ORIGINAL RESEARCH ARTICLE

Glioma 2021 WHO Classification: The Superiority of NGS Over IHC in Routine Diagnostics

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Abstract

Introduction The accurate detection of genetic variants such as single substitutions (*IDH1/2*, *TERT*), chromosomal abnormalities (*CDKN2A*, 1p/19q deletions, and *EGFR* amplifications), or promoter methylations (*MGMT*) is critical for glioma patient management, as emphasized in the World Health Organization's (WHO's) most recent classification in 2021 (WHO CNS5). The purpose of this study was to evaluate novel innovative methods for determining *IDH1/2* status in the context of WHO CNS5.

Methods Multiple biomarkers were simultaneously screened using next-generation sequencing (NGS) on 34 glioma samples. In cases where the *IDH1/2* status determined by immunohistochemistry (IHC) or multiplex ligation-dependent probe amplification (MLPA) was inconsistent with the NGS results, quantitative polymerase chain reaction (qPCR) and Sanger sequencing were performed to resolve the adjudicated discrepancy.
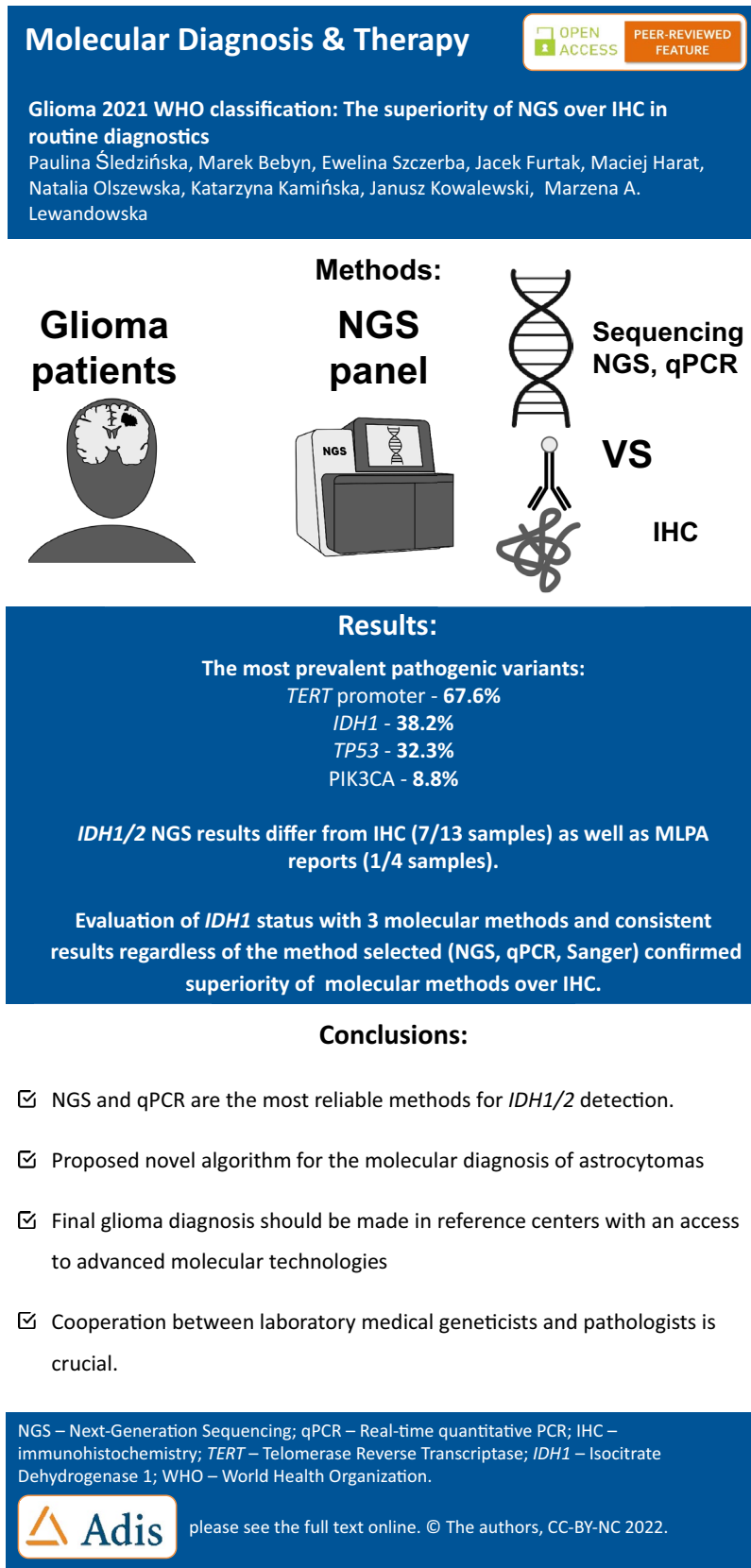
Results *IDH1/2* NGS results differ from IHC (7/13 samples) as well as MLPA reports (1/4 samples). All NGS findings were confirmed by qPCR and Sanger sequencing. WHO CNS5 requires assessment of multiple mutations for glioma classification.

Conclusions We demonstrated that qPCR or NGS performed in reference genetic laboratories, rather than IHC, is the most reliable method for *IDH1/2* analysis. Clinicians should be aware of discrepancies in MLPA or IHC results and seek reconsultation in facilities with extensive access to advanced molecular technologies. Moreover, we proposed a new algorithm for the molecular diagnostic procedures in glioma patients based on the WHO CNS5.

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Graphical abstract

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Glioma 2021 WHO classification: The superiority of NGS over IHC in routine diagnostics

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Methods:

Glioma patients

NGS panel

Sequencing NGS, qPCR

VS

IHC

Results:

The most prevalent pathogenic variants:
TERT promoter - **67.6%**
IDH1 - **38.2%**
TP53 - **32.3%**
PIK3CA - **8.8%**

IDH1/2 NGS results differ from IHC (7/13 samples) as well as MLPA reports (1/4 samples).

Evaluation of *IDH1* status with 3 molecular methods and consistent results regardless of the method selected (NGS, qPCR, Sanger) confirmed superiority of molecular methods over IHC.

Conclusions:

☑ NGS and qPCR are the most reliable methods for *IDH1/2* detection.

☑ Proposed novel algorithm for the molecular diagnosis of astrocytomas

☑ Final glioma diagnosis should be made in reference centers with an access to advanced molecular technologies

☑ Cooperation between laboratory medical geneticists and pathologists is crucial.

NGS – Next-Generation Sequencing; qPCR – Real-time quantitative PCR; IHC – immunohistochemistry; *TERT* – Telomerase Reverse Transcriptase; *IDH1* – Isocitrate Dehydrogenase 1; WHO – World Health Organization.

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Key Points

Next-generation sequencing (NGS) and quantitative polymerase chain reaction (qPCR) are the most reliable methods for *IDH1/2* detection.

We propose a novel algorithm for the molecular diagnosis of astrocytomas.

Final glioma diagnosis should be made in reference centers with access to advanced molecular technologies.

1 Introduction

Gliomas are the most common primary central nervous system (CNS) tumors, with an incidence of 1.9–9.6 per 100,000, depending on age, sex, ethnicity, and geographic location [1, 2]. The 2021 World Health Organization Classification of Tumors of the Central Nervous System (WHO CNS5) has fundamentally changed the classification of gliomas and incorporated many molecular biomarkers [3]. In this classification system, the primary genetic markers for gliomas are *IDH1/2* mutation status, 1p/19q co-deletion, *H3F3A* alterations, *ATRX* gene mutations, *MGMT* promoter methylation status, loss of *CDKN2A*, and *EGFR* amplification, a combined gain of chromosome 7 and loss of chromosome 10, and *TERT* promoter pathogenic variants. In the new classification, there are three genetic parameters, *TERT* promoter mutation, *EGFR* gene amplification, or combined gain of entire chromosome 7 and loss of entire chromosome 10 [+7/−10]), that, in the case of IDH-wildtype diffuse astrocytic tumor in adults, change the diagnosis to glioblastoma (see Fig. 1) [3, 4].

Depending on the genetic aberration, numerous molecular methods can be used, such as fluorescence in situ hybridization (FISH), quantitative polymerase chain reaction (qPCR) including methylation-specific polymerase chain reaction (MS-PCR), and next-generation sequencing (NGS) extended with copy number variation (CNV) analysis. The resolution, sensitivity, and specificity of molecular techniques vary. The limit of detection (LOD) for NGS and qPCR depends on the workflow and type of targeted genomic change. Usually, the detection limit (LOD) varies between 1% and 5% for qPCR and 5% and 10% for NGS; however, the LOD for qPCR- and NGS-based analyses is not consistently defined or determined and usually varies between 1% and 10% and 2% and 15%, respectively [5]. The LOD for NGS and qPCR depends on the workflow and type of targeted genomic

change. Sanger sequencing methodology allows the detection of variants at a frequency of 15–20% and above [6]. It is critical to choose an appropriate approach for detecting genetic changes and to conduct molecular analysis in an oncological reference center. The use of monoclonal antibodies specific to the mutant protein enabled immunohistochemistry (IHC) to be incorporated in standard *IDH1/2* point mutation screening protocols [7]. Even though the cost of analyzing a single sample is significantly higher for NGS than for other methods, it is NGS that enables comprehensive analysis, and thus methods such as FISH, qPCR, Sanger sequencing, or mutant-specific IHC will be replaced in the near future in favor of NGS extended for CNV [8–10].

Taking into account the latest diagnostic recommendations and the growing number and significance of genetic markers in clinical management, the purpose of this study was to consider NGS as the major method that allows for the analysis of many genetic biomarkers in glioma in one simultaneous step. In addition, we compare NGS and IHC methods for determining *IDH1/2* status in the context of WHO CNS5.

2 Materials and Methods

2.1 Patient Selection

A retrospective analysis of the medical records of 86 patients diagnosed with gliomas referred to the Oncology Center in Bydgoszcz between January 2017 and June 2021 was conducted (Table S1, see the electronic supplementary material). Histopathological evaluation and IHC were performed in non-reference, non-oncology centers with an anatomical pathology unit, however, without access to advanced technologies such as NGS and without significant exposures to genetic analysis of glioma. Whereas, data such as age, gender, prior radiotherapy and chemotherapy, as well as the results of genetic tests come from the oncology reference center. NGS was performed on 34 patients' samples. The inclusion criteria for NGS examination were as follows: patients diagnosed with glioma; biological archive material (the same used for histopathological evaluation) that was of adequate quality for analysis. Exclusion criteria were as follows: a lack of biological material or double stranded DNA (dsDNA) not of sufficient quality and quantity for NGS analysis. From 34 gliomas that were tested using NGS, 17 had previous *IDH1/2* status assessed in non-oncological centers using IHC or multiplex ligation-dependent probe amplification (MLPA) (Fig. 2).

The research evaluated surgical materials diagnosed in 12 different facilities with varying levels of IHC and genetic testing availability. The Ethics Committee of the Nicolaus
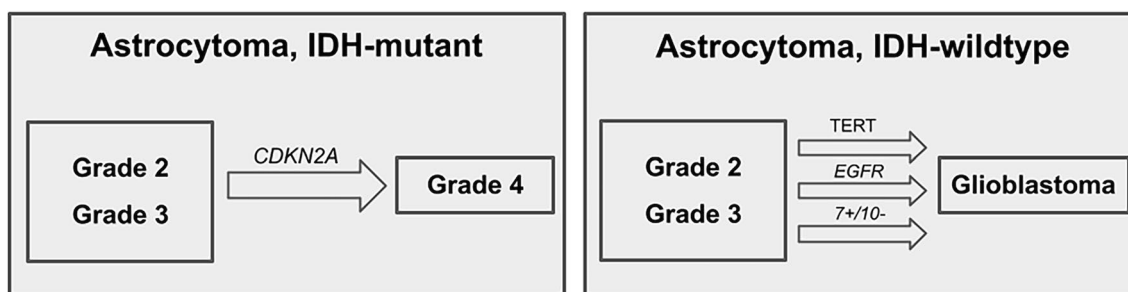
Fig. 1 Astrocytoma grading based on genetic alterations according to WHO CNS5 2021. Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) homozygous deletion, in IDH-mutant astrocytomas, is a marker of the highest malignancy grade. In IDH-wildtype diffuse astrocytic gliomas, the presence of one or more of three genetic parameters (*EGFR* gene amplification, *TERT* promoter pathogenic variants, or a combination of chromosome 7 gain and 10 loss [+ 7/− 10]) obligate to assign the highest CNS WHO grade and classify them as glioblastoma. Glioblastoma, IDH-mutant, does not exist in the current classification. *WHO CNS5 2021* the fifth edition of the WHO Classification of Tumors of the Central Nervous System, *TERT* human telomerase reverse transcriptase, *WHO* World Health Organization

Copernicus University, Collegium Medicum in Bydgoszcz, Poland, approved the study protocol (KB 265/2012).

2.2 DNA Isolation

The percentage of tumor cells in material qualified by a pathologist ranged from 5 to 95%. DNA was isolated from brain cancer tissue fixed in formalin and embedded in paraffin (FFPE) using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The initial concentration and quality of DNA were measured using NanoDrop1000 (Thermo Scientific, Waltham, MA, USA).

2.3 DNA Quality Assessment

The quality and quantity of DNA were evaluated using real-time polymerase chain reaction (PCR) with Fragmentation Quantification Assay (FQA) CE-IVD (EntroGen) or/and by fluorometric methods using Quantus (Promega) with QuantiFluor® dsDNA System (Promega). The FQA assay uses three amplicon sizes (37, 150, 301 bp) of a reference gene and five standards to calculate the absolute copy number of amplifiable DNA and determine sample integrity through the fragmentation ratio (*F*-ratio). The two *F*-ratios, 150 bp/37 bp and 301 bp/37 bp, are indicative of sample quality and help predict the optimal amount of input sample for downstream assays. Archive FFPE material was found to be significantly degraded, but the lack of other biological material forced us to use DNA with an *F*-ratio of < 0.5, below the manufacturer's recommendations.

2.4 Library Preparation and NGS

Libraries for NGS were prepared according to the manufacturer's instructions using a 16-gene panel, the EntroGen NGS Targeted Hotspot Panel (CE-IVD). It includes hotspot exons for genes: *BRAF*, *EGFR*, *ERBB2*, *HRAS*, *C-KIT*, *IDH1*, *IDH2*, *JAK2*, *KRAS*, *MET*, *NRAS*, *PDGFRA*, *PIK3CA*, *RET*, the promoter region of *TERT*, and the entire exome of *TP53*. The libraries were quantified using the Quanti-Fluor dsDNA System (Promega) on Quantus (Promega), denatured, and diluted to 5 pM together with the control library PhiX. Sequencing was performed on a MiniSeq platform (Illumina) using the MiniSeq Mid Output Kit, 2 × 150 cycles, to obtain reads for both strands. Sequencing results were analyzed using EntroGen Variant Reporter v1.3 and Illumina Variant Studio 3.0 [11]. Single nucleotide variant (SNV) pathogenic alterations detected by NGS are listed in the supplementary data: Table S2 (see the electronic supplementary material).

2.5 NGS *IDH1/2* Validation by Two Independent Methods, qPCR and Sanger Sequencing

In all cases where the *IDH1/2* status was determined by IHC or MLPA, qPCR and Sanger sequencing were performed to resolve adjudicated discrepancies between different methods. *IDH1/2* genotyping was validated using allele-specific qPCR (IDH-RT38, EntroGen) and Sanger resequencing. For qPCR analysis, the *IDH1/2* Mutation Analysis Kit (Entro-Gen) was used, which is a screening test for the detection of the most prevalent mutations in codons 100 and 132 of the *IDH1* gene and in codons 140 and 172 of the *IDH2* gene. Sanger sequencing was performed using AmpliTaq Gold, Fast PCR Master Mix (ThermoScientific, USA), ExoSAP-IT™ (ThermoScientific, USA), BigDye™ Terminator v3.1 Cycle Sequencing Kit (ThermoScientific, USA), BigDye XTerminator™ Purification Kit (ThermoScientific, USA), and sequencing primers (forward: 5′ TCA CCA AAT GGC ACC ATA CGA 3′; reverse: 5′ TGT GTT GAG ATG GAC
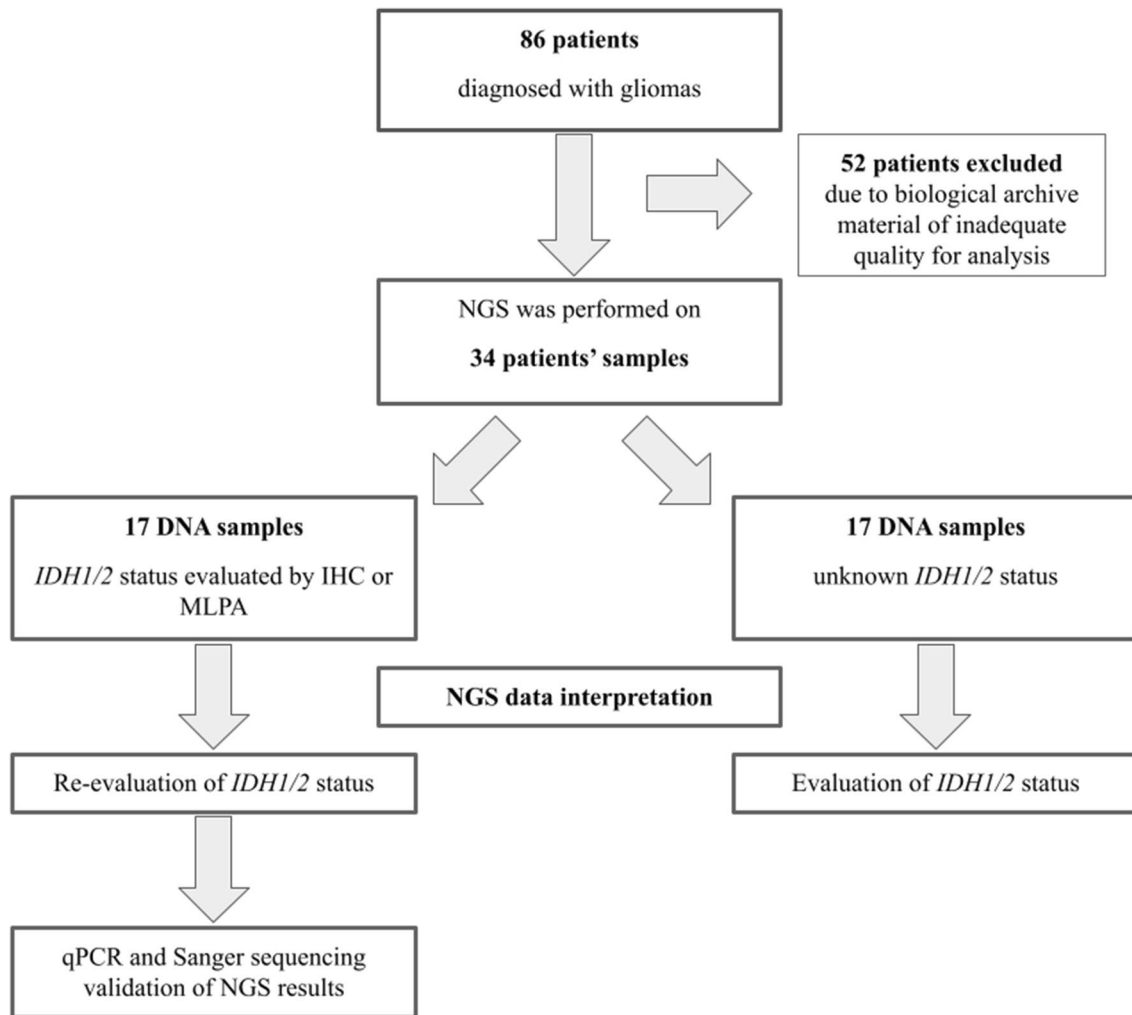
Fig. 2 Flow chart of the patient group. *IHC* immunohistochemistry, *MLPA* multiplex ligation-dependent probe amplification, *NGS* next-generation sequencing, *qPCR* quantitative polymerase chain reaction

GCC TAT TTG TA 3′), receiving a 353-bp amplicon size sequenced on SeqStudio Genetic Analyzer (ThermoScientific, USA). Verified and validated pathogenic variants were used for subsequent WHO CNS5 classification.

2.6 Assessment of *CDKN2A* Homozygous Deletion

The biallelic deletion of the *CDKN2A* gene was performed using the Vysis Paraffin Pretreatment Reagent Kit (Abbott Molecular) according to the manufacturer's protocol described before [12]. In hybridization, applied Vysis CDKN2A/CEP 9 FISH Probe Kit (Abbott Molecular). Assessment of copy number variants to evaluate the presence or absence of *CDKN2A* homozygous deletion through NGS was performed on Nextseq550 using SOLIDaccuTest CE-IVD (NGeneBio Co) according to the manufacturer's protocol.

3 Results

3.1 Frequency of Pathogenic and Likely Pathogenic Variants

We performed NGS analysis using DNA isolated from 34 glioma patients. The *TERT* promoter pathogenic variants were the most prevalent (found in 23/34, 67.6% of patients), with 19 out of 23 patients having NC_000005.9:g.1295228G>A (commonly called C228T) and four out of 23 having NC_000005.9:g.1295250G>A (C250T). C250T and C228T variants were mutually exclusive (Table S2, see the electronic supplementary material). The highest rate of *TERT* promoter pathogenic variants was observed in oligodendroglioma (80%, 4/5 tested patients), followed by glioblastoma (72%, 13/18 patients). The *IDH1/2* pathogenic variants were the second most prevalent in our sample (present in 13/34, 38.2% of patients), followed by the *TP53* pathogenic variants

(11/34, 32.3% of patients) and *PIK3CA* pathogenic variants (3/34, 8.8% of patients) (see Fig. 3). Figure 4 shows the coexistence of pathogenic variants. We can note that the pathogenic variants in *EGFR* did not occur in any case with the pathogenic variants in *IDH1/2*. In addition, pathogenic variants in *IDH1/2* often coexisted with pathogenic variants in *TP53*. Examples of the most common pathogenic variants detected by NGS can be found in Fig. 5.

3.2 Comparison of IHC or MLPA Results with NGS, qPCR, and Sanger Sequencing for *IDH1/2* Status Assessment

To determine the optimal method for assessing *IDH1/2* status, NGS findings acquired at the reference F. Łukaszczyk Oncology Center were compared to IHC or MLPA reports obtained at 12 different hospitals in Poland, none of which were oncology reference centers. The status of *IDH1/2* was previously assessed using IHC in 13 cases and MLPA in four. The same FFPE material used for IHC or MLPA testing at other hospitals was used for NGS analysis (EntroGen NGS Targeted Hotspot Panel, CE-IVD) at the oncology reference center.

According to IHC reports from a non-oncological center, four samples were classified as IDH-wildtype, while NGS results revealed pathogenic variants. Moreover, three samples were classified as IDH-mutant, but no pathogenic variants were detected during the NGS examination (Table 1). Additionally, the reports did not specify the kind of antibody

that was used in the IHC tests, nor whether they were CE-IVD antibodies. On the other hand, in the MLPA reports, there was one out of four findings, which was not consistent with the NGS. In this case, the IDH-wildtype result was obtained using MPLA, whereas NGS revealed the presence of the pathogenic variant not tested by MLPA.

Due to the fact that *IDH1/2* NGS results differ from IHC (7/13 samples) as well as MLPA reports (1/4 samples), NGS results were validated using two independent methods: qPCR and Sanger sequencing. Firstly, the *IDH1/2* Mutation Detection Kit (Entrogen CE-IVD), which allows the detection of eight *IDH1* and nine *IDH2* variants, was used. Secondly, the Sanger sequencing was conducted using the same set of primers [7], but a newer SeqStudio Genetic Analyzer. A direct comparison of NGS, qPCR, and Sanger sequencing with 5, 1, and 20% LOD, respectively, revealed 100% concordance, 100% specificity, and 100% sensitivity for the 34 (qPCR) and 31 (Sanger sequencing) samples analyzed in the oncology reference center by laboratory medical genetics specialists. However, we were unable to confirm the status of *IDH1/2* with Sanger sequencing of three samples due to a lack of amplicons, as DNA was significantly degraded and quality was not sufficient for Sanger sequencing. However, the same three samples passed internal controls for qPCR and those qPCR final results were consistent with NGS analysis.

Moreover, we compared four available MLPA reports to our NGS, qPCR, and Sanger sequencing results. Due to the limited scope of the tested mutations by MLPA (R132C,
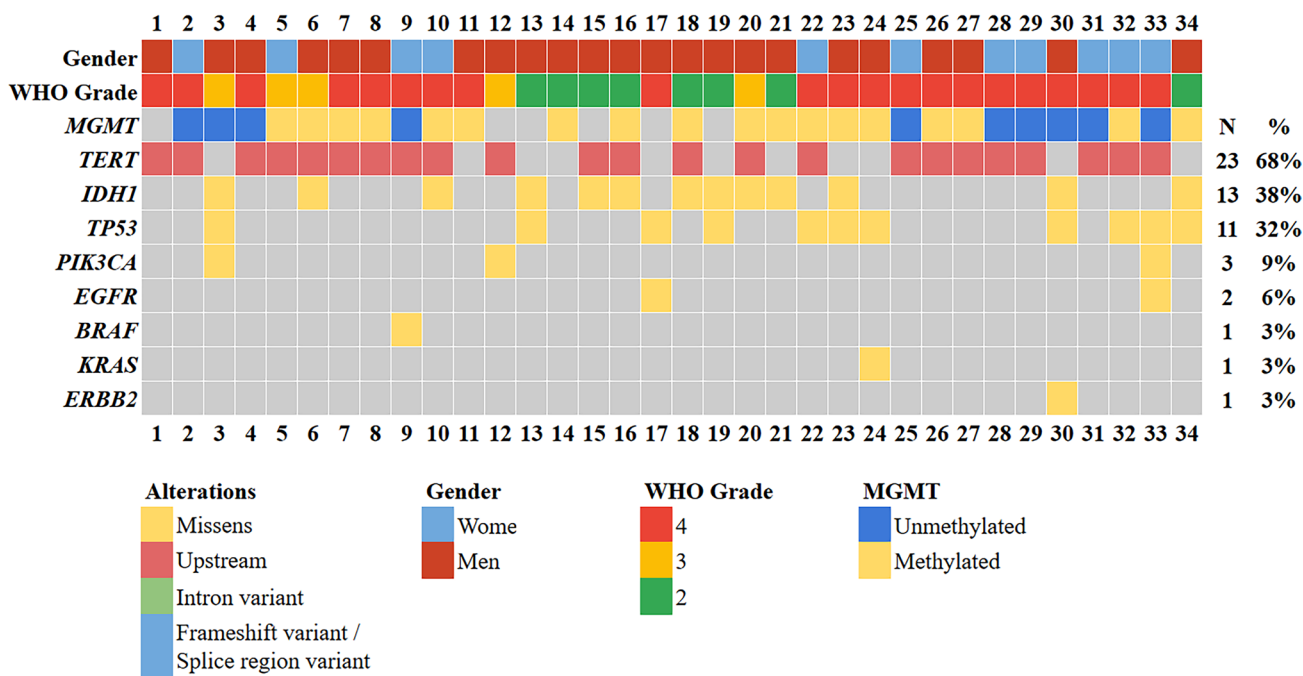

Fig. 3 The genetic landscape of gliomas and the clinical features of patients. *WHO* World Health Organization
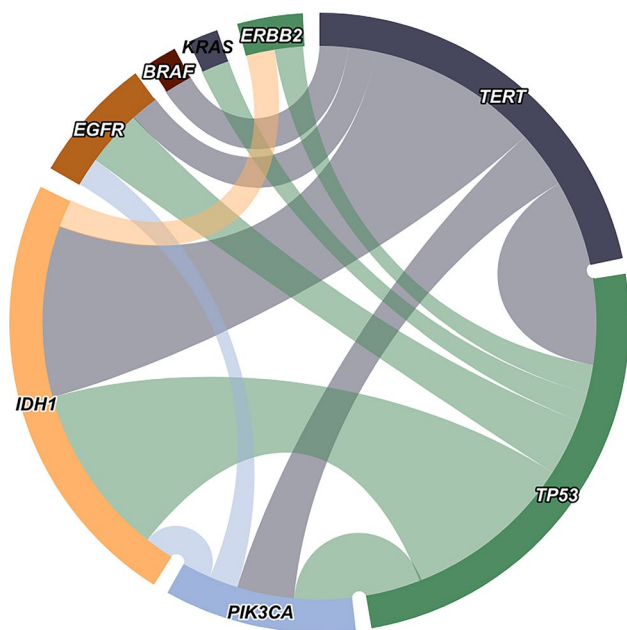
Fig. 4 The dependency wheel of the patients' mutations

R132H, R172K, and R172M), one of four was a false-negative result, as the R132L variant was not included in the commercial test to be detected by MLPA.

The number of *IDH1/2* variants identified by the MLPA detection kit is limited, resulting in a concordance with NGS of 75%, a sensitivity of 66.6%, and a specificity of 100%. However, those results are not representative, because of the limited number of MLPA samples (only four cases; Table 2).

Unfortunately and surprisingly, for IHC reports, we observed a concordance with NGS of only 38.5%, with a sensitivity as low as 33.3% and a specificity as low as 42.9%. Due to the limited study sample and the fact that the reports came from five different facilities, estimated sensitivity and specificity values do not adequately reflect the IHC method's reliability (Fig. 6).

3.3 Reevaluation of Diagnosis Based on WHO CNS5

Taking into account the growing significance of genetic markers and WHO CNS5 diagnostic recommendations, NGS and FISH, if needed, were conducted to reevaluate the diagnosis.

TERT promoter pathogenic variants were identified in all (3/3) patients with WHO grade 3 IDH-wildtype glioma using NGS. The new WHO CNS5 classification recommends classifying these patients as grade 4 glioma rather than grade 3 (see Table 3). *CDKN2A* analysis was performed on samples derived from patients diagnosed with astrocytoma grade 2 (A2) or A3 who had *IDH1/2* pathogenic variants.

Due to the fact that neither of the two tested samples indicated a homozygous deletion, no adjustment in classification was necessary in the analyzed group of glioma patients (Fig. 7). Changes from WHO grade 2 to grade 4 are crucial in terms of treatment and follow-up strategies. However, we were not able to provide an example of IDH-wildtype and *TERT* promoter mutated grade 2 glioma in our group.

4 Discussion

4.1 The Superiority of the NGS Method over the IHC or MLPA

IDH1/2 status can be assessed in multiple ways. Ten years ago, our group examined the prognostic value of *IDH1/2* using various techniques, including IHC and molecular genetic testing (PCR, Sanger) [7]. Over the years, the majority of laboratories have implemented IHC, pyrosequencing, qPCR, or MLPA as routine glioma diagnosis procedures. In this study, we used NGS for detecting all known and novel variations and compared it to technologies that rely on IHC, Taqman-specific probes (qPCR), hybridization (MLPA), and Sanger sequencing. While evaluating final reports from non-reference non-oncological centers where *IDH* status was obtained (IHC or MLPA), we identified clinically substantial discordance that had an impact on WHO classification and treatment. Following the NGS analysis, we observed differences in the *IDH1/2* status in seven out of 13 and one out of four of the patients determined by IHC and MLPA, respectively. To better understand how this discrepancy may affect clinical decisions, qPCR and Sanger sequencing were performed, and all NGS-based diagnoses were confirmed.

It is vital to realize that non-canonical *IDH* mutations occur at an 80% incidence in infratentorial astrocytomas, compared to 10% in supratentorial astrocytomas [13]. As a result, procedures such as IHC, MLPA, or qPCR may be employed as screening tests, with negative results requiring re-evaluation by NGS. In fact, in our study, there was a patient who had a non-canonical mutation; therefore, no mutation was found by IHC.

We found that the *IDH1/2* status of seven out of 13 IHC results obtained in separate non-reference non-oncological centers was incorrectly classified. While the stereotactic biopsy is frequently used to diagnose brain tumors, only a tiny amount of material is collected. This could interfere with the IHC assessment, leading to false-negative results [14]. Moreover, preanalytical standardization of human tissues (both surgery or stereotactic biopsy), as well as histopathological staining and IHC processes, is extremely difficult to implement in hospitals [15]. Therefore, artifacts in IHC findings and challenges in tissue fixation and embedding decrease the sensitivity of the IHC test and make it
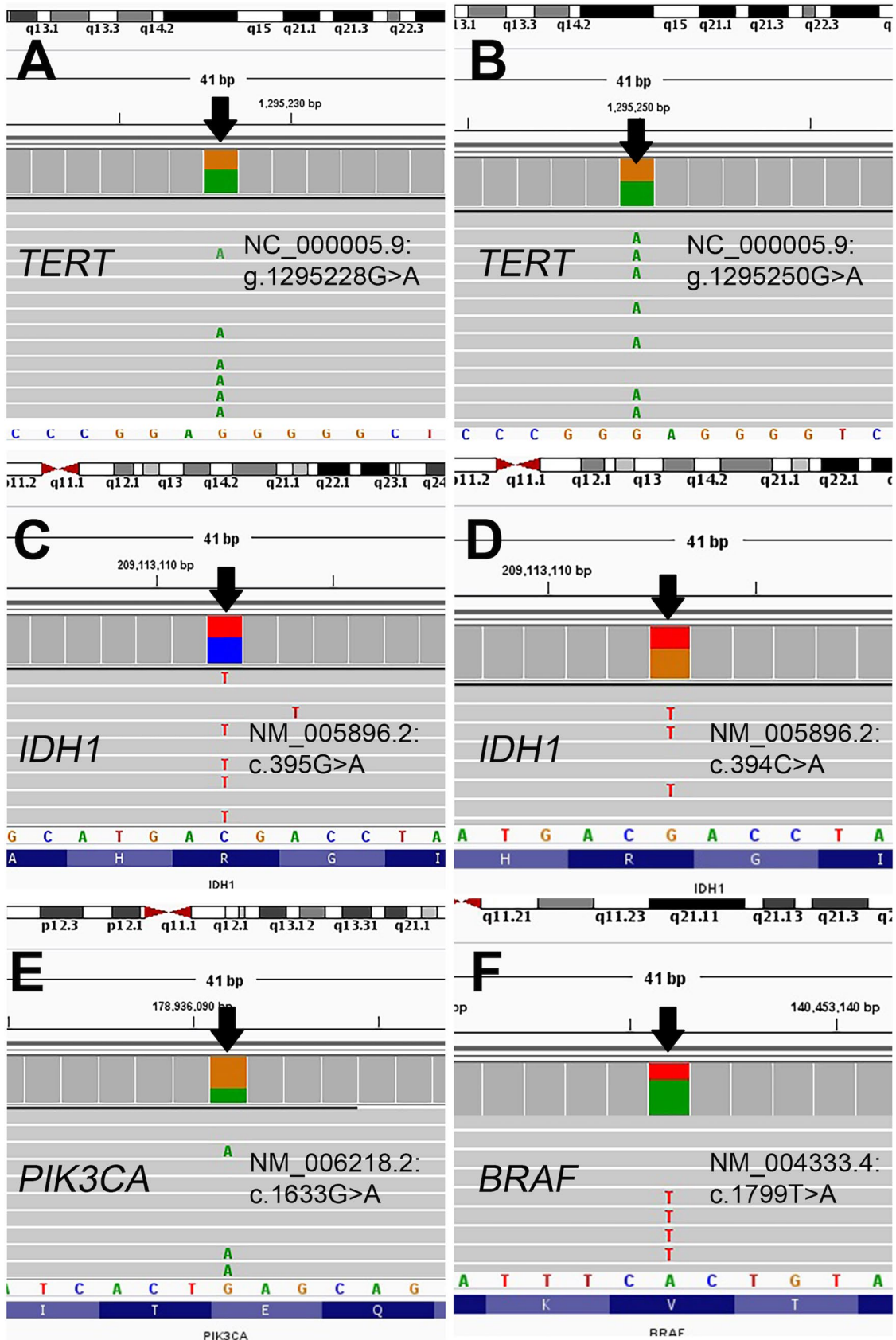
◄**Fig. 5** Examples of the most common pathogenic variants detected by NGS. The *black arrows* show the position of the detected variant. Abnormalities were assessed using TIER 2017 Variant Classification and AMCG 2015. **A** *TERT* promoter upstream gene variant NC_000005.9: g.1295228G>A TIER I; pathogenic. **B** *TERT* promoter upstream gene variant NC_000005.9:g.1295250G>A TIER I; pathogenic. **C** *IDH1* R132H missense variant NM_005896.2:c.395G>A (p.Arg132His) TIER I. **D** *IDH1* R132S missense variant NM_005896.2:c.394C>A (p.Arg132Ser) TIER II; Likely pathogenic. **E** *PIK3CA* missense variant NM_006218.2:c.1633G>A (p.Glu545Lys) TIER I; pathogenic. **F** *BRAF* missense variant NM_004333.4:c.1799T>A (p.Val600Glu) TIER I; pathogenic. *NGS* next-generation sequencing

more difficult to interpret the data unequivocally [16]. In contrast, during NGS, Sanger sequencing, and qPCR, DNA is amplified, enabling the detection of alterations in small tumor specimens. It could explain the inconsistent results in three cases where IHC identified them as IDH-wildtype. However, NGS and qPCR detected mutations in the *IDH1/2* genes.

Taking into account the current 2021 WHO classification, which requires the analysis of multiple genetic alterations, clinical practice should be based on high-throughput technologies such as NGS in glioma management instead of time-consuming cascade diagnostics. Moreover, it is possible that the total cost of different routinely used techniques such as IHC, Sanger sequencing, PCR, or FISH (to evaluate a broad range of somatic *IDH1/2*, *TERT*, *EGFR*, *CDKN2A*, and 1p19q variants) will be more costly than single NGS analysis. On the other hand, implementing NGS in some clinical molecular diagnostic laboratories may be challenging and is unlikely to be accessible for smaller pathology laboratories in general [17]. Therefore, we recommend that a complete glioma diagnosis should be performed using NGS in molecular genetics laboratories located in reference oncological centers and supervised by experienced specialists in laboratory medical genetics. Collaboration between experts in neurosurgery, medical genetics, and pathomorphology is essential in the context of the WHO 2021 classification. Focusing on the strengths and minimizing the disadvantages is the best strategy for any individual specialist.

4.2 Integrated Interdisciplinary Diagnostics Algorithm in Adult Astrocytoma Patients

Advanced molecular techniques have developed significantly over the years. Therefore, molecular parameters have been added for more precise glioma grading and prognosis estimation. In 2016, the WHO CNS classification for the first time used molecular markers to classify gliomas, and in 2021, placed even more emphasis on them [3, 18]. Due to the fact that WHO CNS5 was published relatively recently, there is no standard for the molecular diagnosis of astrocytomas in the literature. In response to this need, we propose the following NGS evidence-based algorithm (Fig. 8).

The first step in diagnosing a brain tumor is to obtain reliable tissue material during surgery. When microsurgical excision is not feasible, stereotactic biopsy should be performed due to its safeness, effectiveness, and high concordance with open surgical specimens in acquiring diagnostic material. In the era of the WHO CNS5 2021 classification, collecting representative samples for histological and molecular investigations is essential [19, 20]. Next, the *IDH* gene status assessment seems to be a crucial step in the management of a patient with astrocytoma. Based on the *IDH1/2* genetic analysis and histopathological findings, the direction of further molecular diagnostics should be set. The diagnostics of patients with astrocytoma should begin with a histopathological grade assessment, especially to differentiate grade 4 gliomas (in the presence of necrosis and microvascular proliferation) from grade 2 or 3. In the case of grade 2 or 3 astrocytoma, we propose performing NGS as the first method of choice. Analysis should include as broad a range of glioma biomarkers as possible. The NGS panel must include *IDH1/2*, and *TERT* and additional analyses of *PIK3CA* and *TP53* are welcome but not obligatory. We also propose the evaluation of CNV in *CDKN2A*, *EGFR*, and +7/−10 chromosomes by NGS analysis. In cases where NGS is chosen without a CNV panel, genetic evaluation of IDH-mutant astrocytoma must be followed by FISH to determine the presence of *CDKN2A* homozygous deletion or *EGFR* amplification.

On the other hand, in IDH-wildtype astrocytoma, further management depends on the presence of *TERT* promoter pathogenic variants and *EGFR* amplification. Therefore, simultaneous NGS analysis of *IDH1/2* and *TERT* promoter together with *EGFR* amplification allows quick, proper WHO CNS5 2021 classification. If *TERT* or *EGFR* alteration was found in previously classified IDH-wildtype astrocytoma, then glioblastoma IDH-wildtype should be diagnosed. In contrast, the absence of *TERT* promoter pathogenic variants and *EGFR* amplification obliges analysis of the presence of + 7/− 10 chromosomal abnormalities, which can be tested during the first NGS analysis with CNV or separately by FISH. While the existence of the combination of aberrations allows for the diagnosis of IDH-wildtype glioblastoma, the absence of + 7/− 10 obliges the diagnosis of IDH-wildtype astrocytoma. In the case of grade 4 astrocytoma, it is sufficient to determine only the presence of an *IDH* gene mutation. If the mutation is identified, WHO CNS5 recommends classifying these tumors as IDH-mutant grade 4 astrocytoma. However, in the absence of the mutation, it is required to diagnose IDH-wildtype glioblastoma.

Last but not least, additional prognostic and predictive biomarkers, such as the assessment of *MGMT* methylation can be performed, especially in glioblastoma IDH-wildtype

Table 1 Evaluation of *IDH1* status obtained by non-reference center (IHC), with 3 molecular methods (NGS, qPCR, Sanger) obtained by reference oncology center

Patient ID	Non-reference center		Reference oncology center					
	Histopathological diagnosis	*IDH1* IHC report	*IDH1* status NGS	First verification qPCR[a]	Second Verification Sanger	Protein change	Concordance in *IDH1* status	Allele frequency
8	GBM	Mutant[b]	wt	wt	wt	p=	No	
18	O2	wt[b]	Pathogenic	Mutant	Mutant	R132H[d]	No	42.49%
20	O3	wt[b]	Pathogenic	Mutant	Mutant	R132H[d]	No	48.48%
23	GBM	wt[c]	Pathogenic	Mutant	Mutant	R132S[e]	No	43.48%
27	A4	Mutant[b]	wt	wt	wt	p=	No	
28	A4	Mutant[b]	wt	wt	wt	p=	No	
30	GBM	wt[b]	Pathogenic	Mutant	NA	R132H[d]	No	38.62%
31	A4	Mutant[b]	Pathogenic	Mutant	NA	R132H[d]	Yes	39.23%
7	GBM	wt[b]	wt	wt	wt	p=	Yes	
11	GBM	wt[b]	wt	wt	wt	p=	Yes	
14	O2	wt[b]	wt	wt	wt	p=	Yes	
15	O2	Mutant[b]	Pathogenic	Mutant	Mutant	R132H[d]	Yes	42.19%
21	A2	Mutant[b]	Pathogenic	Mutant	NA	R132H[d]	Yes	21.96%

A2/4 astrocytoma grade 2/4, *GBM* glioblastoma, *IDH1* isocitrate dehydrogenase 1, *IHC* immunohistochemistry, *NA* not available (lack of results of Sanger sequencing due to DNA degradation), *NGS* next-generation sequencing, *O2/3* oligodendroglioma grade 2/3, *p=* no protein change has been detected in analyzed sample, *qPCR* quantitative polymerase chain reaction, *wt* wildtype

[a]*IDH1/2* Mutation Analysis Kit (EntroGen) is a screening qPCR test for the detection of the most common mutations in codon 132 and 100 of the *IDH1* gene and in codon 172 and 140 of the *IDH2* gene. *IDH1*: codon 132 (the test cannot distinguish between the following mutations: c.395G>A; c.394C>T; c.394C>A; c.394C>G; c.395G>T; c.395G>C; c.394_395delinsGT); codon 100 (c.299G>A)

[b]Unspecified IHC method

[c]IHC detecting the presence of a change only in R132H

[d]NM_005896.2:c.395G>A

[e]NM_005896.2:c.394C>A

Table 2 Evaluation of *IDH1* status received from non-reference center (MLPA), with 3 molecular methods (NGS, qPCR, Sanger) received from reference oncology center

Patient ID	Non-reference center		Reference oncology center				
	Histopathological diagnosis	*IDH1* status MLPA	*IDH1* status NGS	First verification qPCR[a]	Second verification Sanger	Protein change	Concordance in *IDH1* status
12	A3	wt	wt	wt	wt	p=	Yes
13	A2	wt	Pathogenic	Mutant	Mutant	R132L[b]	No*
19	A2	Mutant	Pathogenic	Mutant	Mutant	R132H[c]	Yes
34	A2	Mutant	Pathogenic	Mutant	Mutant	R132H[c]	Yes

A2/3 astrocytoma grade 2/3, *IDH1* isocitrate dehydrogenase 1, *MLPA* multiplex ligation-dependent probe amplification to detect 4 mutant variants (R132C, R132H, R172K, R172M), *NGS* next-generation sequencing, *p=* no protein change has been detected in analyzed sample, *qPCR* quantitative polymerase chain reaction, *wt* wildtype

[a]*IDH1/2* Mutation Analysis Kit (EntroGen) is a screening qPCR test for the detection of the most common mutations in codon 132 and 100 of the *IDH1* gene and in codon 172 and 140 of the *IDH2* gene. *IDH1*: Codon 132 (the test cannot distinguish between the following mutations: c.395G>A; c.394C>T; c.394C>A; c.394C>G; c.395G>T; c.395G>C; c.394_395delinsGT); codon 100 (c.299G>A)

[b]NM_005896.2:c.395G>T

[c]NM_005896.2:c.395G>A

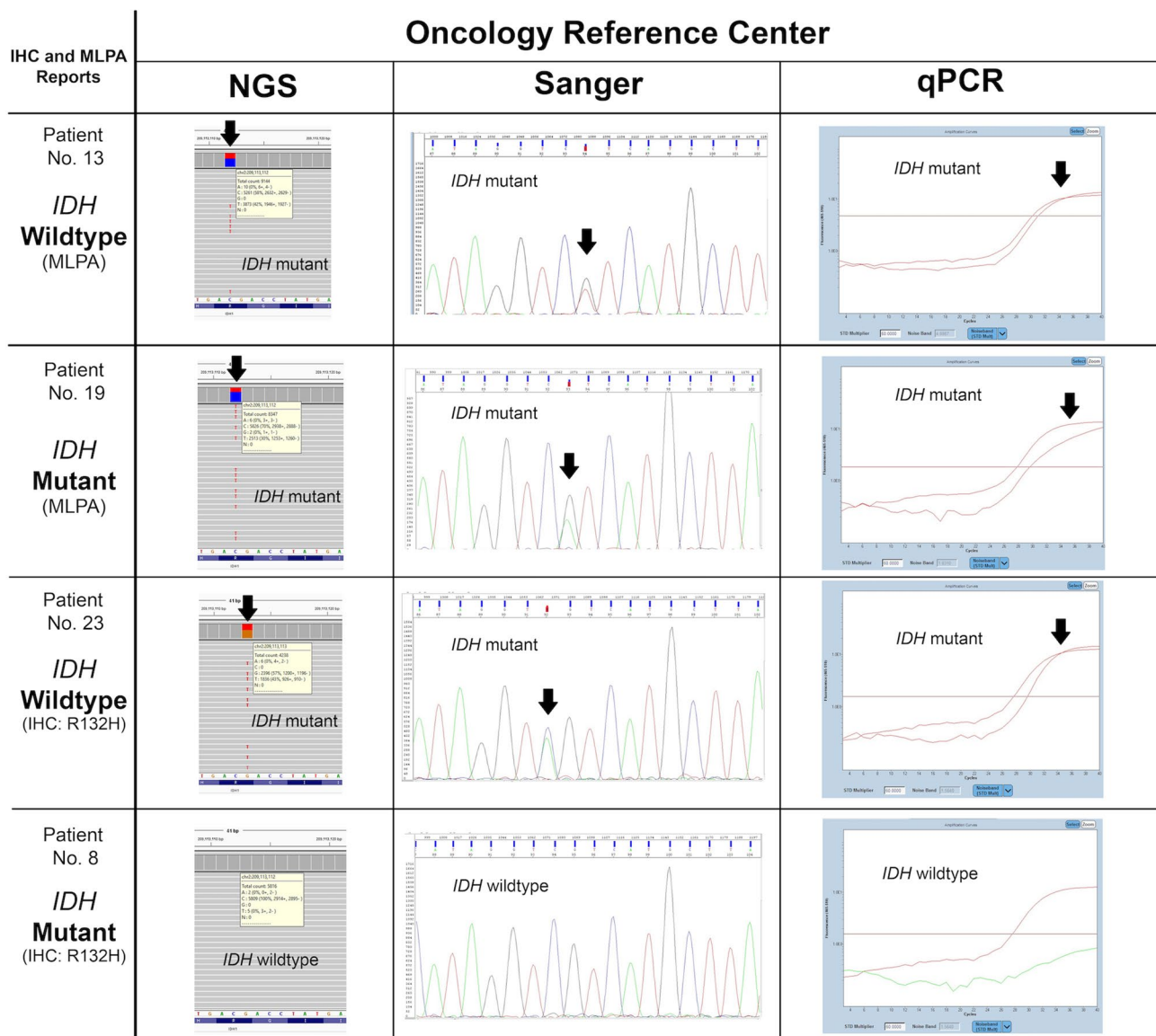*MLPA test does not detect R132L variant

Fig. 6 Examples of IHC and MLPA reports from different non-oncological reference centers versus NGS, qPCR, and Sanger sequencing results from the oncology reference center. Two false positives were detected by MLPA and IHC, and one false negative by IHC. Pathogenic variants detected by NGS were additionally validated by two independent methods: Sanger sequencing and qPCR. The *black arrows* indicate mutations detected using next-generation sequencing (NGS Targeted Hotspot Panel, Entrogen), Sanger sequencing (Applied Biosystems SeqStudio Genetic Analyzer), and qPCR (*IDH1/2* Mutation Detection Kit, Entrogen), with a limit of detection of 5, 20, and 1%, respectively. Two *red curves* represent (1) positive control and (2) amplicon with mutation detected (*arrow*). The patient no. 13 – MLPA report indicates IDH-wildtype, while NGS, qPCR, and Sanger sequencing confirmed mutation in the *IDH1* gene. *IHC* immunohistochemistry, *MLPA* multiplex ligation-dependent probe amplification, *NGS* next-generation sequencing, *qPCR* quantitative polymerase chain reaction

and A4 IDH-mutant. Finally, by integrating histopathological findings and molecular testing, a reliable and unequivocal diagnosis that enables targeted treatment can be made.

In non-reference hospitals, where NGS is not available, we suggest modifying the algorithm. Then, we recommend that the first-choice test should be a qPCR analysis of the *IDH* gene status. Subsequently, in the case of IDH-wildtype, qPCR would be used to test *TERT* and *EGFR* status.

Considering patients with histological diagnosis of grade 4 astrocytoma, when the availability of advanced molecular techniques is limited, assessing the existence of the *IDH1/2* mutations using qPCR would be sufficient to establish a precise diagnosis.

Although in grade 4 glioma patients WHO CNS5 only recommends evaluating the *IDH* gene, which could be done

Table 3 Change of diagnosis based on NGS findings and new WHO CNS5 2021 classification

N	Prior diagnosis (WHO 2016)	% of tumor cells	Gene	Change	Variant	Final diagnosis (WHO 2021)	Change of diagnosis
1	AA	85	TERT	NC_000005.9:g.1295228G>A[a]	Pathogenic	GBM	Yes
2	AA	80	TERT	NC_000005.9:g.1295228G>A[a]	Pathogenic	GBM	Yes
3	AA	100	TERT	NC_000005.9:g.1295228G>A[a]	Pathogenic	GBM	Yes

AA anaplastic astrocytoma, IDH-wildtype, *WHO CNS5 2021* the fifth edition of the WHO Classification of Tumors of the Central Nervous System, *GBM* glioblastoma, IDH-wildtype, *N* patient number, *NGS* next-generation sequencing, *TERT* human telomerase reverse transcriptase, *WHO* World Health Organization

[a]C228T

Fig. 7 Examples of FISH and NGS CNV for homozygous deletion of *CDKN2A* gene in glioma. **A** Representative FISH photo of a single glioma cell with *CDKN2A* homozygous deletion. **A′** *CDKN2A*, normal fluorescent signals in glioma cell. **B** CNV analysis for *CDKN2A*. Lack of homozygous deletion. *CNV* copy number variation, *FISH* fluorescence in situ hybridization, *NGS* next-generation sequencing
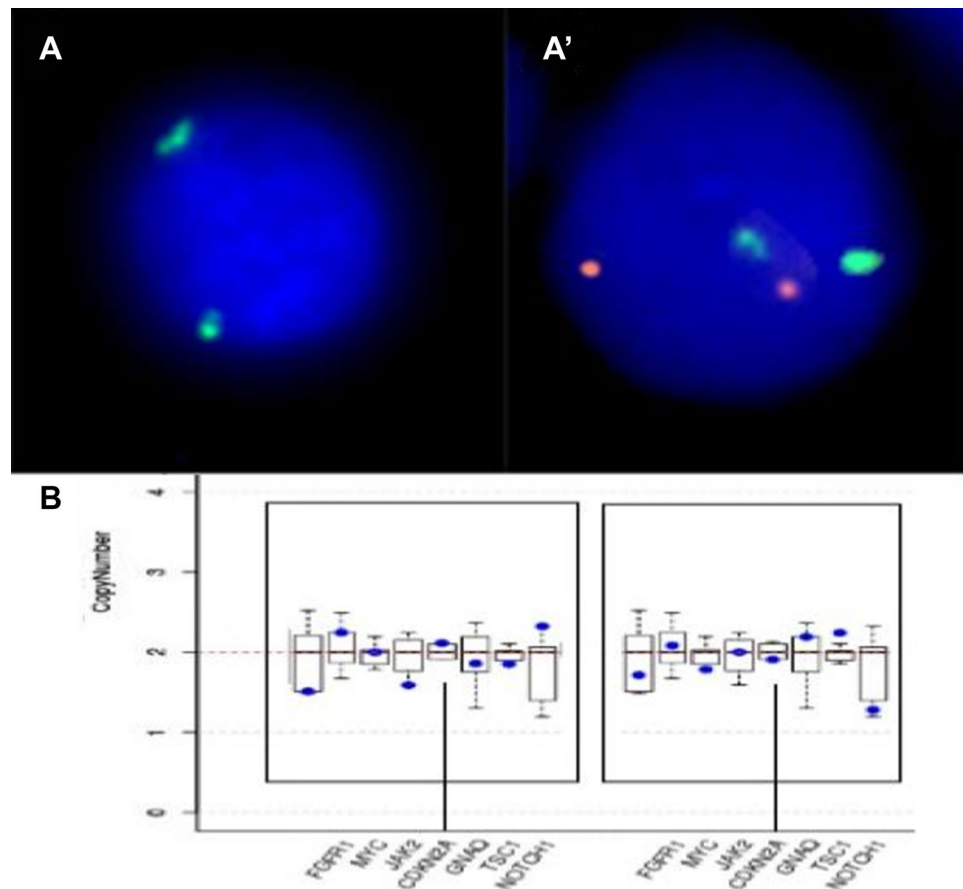
with qPCR, we suggest performing NGS using a panel that consists of the most important and the most frequently mutated genes in astrocytomas (such as *IDH1/2*, *TERT*, *EGFR*, *TP53*, and *PIK3CA*). This enables the diagnosis to be made not only based on histological findings (necrosis and vascular invasion), but also on molecular changes (the presence of genetic alterations that are proven to be related to shorter survival and are included in the WHO CNS5). As a result, assessing many genes using NGS enables a more precise prognosis.

According to the European Association of Neuro-Oncology (EANO) guidelines published before WHO CNS5, molecular analysis of *IDH* needs to be undertaken if the IHC result was IDH-wildtype [21]. It is critical to remember that these IHC guidelines were established prior to the introduction of the entire list of novel genetic biomarkers in WHO CNS5. As a result, these recommendations should be modified to incorporate NGS, which enables not only extensive analysis but also single-phase usage of the material. Therefore, in the case of an increasing number of biomarkers that need to be examined for the proper classification of gliomas,
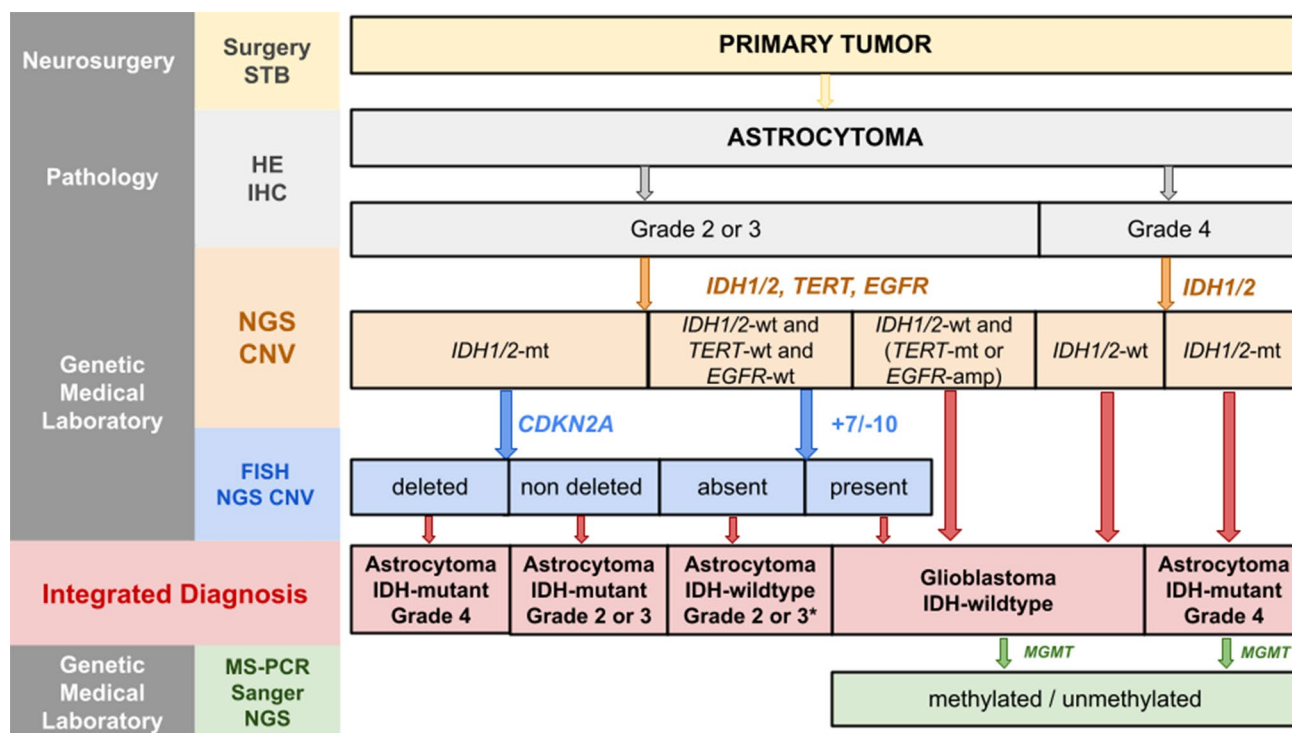
Fig. 8 Algorithm of integrated interdisciplinary diagnostics in astrocytoma patients. *CDKN2A* cyclin-dependent kinase inhibitor 2A homozygous deletion, *CNV* copy number variation, *EGFR-amp* epidermal growth factor receptor amplification, *FISH* fluorescence in situ hybridization, *HE* hematoxylin and eosin staining, *IDH* isocitrate dehydrogenase, *IHC* immunohistochemistry, *MGMT* O6-methylguanine-DNA methyltransferase, *MS-PCR* methylation-specific PCR, *NGS* next-generation sequencing, *PCR* polymerase chain reaction, *STB* stereotactic biopsy, *TERT* telomerase reverse transcriptase promoter pathogenic variants, *mt* mutant, *wt* wildtype, + 7/− 10 combination of chromosome 7 gain and 10 loss. *Not elsewhere classified (NEC); NEC diagnosis is facilitated by the use of layered integrated reports; integrated diagnosis combined tissue-based histological and molecular diagnosis

a comprehensive analysis should be conducted upfront, especially with a limited amount of biological material.

In conclusion, we suggest that NGS should be primarily used, firstly to assess *IDH* status, as our observations indicate that this method provides more reliable results [21], and secondly to assess a possibly broad range of biomarkers. It is expected that *CDKN2A* homozygous deletion, the combination of + 7/− 10, or *EGFR* amplification usually evaluated by FISH, will be replaced by single-step NGS CNV in the near future.

4.3 Limitations

Our study has several limitations. We did not have information about the methods of *IDH1/2* determination in every patient, as the material was evaluated in 12 different centers. Since the type of IHC antibody was not specified in the histopathology data, we are unable to determine which specific antibody was responsible for the false-positive and false-negative results. Moreover, we performed NGS only on 34 glioma samples because only those materials met the quality criteria. The diagnostic algorithm is based on experience working with a small 16-gene panel (EntroGen NGS Targeted Hotspot CE-IVD) and a broader CNV-targeted (SOLI-DaccuTest CE-IVD) NGS 84 gene panel for adult glioma; we did not focus on *FGFR*, the *NTRK* gene family, *NF1*, or *H3 H3F3A*, which are also a necessary focus, particularly for pediatric glioma classification.

5 Conclusions

Integration of histopathological findings and genetic aberrations provides an accurate and unequivocal diagnosis that enables targeted treatments. We evaluated how the use of NGS could be considered as a novel method to improve the classification and molecular characterization of glioma samples, particularly astrocytomas. Indeed, the WHO CNS5 guidelines express and confirm the significance of an integrated histogenetic classification of malignant gliomas,

which should combine histological tumor subtyping and grading with various genetic biomarkers, such as gene mutations and CNVs. Additionally, we developed a new algorithm for molecular diagnosis of glioma patients using NGS based on the WHO CNS5.

Declarations

Ethics and publication participation & consent The study was conducted in accordance with the Declaration of Helsinki, and the Ethics Committee of the Nicolaus Copernicus University, Collegium Medicum in Bydgoszcz, Poland approved the study protocol (KB 265/2012).

Informed consent statement Informed consent was obtained from all participants involved in the study.

Data availability statement All data relevant to this study are reported within the manuscript.

Code availability Not applicable

Conflict of interest The authors declare no conflict of interest.

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