**ANALYSIS REPORT**

PATIENT

Patient ID: JM1629

Diagnosis: Diffuse midline glioma, H3 K27-altered

Report date:

**SUMMARY**

<<summary\_table>>

\*Details regarding the methodology are listed on page 4.

**RESULTS**

**Germline mutations**

<<germline\_table>>

**Somatic mutations**

<<somatic\_table>>

**Gene fusions**

<<fusion\_table>>

**Mutational signatures**

<<mutational\_sign\_table>>

**Expression profile**

<<expression\_table>>

<<plot1>>

**METHODOLOGY**

**Library preparation and sequencing**

**Somatic small variant calling**

DNA for whole-exome sequencing was extracted from peripheral blood using the QIAmp DNA Micro Kit (Qiagen, Germany). Sequencing libraries were prepared using KAPA HyperExome Kit (Roche, Switzerland). Sequencing was carried out on the NextSeq 500 device using NextSeq 500/550 Mid Output Kit v2,5 (150 cycles) (Illumina, CA, USA).

**Germline small variant calling**

DNA for whole-exome sequencing was extracted from FFPE tissue using QIAmp DNA FFPE Tissue Kit (Qiagen, Germany) and treated with NEBNext FFPE DNA Repair Mix (New England Biolabs, MA, USA). Sequencing libraries were prepared using KAPA HyperExome Kit (Roche, Switzerland). Sequencing was carried out on the NextSeq 500 device using NextSeq 500/550 Mid Output Kit v2.5 (150 cycles) (Illumina, CA, USA).

**Fusion genes detection and expression profiling**

RNA for targeted RNA sequencing was extracted from frozen tissue using MiRVana miRNA Isolation Kit and treated with a DNA-free DNA Removal Kit (ThermoFisher Scientific). Messenger RNA was purified using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). Sequencing libraries from polyA-selected mRNA were prepared using NEBNext Ultra II Directional RNA Library Prep Kit (New England Biolabs). Sequencing was carried out on the NextSeq 500 device using NextSeq 500/550 Mid Output Kit v2.5 (75 cycles) (Illumina).

**Patient-specific information regarding the analysis**

DNA was extracted from FFPE tissue. DIN was 2.9. The sequencing was performed on the 26th of April, 2023. 96% of the target regions were covered at least 20 times. (*somatic var call)*

The sequencing was performed on the 26th of April, 2023. 91% of the target regions were covered at least 30 times. (*germline var call)*

The sequencing was performed on the 3rd of May, 2023. The manufacturer´s recommendations on input RNA quality state that the RNA integrity number (RIN) should be ≥7. The RIN of RNA extracted from the patient´s tumor was 8,9. (*expression profile)*

**Bioinformatic workflow**

**Somatic small variant calling**

Data processing: BWA (alignment to GRCh38), Picard (marking duplicates), Samtools (sorting/indexing)

Quality Control: Qualimap, PicardTools CollectHsMetrics, FastQC (aggregated in MultiQC)

Variant Calling: Strelka, VarDict, Mutect2, SomaticSniper, LoFreq, MuSE, VarScan (SomaticSeq pipeline, consensus approach – calls made by 3 or more callers out of 7)

Annotation: Ensembl Variant Effect Predictor + in-house annotation scripts for Gene, Transcript assignment, Variant Consequence, Population databases (1000 Genomes, GnomAD, ExAC), Clinical databases (dbSNP, CancerGeneCensus, COSMIC, HGMD, NHLBI ESP, TruSight, ClinVar, MD Anderson, Foundation One CDx), Protein structure predictors (SIFT, PolyPhen2)

TMB computation: Count only non-synonymous exonic SNVs with VAF > 5%.

TMB = variant count × 10⁶ / exome size

**Germline small variant calling**

Data processing: BWA (alignment to GRCh38), Picard (marking duplicates), Samtools (sorting/indexing)

Quality Control: Qualimap, PicardTools CollectHsMetrics, FastQC (aggregated in MultiQC)

Variant Calling: GATK HaplotypeCaller, VarDict, Strelka (union approach)

Annotation: Ensembl Variant Effect Predictor + in-house annotation scripts for Gene, Transcript assignment, Variant Consequence, Population databases (1000 Genomes, GnomAD, ExAC), Clinical databases (dbSNP , CancerGeneCensus, COSMIC, HGMD, NHLBI ESP , TruSight, ClinVar, MD Anderson, Foundation One CDx), Protein structure predictors (SIFT, PolyPhen2)

**Fusion genes detection**

Data processing: STAR (alignment to GRCh38)

Quality Control: Qualimap, PicardTools

Fusion calling: Arriba, STARFusion

Manual verification: Integrative Genomics Viewer

**Expression profile**

Raw sequencing reads are quality-checked with FastQC and then aligned against GRCh37. The gene counts are estimated with Gencode gene definitions. The gene counts are compared with reference gene counts from the GTEX database of non-disease tissue-specific samples. The comparison is performed against a selection of samples of the selected tissue type. The gene counts are TMM normalized, and a t-test statistic is used. The report contains selected cancer-associated genes with respective pathways and fold change values.

**Limitations**

Mutations outside of the coding regions, in other genes, copy number changes, and changes that cannot be detected at present technical possibilities, and the current level of knowledge cannot be excluded.

Not all detected variants are reported. Reported variants were pre-selected based on their known or potential significance in the disease. All variants found are listed in the protocol supplement.

Not all genes with deregulated gene expression are reported. Reported genes were pre-selected based on their role in important cellular processes and signaling pathways.

**VARIANT CLASSIFICATION SYSTEM**

The classification system used in this report is based on recommendations of the American College of Medical Genetics and Genomics (ACMG), initially published in 2015 by Richards et al. (PMID: 25741868). Gene selection is focused solely on cancer-associated genes if not specified otherwise. Additionally, 72 genes defined by ACMG that should be evaluated in individuals undergoing clinical exome/genome sequencing based on the medical actionability of the associated condition were evaluated as well (for more details, see Miller et al., 2022; PMID: 35802134). Any potential incidental/secondary findings in such genes are reported and consulted directly with a clinical geneticist.

**Pathogenic** – the variant has sufficient evidence to classify as pathogenic (capable of causing disease). Targeted testing of at-risk family members and appropriate changes in medical management (i.e., high-risk surveillance) for pathogenic mutation carriers are recommended. Pathogenic variants are always included in results reports.

**Likely pathogenic** – the variant has strong evidence in favor of pathogenicity. Targeted testing of at-risk family members and appropriate changes in medical management (i.e., high-risk surveillance) for carriers are recommended. Likely pathogenic variants are always included in results reports.

**Variant of uncertain significance (VUS)** – the variant has limited and/or conflicting evidence regarding pathogenicity. VUS in cancer-associated genes is typically included in results reports, however, can be omitted for heterozygous variants in genes associated with autosomal recessive disorders that predict rather benign by available prediction algorithms.

**Likely benign** – the variant has strong evidence against pathogenicity. Likely benign variants are not included in the results reports.

**Benign** – the variant has very strong evidence against pathogenicity. Likely benign variants are not included in the results reports.

**Disease-associated and/or functional polymorphism** – this category was defined by the HGMD database and describes variants that are either (i) disease-associated and of likely functional significance, or (ii) of clear functional significance even though no associated clinical phenotype may have been identified to date.