**Abstract:** Patients below 55 years were genetically studied because the prevalence of isocitrate dehydrogenase 1 (IDH1) decreases in older patients and on grounds of cost-effectiveness, as suggested by the World Health Organization (WHO) in 2016. The aim of our study was to use novel massively parallel sequencing (MPS) approaches to examine rare variants of IDH1/2 in Czech diffuse astrocytic and oligodendroglial tumors (gliomas) below 55 years of age who had been immunohistochemically (IHC) diagnosed as IDH1 R132H negative. The IHC IDH1 status (wild type or mutant) of 275 tissue samples was analyzed using antibodies against the IDH1 R132H protein. Sixty-three samples of 55 years old patients with IHC IDH1 WT status were genotyped using two different MPS technologies to detect rare IDH1 and IDH2 variants. The tiered IHC (60 positive) and molecular (10 positive) approach thus revealed that 70 of the 275 samples (25%) bore IDH1/IDH2 mutations. The combined molecular and IHC approach thus revealed that 70 of the 275 samples (25%) considered in the study bore IDH1/IDH2 mutations. IHC detection of the IDH1 R132H variant should be routinely complemented with MPS to detect rare IDH1/2 variants in glioma patients below 55 years of age with negative IHC result of IDH1 R132H variant.

**Key Words:** IDH1, IDH2, fast sequencing, immunohistochemistry, diffuse gliomas

T he World Health Organization (WHO) has historically classified tumors of the central nervous system (CNS) based on their histologic features. In 2016, the WHO incorporated selected molecular parameters into the tumor classification system, improving diagnosis and patient care. In particular, the use of genetic markers (IDH1/2, ATRX, TP53, and the 1p/19q co-deletion) as diagnostic tools has led to the development of robust and reproducible algorithms that predict patients’ survival better than histology alone. In 2008, exome sequencing studies on glioblastomas identified missense mutations in isocitrate dehydrogenase 1 (IDH1), a Krebs cycle gene. This led to the elucidation of common genetic alterations during early formative stages of progressive gliomas and secondary glioblastomas, significantly improving the understanding and classification of gliomas. IDH1 and 2 are important in cellular processes such as the response to glucose, glutamine metabolism, lipogenesis, and the regulation of cellular redox status. Under normal conditions, the IDH enzyme catalyzes the conversion of isocitrate to α-ketoglutarate by decarboxylation. The residues of IDH1 and IDH2 most frequently mutated in gliomas are Arg132 and Arg172, respectively. Mutations of these residues induce neomorphic enzyme activity: instead of the wild type activity, the mutated proteins catalyze the reduction of α-ketoglutarate into the oncometabolite D-2-hydroxyglutarate. The aberrant production of D-2-hydroxyglutarate leads to a cellular state of malignant transformation involving both epigenetic changes and aberrant differentiation. The presence of IDH1/2 mutations was identified as a marker of survival, and these
mutations have become critical diagnostic tools that are used to guide clinical decision making relating to gliomas.2

The incidence of IDH1 mutations in glioblastomas is ~12%, but studies on grade II to III gliomas and secondary glioblastomas found these mutations in ~80% of samples.9-13 IDH2 mutations are less common and are mutually exclusive with IDH1 mutations.13,14 All IDH1 and IDH2 mutations observed in glioblastomas are single amino acid missense mutations at arginine 132 (R132) or the analogous arginine 172 (R172), respectively. The most frequent variant, IDH1 R132H, is found in over 85% of gliomas15 and features a heterozygous missense mutation of arginine to histidine (CGT→CAT). This mutation changes the enzyme’s active site, reducing its catalytic activity and its affinity for isocitrate.16

The detection of R132 and R172 variants has implications for glioma diagnosis,17 prognosis14,18 and potentially treatment.19,20 However, the only variant that can be detected by immunohistochemistry (IHC) is IDH1 R132H; the other variants are currently detected by follow-up genetic sequencing using Sanger or next-generation technology.2 On the basis of studies examining the effects of variables such as patient age, tumor grade, and IDH1 R132H IHC, the WHO recommended in 2016 that only glioma patients below 55 years of age should undergo sequencing for rare IDH1 mutations following a negative IDH1 R132H IHC analysis.2,21,22 Screening for IDH mutations has thus become a key diagnostic tool for brain tumors but is not cost-effective for all patients.22

Since the IDH mutations status is crucial for diagnostic algorithm for integrated classification of diffuse astrocytic and oligodendroglial tumors, the revelations true positive/negative samples is a necessity these days. Our aim was to reveal samples by massively parallel sequencing (MPS) approaches that were signed as false negative samples by the IHC methodology and if there is a space for reduction of expenses and/or increasing the efficiency of genotyping when using different approaches of next-generation sequencing (NGS) methodology. Our fast IDH method is suitable for genotyping of known hotspots for somatic mutations with concordant results validated by the commercially available kit (Nextera XT kit, Illumina).

MATERIAL AND METHODS

Study Group and Tissue Specimens

The study cohort consisted of 275 patients with gliomas who had undergone surgical intervention at the Department of Neurosurgery in Olomouc between the years 2011 and 2017. Formalin-fixed paraffin-embedded (FFPE) sample was collected from each participating patient, and all samples included in the study were validated by a pathologist experienced with CNS tumors.

IHC

The 1 to 2 μm thick tissue sections were pretreated using the system PT Link (Agilent) at 97°C, pH9 for 20 minutes to ensure epitope retrieval. Hydrogen peroxide was used to block endogenous peroxidase activity. The sections were subsequently treated for with primary antibody, Anti-IDH1 R132H, clone H09 (Dianova, Hamburg, Germany), dilution 1 : 100 for 20 minutes at room temperature. EnVision Flex+, Mouse, High pH (Agilent DAKO) was used to amplify the signal of primary antibody. After the application of the secondary antibody EnVision, Flex/HRP (Agilent DAKO) for 20 minutes, the reaction was finally visualized using DAB+ Substrate Chromagen System (Agilent DAKO).

Fluorescence In Situ Hybridization (FISH)

The 1p/19q gene co-deletion was detected by FISH, which was performed in accordance with the manufacturer’s protocol for FFPE tissue sections (IntellMed Ltd., Olomouc, Czech Republic). The locus-specific identifiers 1p36.3 and 19q13 were used for chromosome copy number enumeration. At least 100 nonoverlapping nuclei were selected for assessment in each sample using fluorescence microscopy.

IDH1 and IDH2 Genotyping by Next-Generation Sequencing

The protocols used for DNA extraction from FFPE tissue sections and IDH1 R132 and IDH2 R172 genotyping are provided in the supplementary material and methods (Supplemental Digital Content 1, http://links.lww.com/AIMM/A328). Two NGS-based methods were used to increase the reliability of the results. Also, the genotyping was repeated in case of discordant results by 2 methods. Briefly, the first commercial method included amplification of specific regions and preparation of library using tagmentation (Nextera XT kit, Illumina). The second Fast method included multiplex amplification comprising IDH1 and IDH2 reactions with specific primers containing overhangs required for sequencing and this process is followed by amplicon purification. These primers ensures skipping the process of tagmentation and indexing resulting in substantial time-saving.

RESULTS

IHC

A total of 275 patient (mean age =60.2 y) samples were histologically evaluated by the pathologist according to the 2016 CNS WHO recommendations by the pathologist, therefrom 11 samples were unable to be IHC examined for IDH R132H (Table 1).2 The samples were then subdivided according to IHC R132H positivity (mutated) or negativity (WT). The data were stratified by tumor subtype; R132H immunoreactivity was observed in 60 of 275 samples (22%).

FISH

No generally accepted cut-off values suitable for analytical validation of 1p or 19q deletions detection in oligodendrogloma have been reported therefore we set the cut-off at 20% of nuclei harboring only 1 copy. FISH analysis indicated the presence of the 1p/19q co-deletion in 6 of 8 IHC R132H positive samples, one sample could not be repeatedly analyzed probably because of tissue processing error.
Sequencing of IHC IDH1 WT Patients Below 55 Years of Age

To maximize cost-effectiveness rising from low prevalence of IDH mutations in patients 55 years or above, only tumors from patients under the age of 55 with WT IDH1 according to IHC (n = 63) were sequenced (Table 2).

The sequencing results indicated that 10 of the 63 samples (16%) were either IDH1 R132 or IDH2 R172 mutated. The 2 sequencing methods gave consistent results for all 10 positive samples; 2 samples could not be successfully analyzed by at least 1 of the methods (Table 3). As expected, we detected rare mutations in IDH1 (R132S and R132C were present in 3 samples). In addition, 3 samples were found to carry rare IDH2 mutations in codon 172 that were not detectable by IHC. Surprisingly, 4 samples were genotyped as being IDH1 R132H positive even though the IHC data indicated that all samples chosen for sequencing contained only WT IDH. Two samples could not be analyzed because of low input DNA quality and poor PCR amplification of targeted regions. Furthermore, 1 sample of 11 that could not be IHC analyzed and fulfilled the age criteria was unequivocally signed as IDH R132H mutated (Table 3, case No. 8).

Quality Control of Sequencing Assay

The typical NGS assays consist of > 30 PCR cycles that include more than billion-fold amplification of targeted DNA segments followed by manipulation of amplicons, leading to concern for amplicon contamination and repeatability of results. In Table 4 we present the results analysis or different types of controls for fast IDH. To increase the contamination possibility, we ranked samples for processing using regular alternation pattern (wt or negative alternating with IDH1 R132H mutation positive) as shown in Table 4. For negative control we used heavily fragmented (< 100 bp) low amount of DNA (~1 ng/µL) isolated from blood of donor, or no template controls where no template was added, however, base calling and data processing were handled as usual. We did not observe any contamination as wt samples contained 0.17% of variant c.395G > A (n = 6), which is expected overall error rate caused by library preparation followed by Illumina based sequencing.24 For no template/fragmented DNA we observed on average less than one c.395G > A variant bearing read and 3 c.395G > A reads were observed at maximum (n = 12). This would translate in < 0.3% contamination when assuming 1000 or more reads are required for the processing. Also we observed high repeatability and reproducibility of c.395G > A containing sample. VAF was equal to 32.1% ± 0.6% (n = 8, average ± SD).

TABLE 1. Histologic Subtypes of Diffuse Gliomas Included in Our Single Center Study Conducted Between 2011 and 2017, Showing Subgroup Characteristics

| Diagnosis                  | Grade | N/Total (%) | M/F | Age Mean/Range (y) |
|---------------------------|-------|-------------|-----|--------------------|
| Oligodendroglioma II      |       | 1/10 (10%)  | 0/1 | 9.3                |
| 1p/19q co-deleted         |       | 0           | 0/1 | —                  |
| Anaplastic oligodendroglioma III |   | 4/12 (33.3%) | 2/2 | 54.1 (30.3-54.1)   |
| Diffuse astrocytoma II    |       | 17/40 (42.5%)| 10/7| 43.0 (22.8-76.1)   |
| Anaplastic astrocytoma III|       | 22/35 (62.9%)| 9/13| 64.6 (33.2-81.6)   |
| Glioblastoma IV           |       | 162/178 (91.0%)| 104/58| 61.8 (23.7-84.3)   |

| Diagnosis                  | Grade | N/Total (%) | M/F | Age Mean/Range (y) |
|---------------------------|-------|-------------|-----|--------------------|
| Oligodendroglioma II      |       | 9/10 (90%)  | 6/3 | 49.3 (33.1-73.0)   |
| 1p/19q co-deleted         |       | 6/8 (75%)   | 4/2 | 51.7 (34.3-73.0)   |
| Anaplastic oligodendroglioma III |   | 8/12 (66.7%)| 3/5 | 49.6 (33.1-68.7)   |
| Diffuse astrocytoma II    |       | 22/40 (55%) | 10/12| 42.3 (23.1-70.8)   |
| Anaplastic astrocytoma III|       | 9/35 (25.7%)| 5/4 | 40.0 (27.6-56.7)   |
| Glioblastoma IV           |       | 12/178 (6.7%)| 7/5 | 47.9 (33.8-75.2)   |

F indicates female; M, male; WT, wild type.

TABLE 2. Characteristics of Patients Aged 55 Years or Below Selected for Sequencing to Detect IDH Mutations in Codons 132 and 172

| Diagnosis                  | Grade | M/F | Age Mean/Range (y) | Mutated IDH Codons R132 and 172 /Total |
|---------------------------|-------|-----|--------------------|----------------------------------------|
| Oligodendroglioma II      | II    | 0/1 | 9                  | 0/1 (0%)                               |
| 1p/19q co-deleted         |       | 0   | —                  | —                                      |
| Anaplastic oligodendroglioma III |   | 1/2 | 46 (30-54)         | 2/3 (67%)                              |
| Diffuse astrocytoma II    | II    | 5/5 | 35 (23-53)         | 6/10 (60%)                             |
| Anaplastic astrocytoma III|       | 4/2 | 43 (33-54)         | 2/6 (33%)                              |
| Glioblastoma IV           | IV    | 26/17| 48 (24-55)         | 2/43 (5%)                              |

F indicates female; IDH, isocitrate dehydrogenase; M, male.
### TABLE 3. Patient Characteristics and Genotyping Results Obtained Using the FastIDH Method and the Nextera XT Library Prep Kit

| Case No. | Age (y) | Sex | Side | Location | Subtype | WHO Grade | IDH | IDH Nextera | IDH Fast | MAF, % | IDH | IDH Nextera | IDH Fast | MAF, % |
|----------|---------|-----|------|----------|---------|-----------|-----|-------------|---------|-------|-----|-------------|---------|-------|
| 1        | 53      | F   | Right | Frontal | Anaplastic oligodendroglioma | III | IDH2 | R172K | 56 | IDH2 | R172K | 48 |
| 2        | 30      | M   | Left  | Frontal | Diffuse (fibrillar) astrocytoma | II | IDH2 | R172M | 59 | IDH2 | R172M | 46 |
| 3        | 32      | M   | Left  | Frontal | Diffuse (fibrillar) astrocytoma | II | IDH1 | R132C | 14 | IDH1 | R132C | 13 |
| 4        | 33      | F   | Left  | Frontal | Anaplastic astrocytoma | III | IDH1 | R132C | 64 | IDH1 | R132C | 48 |
| 5        | 33      | F   | Left  | Frontal | Diffuse (fibrillar) astrocytoma | II | IDH1 | R132S | 35 | IDH1 | R132S | 34 |
| 6        | 33      | F   | Left  | Frontal | Diffuse (fibrillar) astrocytoma | II | IDH1 | R132H | 23 | IDH1 | R132H | 29 |
| 7        | 35      | M   | Left  | Temporal | Diffuse astrocytoma | II | IDH1 | R132H | 15 | IDH1 | R132H | 35 |
| 8        | 38      | M   | Left  | Temporal | Diffuse (gemistocytic) | II | IDH1 | R132H | 26 | IDH1 | R132H | 28 |
| 9        | 54      | M   | Left  | Temporal | Anaplastic oligoastrocytoma | III | IDH2 | R172K | 50 | IDH2 | R172K | 38 |
| 10       | 47      | F   | Right | Frontal | Diffuse astrocytoma | II | IDH1 | R132H | 15 | IDH1 | R132H | 15 |
| 11       | 45      | M   | Right | Frontal | Glioblastoma | IV | Not analyzable | Not analyzable |
| 12       | 53      | M   | Right | Frontal | Glioblastoma | IV | wt | Not analyzable | Not analyzable |

Nucleotide substitutions in our samples for IDH mutations: IDH1 R132H-c.395G>A; IDH1 R132C-c.394C>T, IDH1 R132S-c.394C>A, IDH2 R172M-c.515G>T, IDH2 R172K-c.515G>A.

F indicates female; IDH, isocitrate dehydrogenase 1; M, male; MAF, mutation allelic fraction in the sample; wt, wild type.

### TABLE 4. Quality Control for FastIDH Assay

| Run ID | Index | Sample | Result (If Available) | c.395A Count (R132H) | Total Read Count | VAF c.395G > A (R132H) |
|--------|-------|--------|-----------------------|----------------------|------------------|------------------------|
| FR124  | i30   | Neg. contr. fr. DNA | 0 | 0 | NA |
| FR124  | i31   | Positive control 1 | IDH1 | R132H | 1990 | 6078 | 32.7% |
| FR124  | i32   | Neg. contr. fr. DNA | 1 | 7 | NA |
| FR124  | i33   | Positive control 1 | IDH1 | R132H | 1695 | 5294 | 32.0% |
| FR124  | i35   | Neg. contr. fr. DNA | 0 | 0 | NA |
| FR124  | i36   | Positive control 1 | IDH1 | R132H | 1853 | 5663 | 32.7% |
| FR124  | i38   | WT control 1 | wt | 5 | 4258 | 0.1% |
| FR124  | i41   | Neg. contr. fr. DNA | 3 | 4 | NA |
| FR124  | i42   | Positive control 1 | wt | 2352 | 7490 | 31.4% |
| FR122  | i30   | WT control 2 | wt | 7 | 3569 | 0.2% |
| FR122  | i32   | Positive control 1 | IDH1 | R132H | 1290 | 3994 | 32.3% |
| FR122  | i36   | WT control 2 | wt | 3 | 1268 | 0.2% |
| FR122  | i38   | Positive control 1 | IDH1 | R132H | 1539 | 4829 | 31.9% |
| FR122  | i38   | WT control 2 | wt | 7 | 4761 | 0.1% |
| FR122  | i42   | Positive control 1 | IDH1 | R132H | 1651 | 5077 | 32.5% |
| FR122  | i31   | Neg. contr. no template | 0 | 15 | NA |
| FR122  | i33   | Neg. contr. no template | 0 | 0 | NA |
| FR122  | i35   | Neg. contr. no template | 0 | 0 | NA |
| FR122  | i30   | Neg. contr. no template | 0 | 5 | NA |
| FR122  | i31   | Neg. contr. no template | 0 | 7 | NA |
| FR122  | i32   | Neg. contr. no template | 0 | 0 | NA |
| FR122  | i33   | Neg. contr. no template | 0 | 1 | NA |
| FR122  | i35   | Neg. contr. no template | 0 | 0 | NA |
| FR122  | i36   | Positive control 2 | IDH1 | R132H | 5744 | 14602 | 39% |
| FR120  | i30   | WT control 3 | wt | 26 | 17907 | 0.1% |
| FR120  | i41   | WT control 4 | wt | 18 | 15300 | 0.1% |

Data from 3 independent runs were used.

For negative control with fragmented DNA we used ultrasound fragmented donor blood DNA with median fragment size <100 bp.

For no template control on DNA library was added to the sequencer in order to see the contamination based on index hopping and/or sequencer overflow.

DNA isolated from glial tumor tissue with known IDH1/2 status was used as positive control and WT control.

IDH indicates isocitrate dehydrogenase 1; NA, not analyzable; VAF, variant allele fraction; WT, wild type.
DISCUSSION

The integration of phenotypic and genotypic parameters in the classification of CNS tumors has improved diagnosis. The frequency of the IDH1 R132H variant is reported to be ~90% (it was 91% in our studied population), and this mutant can be detected by IHC.25 IHC detection of the IDH1 R132H variant was performed using the DIA-H09 antibody, for which the expected true positive rate is 88% to 99%. If we assume that all samples found to be IDH1 R132H positive by IHC would also be IDH1 R132H positive by sequencing, the concordance rate of the sequencing and IHC methods would be 94% (60/64). A number of other IDH1 variants are known, including R132C (whose frequency in glioma patients is reportedly around 3%; in the studied population, it was 3%), R132S (frequency in this work: 1%), and R132G and R132L (whose reported frequencies are both around 1%; neither was detected in this work). IDH2 variants are less common, with R172K being observed in 3% of glioma patients (3% in our study), R172M (1% in our study), R172W (none in our study) and R172S (none in our study) having frequencies of ~1% each.26 Our data agree with the previously reported stratification and frequency of IDH mutations in gliomas.

IDH1 status determination is crucial for diagnosis and selecting an appropriate treatment strategy. Typically, the first step in treating a glioma is to perform the safest radical resection that will provide enough tumor tissue for reliable diagnosis. Regardless of tumor grade, any glioma expressing wild type IDH should be regarded as a glioblastoma and treated with aggressive chemoradiotherapy according to the Stupp protocol. The treatment of gliomas expressing mutated variations of IDH should be guided by the presentation of clinical and molecular features. For radically resected low-grade tumors exhibiting both the 1p/19q co-deletion and an IDH mutation, one might even consider omitting oncotherapy altogether and simply recommend watchful follow-up.27

As noted above, accurate determination of IDH status is vital for selecting effective treatment strategies for diffuse glioma patients and thus for their prognosis. The financial burden of diagnosing gliomas is increasing because of the multitude and complexity of current laboratory methods. WHO recommends testing all IDH negative samples from patients below 55 years old, in our study it was 63 patients of 275 (23%), %). IHC was analytically false negative for IDH1 R132H in 4 specimens, and an additional 6 specimens had clinical false negative IHC by virtue of alternate IDH1/2 mutations. The data validate the notion that while IHC is the standard method for detecting IDH, genetic sequencing should also be used to confirm negative IHC results and minimize the risk of false negatives. Using 2 different molecular approaches, this work confirmed the high incidence of IDH1 variants in glioma patients below 55 years old.

The IHC determination of IDH1 R132H mutation failed in 11/275 (4%) samples included in our cohort. This failure might be caused by laboratory errors or possible inadequate tissue handling. Therefore, second step control incorporating sequencing is fundamental for crucial genes involved in the molecular-histologic definition of gliomas. This 2-step procedure could be applied also for other genes examined in gliomas like alpha-thalassemia/mental retardation syndrome X-linked (ATRX) or telomerase reverse transcriptase (TERT).

After recent classification of tumors of the CNS and the development of MPS techniques, it starts to be feasible to use a glioma-tailored customized gene panels for understanding the molecular background.28,29 In our work, we showed that precision medicine information is increased even by testing IDH1 and IDH2 genes only. Our homemade Fast IDH MPS method shows concordant results as Nextera XT based method. For library preparation FAST IDH includes just 1 step (PCR amplification with purification, ~3 h in total) in contrast to Nextera XT method (1. PCR amplification with purification, 2. tagmentation and 3. indexing PCR amplification with purification, ~7 h in total) and the likelihood of the technical error is and hands-on time thus reduced. Overall Fast IDH method provides higher cost efficiency because of faster sample processing and will be licenced to a company to be available world-wide. Because of the high cost of sequencing chemistry we used in our laboratory the smallest flow-cell (MiSEQ nano) with pricing 380 to 500 euros to achieve cost efficiency of the method. Usually in routine diagnostics 2 to 4 samples for IDH1/2 genotyping are sequenced in each run (once a week or every second week). Therefore it is necessary to combine IDH1/2 sequencing with other sequencing genotyping methods such as KRAS, NRAS, BRAF (colon cancer), EGFR and BRAF (lung cancer), or BRCA1/2 (ovarian cancer). When sequencing 16 samples, the cost sequencing is around 30 euros per sample for both methods. About 15 euros per sample (half of total costs) includes library preparation, work cost along with running costs. Library preparation using Nextera XT costs around 50 euro per sample. When the fastIDH kit is commercially available, expected costs per sample are 30 to 60 euros. Total cost of IDH1/2 sequencing would nowadays thus be about or even under 100 euro. This opens the question over the current validity of the model published by DeWitt in 2017 which is based on 1800 USD (~1500 euros) as NGS costs. Although it might sound controversial, we assume that nowadays even tumors of patients over 55 years should be analyzed by MPS and gradually NGS could replace IDH IHC entirely. This data suggests that more than two-thirds of costs are saved and less common mutations and false negatives are revealed with propriate efficacy. Limitation of our work is that we did not sequenced IHC wt samples of patients over 55 to see if there are any noncanonical or false negative samples.

CONCLUSION

The correct identification of diffuse gliomas is crucial for identifying appropriate tailored therapies. Gliomas are classified using the 2016 WHO system, which is based on the presence of validated biomarkers including IDH mutations and the 1p/19 co-deletion. We have shown that rare variants of IDH1/2 occur in patients with CNS tumors, corroborating...
previous findings. Our laboratory performs DNA sequencing of tumors identified as IDH1-negative by IHC in patients below 55 years old, and we recommend this approach to other laboratories interested in precise molecular diagnostics.

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