Application of targeted Next Generation Sequencing in molecular classification of diffuse gliomas

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Background and objectives

With the rapid expansion in knowledge of the molecular basis of CNS tumour in recent years, the latest WHO Classification of Tumors of the Central Nervous System (CNS) (2021, 5th edition) emphasizes the importance of molecular diagnostics in tumour classification. Integration of clinical-radiological information, histological features and molecular biomarkers is required to formulate an integrated diagnosis. As the sample nature of CNS tumours are often tiny and limited, there is a clinical demand of diagnostic techniques that can reliably detect multiple relevant biomarkers simultaneously with small amount of tumour DNA/RNA input. We report on the establishment and validation of amplicon-based targeted next generation sequencing (NGS) using a large gene panel combined with customized filter for the molecular characterization of diffuse gliomas.

Methods

Sample selection and nucleic acid extraction

- Diffuse glioma samples were collected from 20 patients surgically treated with pathological diagnosis in PYNEH between January 2018 and December 2022.
- Sample DNA and RNA were extracted from the formalin-fixed paraffin-embedded (FFPE) tissue using AllPrep DNA/RNA FFPE Kit (Qiagen).
- Microdissection was performed on sample with tumor content ≤30% for tumor enrichment.
- Extracted DNA and RNA were quantified using Qubit™ ds DNA High-Sensitive Assay kit (Thermo Fisher Scientific) on Qubit fluorometer (Thermo Fisher Scientific) and in-house developed RNA expression assay respectively.

Library preparation and sequencing

- Pretreatment of sample DNA using Uracil-DNA Glycosylase (UDG) was performed prior to polymerase chain reaction (PCR).
- Library preparation and sequencing were performed using Oncomine Comprehensive Assay Plus (OCAplus) on Ion 550 Chips on Ion GeneStudio S5 Prime System (Thermo Fisher Scientific). The assay is a targeted NGS assay that provides comprehensive genomic profiling covering more than 500 genes, which is capable of detecting single-nucleotide variants (SNVs), short insertions and deletions (indels), copy number variations (CNVs), known and novel fusions, and splice site variants.

Data analysis

Ion Reporter Software (Thermo Fisher Scientific) was used for bioinformatics analysis for sequencing data. Oncomine Comprehensive Plus—w2.0—DNA—Single Sample was used as analysis workflow. Data was mapped to the human reference genome assembly hg19. Custom filters were created for variant filtering based on variant allele frequency, location, variant effect, variant type. Variants were also flagged based on p-value, low coverage <100, public variant database ClinVar, UCSC.

Comparative analysis between NGS and conventional orthogonal techniques

- Genetic alteration with diagnostic and potentially prognostic relevance to diffuse gliomas were selected for comparative analysis.
- Single-nucleotide variants (SNVs) affecting codon 132 of *IDH1* and *TERT* promoter hotspot mutations located at chromosome 5 nucleotide 1,295,228 (“C228T”) or nucleotide 1,295,250 (“C250T”) detected by NGS were validated by Sanger Sequencing. Negative IDH1-R132H mutation detection result on NGS was compared with immunohistochemical study using a monoclonal antibody clone H09 (Dianova, Hamburg, Germany) (lack of cytoplasmic staining).
- Copy number variations (CNVs) of 1p19q and EGFR gene (amplification) detected by NGS were validated by Florescence In-Situ Hybridization (FISH) using Vysis 1p36/1q25 with 19q13/19p13 FISH probe kit and Vysis EGFR/CEP 7 FISH probe kit respectively.
- *TP53* mutations detected by NGS were validated by immunohistochemical study using a mouse monoclonal antibody (D07, Dako, Glostrup, Denmark).
- *ATRX* gene mutations detected by NGS were validated by immunohistochemical study using a rabbit polyclonal antibody (HPA001906, Sigma-Aldrich, St. Louis, MO, USA) (loss of nuclear staining).
- Histone H3 c.83A>T pK28M (K27M) substitution detected by NGS was validated by Sanger Sequencing.

Results

Concordance rate of positive results between NGS and orthogonal techniques (OTs)

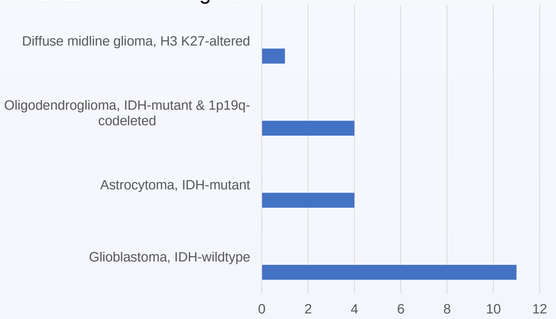
	NGS-detected positive cases	Concordance rate between NGS & OTs
IDH1 mutation	8	8/8 (100%)
TERT promoter hotspot mutations	11	11/11 (100%)
TP53 mutation	6	6/6 (100%)
1p19q co-deletion	4	4/4(100%)
EGFR amplification	3	3/3 (100%)
ATRX gene mutation	5	Diffuse loss of staining 3/5 (60%) Focal loss of staining 2/5 (40%)
Histone H3 c.83A>T pK28M (K27M) substitution	1	1/1 (100%)

Concordance rate of negative results between NGS and orthogonal techniques

- Concordance rate between negative result of *IDH1*-R132H mutation in NGS and immunohistochemistry: 11/11 = 100%

Integrated tumour diagnosis

Combining the histological features and molecular findings detected by NGS, the 20 samples of diffuse gliomas were successfully characterized into 4 distinct types following the 2021 CNS WHO guideline.



Discussion and conclusion

In this study, we established and validated the application of an amplicon-based gene panel NGS for detection of common genetic aberrations in diffuse glioma, including diagnostically important mutations in IDH1/2, ATRX, TP53, H3F3A and TERT promoter; and diagnostically important DNA copy number variations (CNVs) namely 1p/19q codeletion and EGFR gene amplification. The results demonstrated excellent concordance with the conventional orthogonal techniques, which proved targeted gene panel NGS as a promising technique allowing for a single assay solution for molecular characterization required for the latest WHO CNS tumour classification. Optimization and validation of NGS detection of diagnostically important DNA CNVs namely chromosome 7+/10- alteration and homozygous deletion of CDKN2A/B are currently in progress.

Reference

1. Zacher, A., Kaulich, K., Stepanow, S., Wolter, M., Köhrer, K., Felsberg, J., Malzkorn, B. and Reifenberger, G. (2017), Molecular Diagnostics of Gliomas Using Next Generation Sequencing of a Glioma-Tailored Gene Panel. Brain Pathology, 27: 146-159.
2. Tirrò E, Massimino M, Broggi G, Romano C, Minasi S, Gianno F, Antonelli M, Motta G, Certo F, Altieri R, Manzella L, Caltabiano R, Barbagallo GMV, Buttarelli FR, Magro G, Giangaspero F and Vigneri P (2022) A Custom DNA-Based NGS Panel for the Molecular Characterization of Patients With Diffuse Gliomas: Diagnostic and Therapeutic Applications. *Front. Oncol.* 12:861078.