samples. To test this hypothesis, we analyzed histone H3 lysine 79 (H3K79) and H3 lysine 9 (H3K9) methylations in glioma samples (Fig. 2d). Our data demonstrated that H3K79 dimethylation was significantly elevated in *IDH1*-mutated cases in which the 2-HG level was higher than 2 mM/g neoplasm tissue (referred to as 2-HG^high, $n = 9$) as compared to tumors with wild-type IDH1 ($P < 0.0001$, $n = 6$). Notably, H3K79 dimethylation was not increased in *IDH1*-mutated glioma samples in which 2-HG level was lower than 0.1 mM/g neoplasm tissue (referred to as 2-HG^low, $n = 10$) when compared to tumors with wild-type IDH1. And H3K79 dimethylation was significantly higher

in the 2-HG^high group than the 2-HG^low group ($P < 0.0001$). Methylation of H3K79 is catalyzed by DOT1L/KMT4 and plays a role in multiple cellular pathways, including telomeric silencing, cellular development, cell-cycle checkpoint, DNA repair, and regulation of transcription [25]. Our results indicate that the activity of putative H3K79 demethylase, which is yet to be identified, appears to be sensitive to the concentration of 2-HG in cells and can only be inhibited when 2-HG is accumulated to high levels.

On the other hand, H3K9 dimethylation was significantly elevated in both the 2-HG^high and 2-HG^low groups harboring *IDH1* mutation as compared to the IDH1 wild-type group ($P < 0.05$ and $P < 0.0001$, respectively). No significant change in H3K9 dimethylation levels was found between the 2-HG^high and 2-HG^low groups with *IDH1* mutation. Bioinformatic analysis from TCGA datasets demonstrated that most of the H3K9 dimethylation-associated histone demethylase genes [26, 27], such as *KDM1A, KDM3B, KDM4B, KDM4C, KDM4D, KDM5B*, and *KDM7A*, were upregulated in *IDH1*-mutated gliomas compared to wild-type tumors (Supplementary Fig. S1). These results support the notion that *IDH1* mutation and 2-HG accumulation lead to inhibition of histone demethylase activity, rather than suppression of their expression.

Inhibition of DNA hydroxymethylation by highly accumulated 2-HG in *IDH1*-mutated gliomas

Beside α-KG-dependent histone demethylases, 2-HG also inhibits the catalytic activity of TET1 and TET2 in vitro and in *IDH* mutant cells [24, 28]. In accord, *IDH* mutation is associated with CpG island methylator phenotype (G-CIMP) in glioma [29], and can singularly establish G-CIMP when ectopically expressed in primary astrocytes [30]. We found that the average relative intensity of 5-methylcytosine (5mC) did not appreciably differ among the 2-HG^high ($n = 9$), 2-HG^low ($n = 10$), and wild-type groups ($n = 6$) (Fig. 2d). The average relative intensity of 5-hydroxymethylcytosine (5hmC) tended to be decreased in the 2-HG^high group compared to the IDH1 wild-type group. The tendency towards reduced 5hmC was, however, not observed in the 2-HG^low group compared to the IDH1 wild-type group. Of note, the 5hmC level was significantly lower in the 2-HG^high group than the 2-HG^low group ($P < 0.05$). Gene expression data from TCGA demonstrated that all there *TET* genes were significantly upregulated in *IDH1*-mutated gliomas compared to IDH1 wild-type tumors (Supplementary Fig. S2), supporting the notion that *IDH1* mutation and 2-HG lead to inhibition of TET activity in gliomas, rather than suppression of their expression. Additionally, these results also suggest that the catalytic activity of TET enzymes can only be inhibited when 2-HG is accumulated to high levels in *IDH*-mutated gliomas.
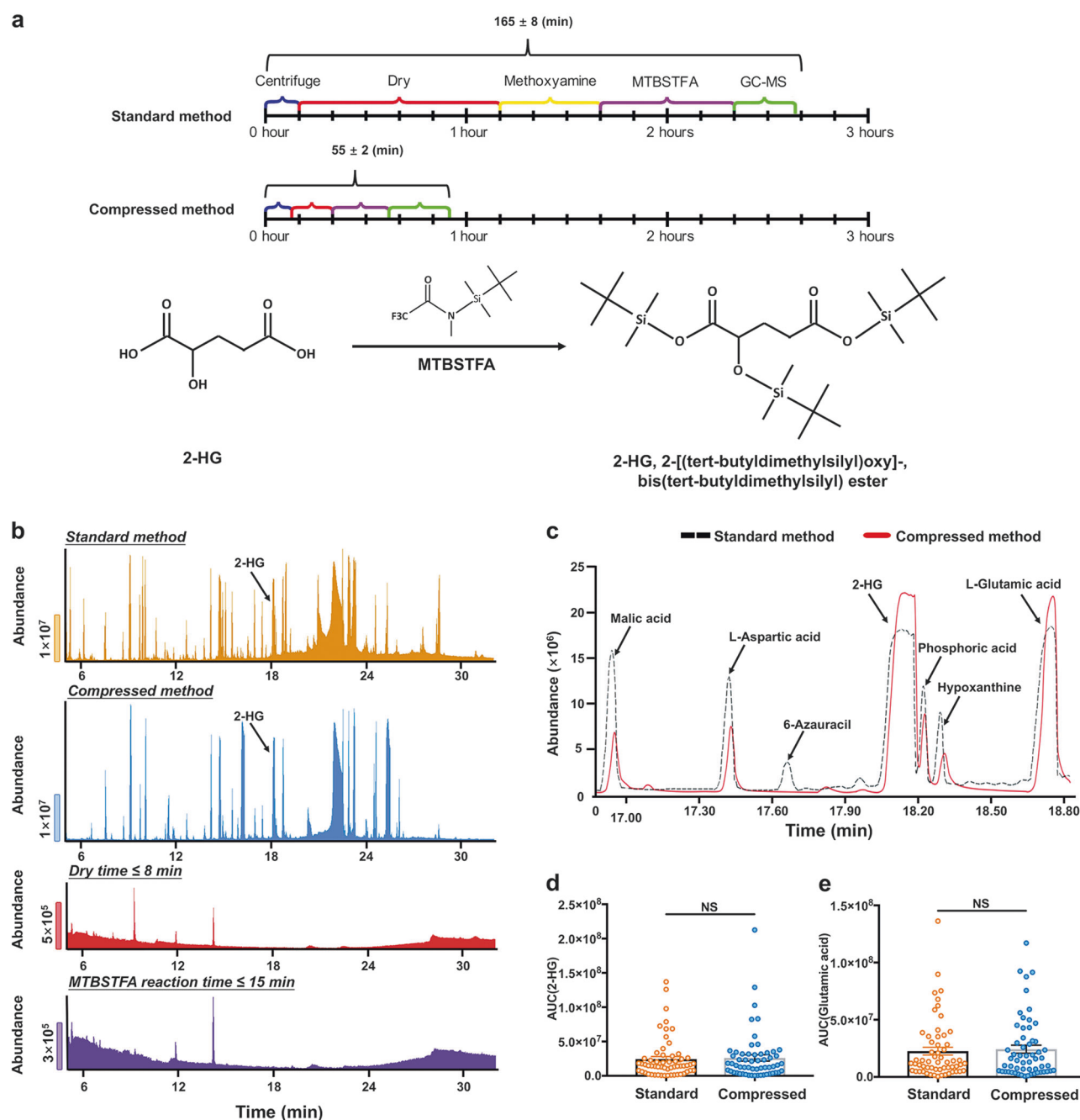
Fig. 3 A compressed GC-MS method for rapid 2-HG detection. **a** Diagram showing the standard and compressed methods. **b** Metabolite profiling in *IDH1*-mutated glioma samples. Arrow pointing the 2-HG peak. When the vacuum-drying time is shortened to 8 min or the MTBSTFA reaction time is cut to 15 min, the 2-HG peak cannot be detected by GC-MS. **c** Comparison of metabolite profiles determined by the standard and compressed methods. The AUC of **d** 2-HG and **e** L-glutamic acid in *IDH1*-mutated glioma samples ($n = 58$) as determined by the standard and compressed GC-MS methods

A compressed GC-MS procedure for rapid 2-HG detection

Both PCR-sequencing and IHC staining methods require usually more than 1 day to complete, making them not applicable for rapid diagnosis of *IDH* mutation in clinical practice. The standard procedure for GC-MS method includes homogenization, centrifugation, vacuum-drying, oximation by methoxyamine hydrochloride (MOX), and derivatization by N-tert-Butyldimethylsilyl-N- methyltri-fluoroacetamide (MTBSTFA), which generally takes about 3 h (i.e., 165 ± 8 min) (Fig. 3a). Of note, 2-HG is a small molecular metabolite without carbonyl groups and thus cannot react with MOX [31]. Considering that 2-HG
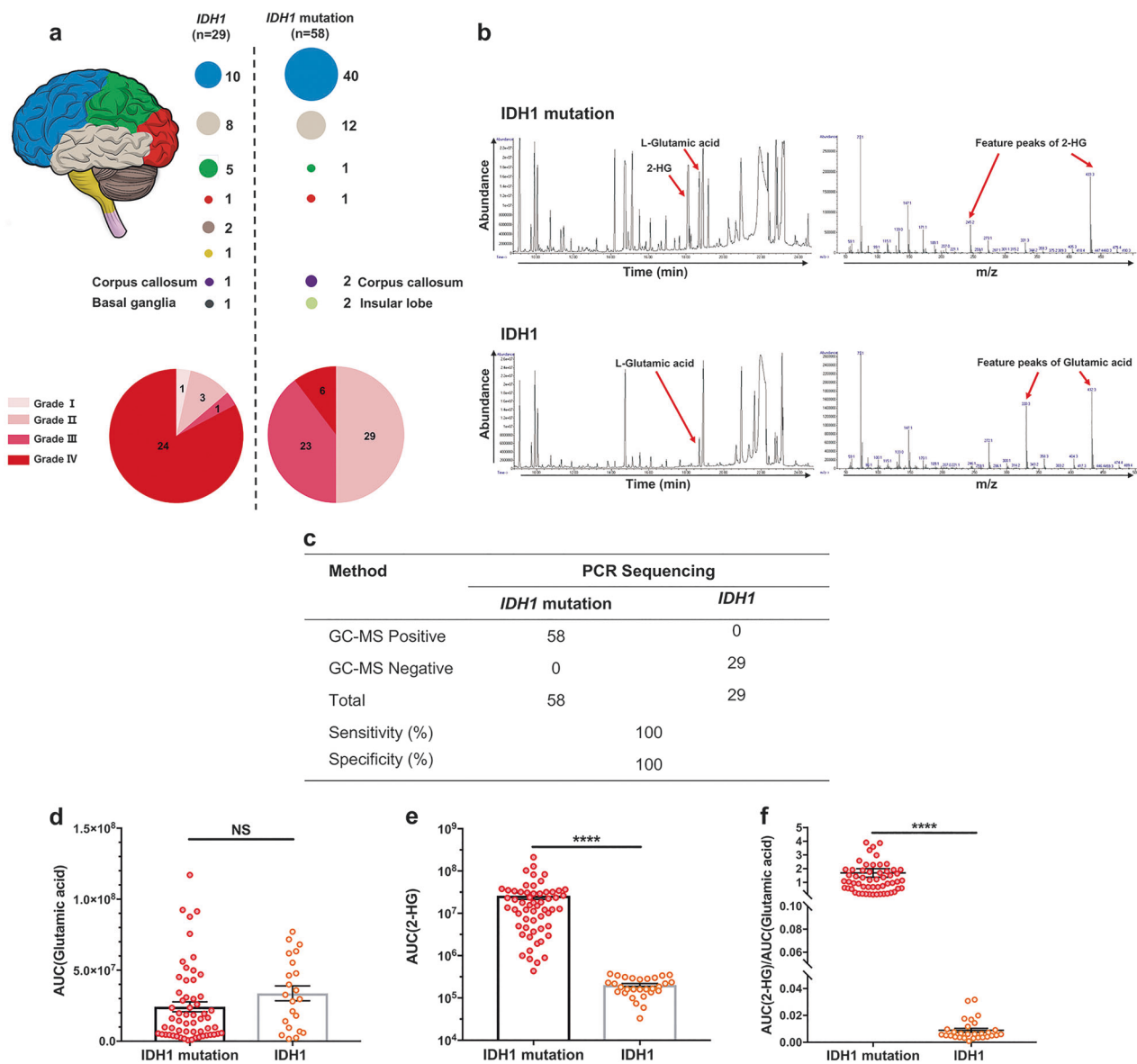
Fig. 4 The compressed method can commendably predict *IDH1* mutation status. **a** The clinical characteristic of patient population. The circle represents the population size and the color corresponds to the location. The distribution of WHO grades (lower panel) is shown. **b** Metabolite profiling in *IDH1*-mutated or WT glioma samples. **c** The sensitivity and specificity of the method. The AUC of **d** L-glutamic acid and **e** 2-HG in *IDH1*-mutated or WT glioma samples was determined by the compressed method. For 2-HG standardization, **f** the ratio of 2-HG/L-glutamic acid is calculated and shown

contains three hydroxyls and thereby can react with MTBSTFA (Fig. 3a), we developed a compressed GC-MS procedure with a goal to detect 2-HG rapidly. To accelerate the GC-MS procedure, we tested various conditions to shorten the time-period for each step of GC-MS and their combinations. We determined that the centrifugation time can be reduced to as short as 6 min, the vacuum-drying time to as short as 10 min, the oximation step by methoxyamine hydrochloride can be omitted, and the MTBSTFA reaction time can be cut to 20 min without losing the detection of 2-HG peak (Fig. 3a, b). As such, the compressed GC-MS procedure can be completed from

tissue sample preparation to 2-HG detection in less than an hour (55 ± 2 min).

Next, we tested the potential for clinical utility of this compressed GC-MS based 2-HG detection method in a cohort of 87 glioma patients (for characteristic of this population, Supplementary Table S2). The workflow of operation starts at the diagnosis based on MRI before surgery and ends at retrospective assessment of *IDH1* mutation by PCR-sequencing and IHC staining. Surgically dissected gliomas samples were directly subjected to metabolite profiling by using the GC-MS based methods (Supplementary Fig. S3). The compressed GC-MS
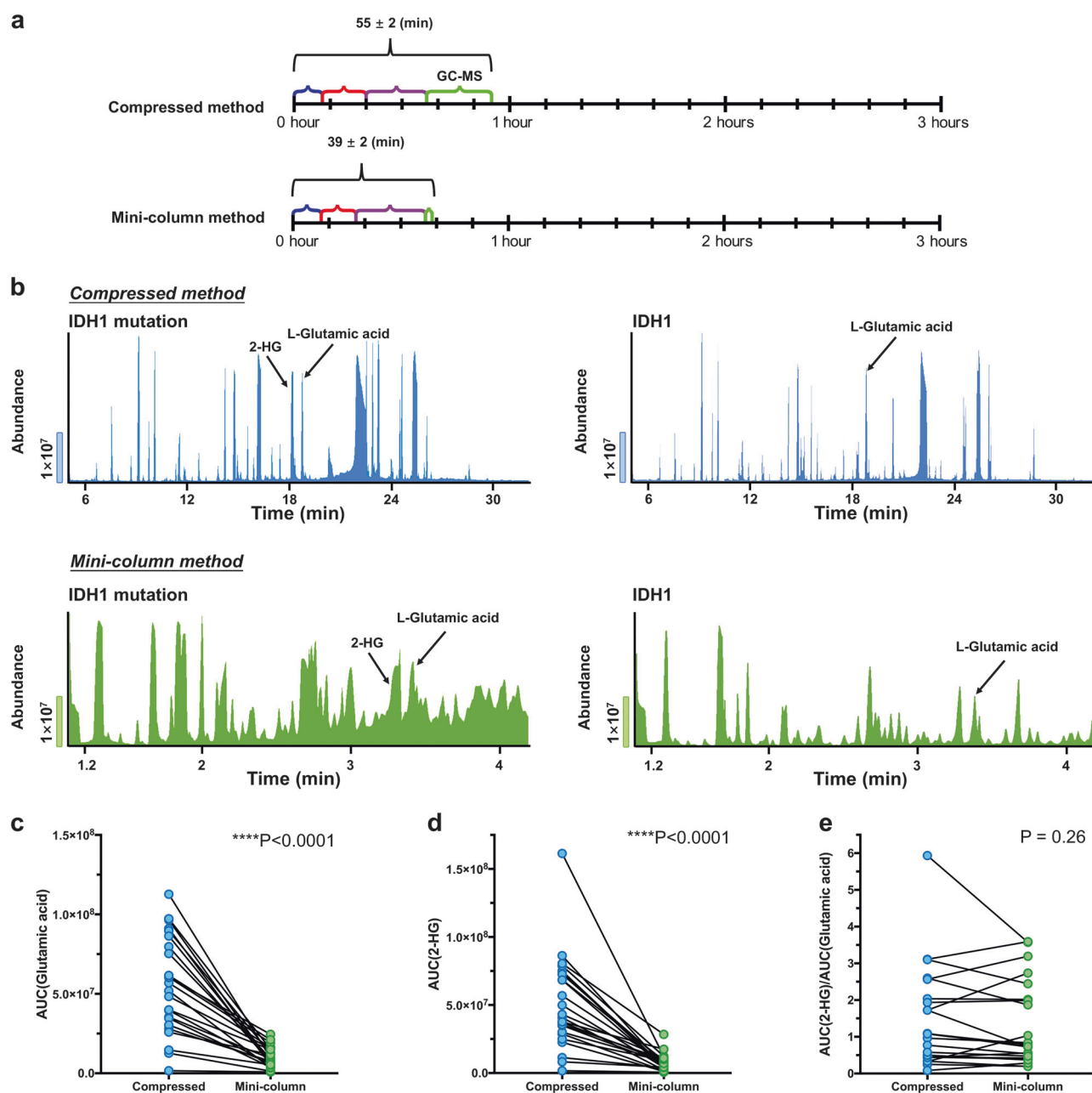
Fig. 5 A mini-column method for rapid diagnosis. **a** The GC-MS running time was shortened dramatically by cutting down the GC capillary column and total detection time was within 40 min. **b** Metabolite profiling in *IDH1*-mutated glioma samples. The AUC of **c** L-glutamic acid **d** 2-HG, and **e** the ratio of 2-HG/L-glutamic acid in 24 cases of *IDH1*-mutated glioma samples was determined by the compressed and mini-column methods

method omits the MOX oximation step and interferes the detection of metabolites containing carbonyl groups. Indeed, the AUC (area under curve) of metabolites with carbonyl groups, such as 6-azauracil and hypoxanthine (Fig. 3c), determined by the compressed GC-MS method was clearly smaller than that determined by the standard GC-MS method. Neither 2-HG nor L-glutamic acid, which is commonly used for 2-HG standardization [32, 33], has carbonyl groups. Accordingly, the AUC of 2-HG or L-glutamic acid determined by the compressed

GC-MS method was similar to that analyzed by the standard method ($n = 58$, paired *t*-test; Fig. 3d, e).

Among these 87 cases, 58 samples were identified to harbor *IDH1* mutation by PCR-sequencing (Supplementary Table S2). By applying the compressed GC-MS method, we detected 2-HG accumulation in all 58 *IDH1*-mutated samples (Fig. 4a, b, c). None of the examined 29 samples, which had been identified to contain wild-type IDH1 by PCR-sequencing, showed 2-HG accumulation when analyzed by the compressed GC-MS method (Fig. 4b). Thus,

these findings indicate a 100% accuracy of the compressed GC-MS method in identifying *IDH*-mutated glioma samples. Consistent with our earlier finding (Fig. 1a), we found that *IDH1*-mutated/2-HG-positive cases were enriched in WHO Grade II and III gliomas and mainly located in the frontal lobe by using the compressed method (Fig. 4a). The AUC of L-glutamic acid determined by the compressed GC-MS remains constant between *IDH1*-mutated and wild-type tumors (Fig. 4d). This, together with the close retention time of L-glutamic acid (GLU, 18.72 min) and 2-HG (18.12 min), provides a convenient and semi-quantitative internal control to the rapid GC-MS procedure. We found that the ratio of 2-HG/GLU was significantly increased in all cases of *IDH1*-mutated cases (1.685 ± 2.367, $n = 58$; $P < 0.0001$) compared to the IDH1 wild-type samples (0.0096 ± 0.0078, $n = 29$, Fig. 4d, e, f). The ratio of 2-HG/GLU, therefore, represents a more accurate measurement to discriminate between *IDH1* mutant and wild-type gliomas: a ratio of 2-HG/GLU below 0.04 indicated wild-type IDH status, a ratio above 0.1 was only found in tumors with *IDH1* mutation, and ratios in the range of 0.04 to 0.1 were not seen in two cohorts of the analyzed samples (Fig. 4f).

A faster and qualitative GC-MS procedure for 2-HG detection and rapid diagnosis of *IDH1*-mutated gliomas

To get a rapid diagnosis of *IDH* mutation, the capillary column in the GC machine was cut down from 30 to 5 meters (mini-column method). As a result, the GC running time was dramatically shortened from 32 min (standard method) to 4.2 min (mini-column method). During GC running period, the 2-HG peaks appear at 18.1 min and 3.3 min when the standard and mini-column methods are applied, respectively. Total detection time (mini-column method) was 39 ± 2 min compared with 55 ± 2 min of the compressed method ($P < 0.0001$, Fig. 5a) (Supplementary Table S3). The separation efficiency and peaks deceased obviously using 5-meter column method, but the 2-HG peak can still be sufficiently separated by this mini-column and clearly identified by MS, appearing at 3.3 min instead of 18.1 min on the standard column (Fig. 5b).

To evaluate the accuracy and clinical utility of the mini-column method in detecting 2-HG and identifying *IDH* mutation status in tumor tissue, a cohort of 43 surgically dissected gliomas were subjected to 2-HG detection by both the compressed method and mini-column method. Of these 43 samples, 24 harbor *IDH1* mutation as determined by PCR-sequencing (data not shown), and all these 24 samples were 2-HG positive as determined by either the compressed or mini-column GC-MS method. The AUC of L-glutamic acid or 2-HG was significantly smaller on the mini-column method ($P < 0.001$, paired *t*-test; Fig. 5c, d). Nevertheless, the ratio of 2-HG/GLU determined by the mini-column

method was similar to that determined by the compressed GC-MS procedure ($P = 0.26$, paired *t*-test; Fig. 5e). Furthermore, the ratio of 2-HG/GLU is commonly above 0.1 in all the examined 24 samples harboring *IDH1* mutation as determined by the mini-column method, while the ratio is < 0.04 in 19 wild-type samples, suggesting that the 2-HG/GLU ratio may represent as a reliable index to judge the *IDH* mutation status in clinical cases (Supplementary Fig. S4). Collectively, these results indicate that the mini-column method, although not suitable for quantification, can reliably and rapidly identify 2-HG and thus *IDH1* mutation status in surgically dissected glioma samples, providing a novel method with potentially broad clinical utility for rapid diagnosis of *IDH* mutation.

Discussion

IDH mutations define distinct subtypes within otherwise heterogeneous gliomas [34, 35], and these subgroups exhibit unique clinical features and response to therapy [3, 36, 37]. Precise diagnosis of *IDH* mutation is critical for patient care management in gliomas. In recent years, rapid progress has been made in the development of mass spectrometry technology, including the identification of small metabolites. The discovery of D-2-HG production by mutant *IDH* provides an excellent example to elucidate the promise of the mass spectrometry-based technology [8].

In this study, we developed GC-MS based methods for 2-HG detection and quantification. Our data demonstrate that D-2-HG concentration varies among *IDH1*-mutated gliomas, ranging from 5.4 μM/g to 8.2 mM/g neoplasm tissue. 2-HG carries an asymmetric carbon atom in its carbon backbone, leading to the formation of two enantiomers: D-2-HG and L-2-HG. Both enantiomers of 2-HG are normal endogenous metabolites but may differ in the potency to bind and inhibit the activity of α-KG-dependent dioxygenases. The activity of D-2-HG to inhibit α-KG-dependent dioxygenases including histone and DNA demethylases has been well recognized. Comprehensive in vitro studies have reported that the activity of D-2-HG to inhibit members of the α-KG-dependent dioxygenase family varies greatly, with histone demethylases KDM4A/JMJD2A and KDM4C/JMJD2C being the most sensitive ($IC_{50} = 24$ μM and 79 μM, respectively), and proline hydroxylase domain protein 2 (PHD2) and γ-butyrobetaine hydroxylase (BBOX) being very resistant ($IC_{50} = 7.3$ mM and 13.2 mM, respectively) [23]. Additionally, D-2-HG also inhibits mouse Tet1 and Tet2 in vitro ($IC_{50} = 4$ mM and 5 mM, respectively) [28, 38]. Therefore, α-KG-dependent dioxygenases are predicted to be differently inhibited by D-2-HG in glioma harboring *IDH* mutation, and ones with the lowest IC_{50} value for D-2-HG will be preferentially inhibited first. Supporting this notion, we show in this study that 2-HG

accumulation in both the 2-HG^{low} and 2-HG^{high} groups are sufficient to inhibit α-KG-dependent KDM4A/JMJD2A and KDM4C/JMJD2C which are highly susceptible to 2-HG inhibition [23]. On the other hands, 2-HG accumulated in the 2-HG^{low} group of gliomas is not sufficient to effectively inhibit TETs, and higher accumulation of 2-HG (e.g., 4 to 5 mM) [38] will be needed for catalytic inhibition of TET. Collectively, these results provide in vivo evidence re-affirming inhibition of α-KG-dependent histone and DNA demethylases by highly accumulated 2-HG in *IDH1*-mutated gliomas. Clinical implications of the observed differential inhibition of diverse α-KG-dependent dioxygenases needs further investigation.

Rapid diagnosis of *IDH* mutation is urgently needed to help neurosurgeons to design strategies for treating brain tumors for several reasons. First, *IDH* mutation can distinguish LGG from gliosis and normal brain tissues. Second, 2-HG concentration usually sharply falls among the margins of gliomas [12], which is very helpful for recognizing the tumor boundary. Third, rapid diagnosis of *IDH* mutation would have potentially significant benefit for deciding the extent of resection. If the tumor is located in non-functional areas of the brain, total resection or maximal surgical resection will definitely benefit patients harboring *IDH1* mutations. If the *IDH*-mutated tumor is next to or located in functional areas of the brain, these tumors should be left to preserve critical neurological functions and are supposed to be highly sensitive to radio-chemotherapy [36]. In the current study, we have developed a new compressed GC-MS-based method, which can accurately and rapidly identify *IDH* mutation status in brain tumors by monitoring 2-HG. After further modification, the GC running time has been successfully shortened to 4.2 min, and thus enables us to detect 2-HG in small amounts of glioma samples within 40 min after surgery. Very recently, desorption electrospray ionization-mass spectrometry (DESI-MS) was reported to identify 2-HG (around 3 min, regardless of the sample preparation time) in frozen tissue smears and help the rapid molecular diagnosis of *IDH* mutation in gliomas [39]. Both DESI-MS and GC-MS are convenient for 2-HG detection, because DESI-MS doesn't need a reference library and GC-MS owns a standard Mass Database, which can be widely applied to establish a standard protocol for detection of metabolites including 2-HG and any other oncometabolites [40]. It has to be noted that establishment of the reference library is needed before conducting the DESI-MS to determine tumor cell percentage(TCP) [41]. Liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI–MS/MS) method was developed to accurately detect 2-HG [42], while it couldnot completely distinguish the mutated from the wild-type. Because there is little 2-HG among wild-type tumor, accurate diagnosis couldnot be made solely dependent on 2-HG. We believe that both ambient ionization methods and GC-MS-based methods will have broad clinical utility by helping to intraoperatively diagnose *IDH* mutation status, expedite clinical decision-making, and improve the care and treatment of neuro-oncologic patients in the near future.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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