nature portfolio

https://doi.org/10.1038/s41596-021-00594-2

Supplementary information

An integrated pipeline for comprehensive analysis of immune cells in human brain tumor clinical samples

In the format provided by the authors and unedited

Supplementary Methods

Isocitrate dehydrogenase (IDH) pyrosequencing

IDH pyrosequencing of the relevant mutational hotspots of *IDH1* and *IDH2* genes was performed at the Service of Clinical Pathology of the Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne. Representative tissue blocks used for pyrosequencing were either from (i) fresh-frozen OCT Compound (Sakura TissueTek; #4583) embedded tissues, (ii) tissues fixed with 4% paraformaldehyde (PFA, E.M.S.; #15714-S) for 24 hours at 4°C before OCT-embedding and freezing at -80°C, or (iii) formalin-fixed paraffin-embedded (FFPE) tissue. Blocks were selected based on hematoxylin and eosin (H&E)-stained slides. For FFPE tissue blocks, if tumor cell content was <80%, the regions of interest were marked on the H&E section and subsequently reported on corresponding FFPE sections stained with toluidine blue. Manual microdissection of the regions of interest was performed under a microscope, by scraping the tissue sections off the slides, followed by genomic DNA extraction (Maxwell 16 FFPE Plus LEV DNA Purification Kit, Promega). For all cases the estimated tumor cell content in the analyzed DNA was ≥80%.

Prior to sequencing, exons 4 of *IDH1* and *IDH2*, including codon 132 and 172 respectively, were amplified by polymerase chain reaction (PCR) using primers with the following sequences: *IDH1* biotinylated forward primer 5'-TGATGAGAAGAGGGTTGAGGAGTT-3' and reverse primer 5'- TTGCCAACATGACTTACTTGATCC-3' (198 bp amplicon); *IDH2* forward primer 5'-ATCCCACGCCTAGTCCCTG-3' and biotinylated reverse primer 5'-CTCCACCCTGGCCTACCT-3' (82 bp amplicon). PCR reactions were performed in duplicate, each in a total volume of 20 µl, containing : 5.9 µl nuclease-free water (Promega; #P119C), 1 µl dNTPs (5 mM; Illustra, dNTP mix set; #28-4065-57), 0.1 µl Platinum Taq DNA Polymerase (2U/ reaction; Invitrogen; #10-966-026), 10 µl of a 2X mix of the appropriate primers (for each *IDH* gene, a primer mix was prepared at 0.5 µM: nuclease-free water + 10X PCR Rnx Buffer (Invitrogen) + 50 mM MgCl2 (Invitrogen) + Forward/Reverse primers as indicated above), and 3 µl of genomic DNA (3 ng/µl) per reaction. Thermal cycling consisted of 38 cycles with denaturing (95°C, 15 s), annealing (57°C, 30 s), and elongation (72°C, 15 s) steps, preceded by an initial denaturation step (95°C, 5 min) and completed with a final elongation step (72°C, 5 min).

Pyrosequencing was performed utilizing a PyroMark Q24 Advanced pyrosequencer (Qiagen). 70 µl of Sepharose bead mix (comprised of 2 µl of Streptavidin Sepharose High Performance beads (GE Healthcare, Piscataway, NJ; #17511301), 40 µl PyroMark Binding Buffer (Qiagen; #979006), and 28 µl of Ultra Pure MilliQ water) were added to a 96-well PCR plate per run. 10 µl of PCR product were added to the wells, and then shaken at room temperature for 8 minutes (1500 rpm) to bind the biotinylated amplicons to the sepharose beads. Biotinylated PCR amplicons captured on the beads were then processed through washes of 70% ethanol (5 to 10 s), 0.2 M NaOH for denaturation (5 to 10 s), and PyroMark Wash Buffer to remove nonbiotinylated DNA strands (10 to 20 s) using the PyroMark Q24 Advanced Vacuum Prep Workstation. Biotinylated DNA strands were then released into the sequencing primer solution (25 µl at 0.3 µM of primer) in a PyroMark Q24 Plate. The sequencing primers were as follows: for *IDH1* codon 132: 5'-TGATCCCCATAAGCA-3' (annealing to the reverse strand) and for *IDH2* codon 172: 5'-AAGCCCATCACCAT-3'. The solution was then heated at 80°C for 2 minutes, and then cooled at room temperature (5 min). PyroMark Q24 Gold Reagents (enzymes, substrates, dNTPs; Qiagen; #971802) were used in the pyrosequencing reactions. Each assay was performed in duplicate, in parallel with a wild-type DNA control and a no template control. The technical sensitivity (limit of detection) of the method is of approximately 10% mutant allele content.

Sequence to analyze (S2A) and dispensation order (DO) using PyroMark Q24 software were designed in order to optimize non-synonymous variant detection at codon 132 of *IDH1* and codon 172 of *IDH2*. *IDH1* codon 132 was interrogated using DO GTGTACTGACGTA and S2A TGAC*N*ACCTATGATGAT (*N,* any base; sequence to be read on the reverse strand). *IDH2* codon 172 was assessed using DO CGTCTACTGTCACG and S2A GGCA*N*GCACGCCCATGGCGACC.

Automated immunohistochemistry (IHC) staining for ATRX

ATRX IHC staining was performed at the Service of Clinical Pathology of the Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, using a DISCOVERY Ultra automated stainer (Roche Ventana). Sections used for staining were either from (i) fresh-frozen OCT Compound embedded tissues, (ii) tissues fixed with 4% PFA for 24 hours at 4°C before OCT-embedding and freezing at -80°C (both cryosectioned at 10 µm), or (iii) FFPE tissue sections (4 µm). FFPE slides were heated for 4 minutes at 72°C and placed in EZ Prep solution (Roche Ventana; #950-102) for deparaffinization. Frozen slides were thawed and air-dried for 5 min, followed by fixation using 4% PFA for 3 min at room temperature (RT; only performed for frozen slides that had not been pre-fixed before OCT-embedding). Slides were then hydrated using Reaction Buffer (Roche Ventana; #950-300). Slides were placed in the DISCOVERY Ultra automated stainer. In the selected staining program, slides were washed 1x with Ultra Liquid Coverslip (LCS) solution (Roche Ventana; #650-210), followed by Reaction Buffer. Antigen retrieval was performed using Cell Conditioning 1 antigen retrieval solution (Roche Ventana; #950-500) for 32 min at 95 °C, followed by a wash with Reaction Buffer. ATRX was detected using a polyclonal rabbit anti-human ATRX antibody (Sigma; #HPA001906, at 1:100 dilution) for 60 min at 37°C, followed by 2 washes of 5 min each with Reaction Buffer. Primary antibody detection was performed using an OmniMap anti-rabbit HRP-conjugated secondary antibody (Roche Ventana; #760-4310) for 16 min at RT. The DISCOVERY ChromoMap DAB detection kit (Roche Ventana; #760-159) was used for detection. Tissue counterstaining was performed using Hematoxylin II solution (Roche Ventana; #790-2208). The stained slides were rinsed with distilled water and dehydrated using an alcohol bath sequence (3 x 5 s per step: ethanol 95% - ethanol absolute (Reactolab SA; #99570) - xylol (VWR Chemicals, #28973.363). Finally, slides were mounted using Glas Mounting Medium (Sakura TissueTek; #1408) and covered (24x50 mm coverslips, VWR, ECN 631-1574) using a TissueTek Glas g2-E2 coverslipper (Sakura Finetek; #6502). Slides were scanned using a digital slide scanner (NanoZoomer S60, Hamamatsu; #C13210-01) with 20x objective lens magnification, 0.75 numerical aperture, and a scanning resolution of 0.46 µm/pixel. Images were acquired in a jpeg compressed file format and analyzed using Leica Biosystems Version 4.0.7 (Leica Biosystems).