Supplementary Methods

Cell and Spheroid culture, generation of spheroids

Spheroids used in this study were obtained from 13th- and 14th-generation implantation events (of in vivo passaging in nude rats). Seven days before intracranial implantation, spontaneous spheroids formed in the agar-overlay culture were harvested and dissociated into single cells using a neuronal dissociation kit (Miltenyi Biotech). The single cells were plated in round-bottomed 96-well plates with 10,000 cells/well in 100 µl DMEM supplemented with 10% FBS, 1% L-glutamine, 100 U/ml Penicillin 100 µg/ml Streptomycin and 4% methylcellulose containing DMEM medium (12 mg/ml methyl cellulose). Plates were centrifuged for 1.5 h at 31°C to generate new spheroids with the defined number of cells. Shortly before implantation, the spheroids were collected in a polypropylene tube, washed with PBS and kept on ice until application. For in vitro GCV-sensitivity assay, collected tumor cells from recurrent tumor mass were dissociated using a similar protocol mentioned above and cultured in neurobasal medium (NB) as reported earlier [1].

Cell viability assessment

Cell viability was assessed by using RealTime-Glo™ MT Cell Viability Assay (Promega) according to the manufacturer's protocol.

Immunofluorescence (IF)

Deparaffinization of paraffin-embedded sections, subsequent antigen retrieval and blocking were performed as mentioned previously [2]. Briefly, after overnight incubation of primary antibody (or antibody mixture) at 4°C, sections were washed thoroughly and secondary antibody (or antibody mixture) was applied for 1 hour at room temperature. After washing

again, the sections were treated with Sudan Black B for 10 minutes followed by mounting under cover slip with ProLong Gold Antifade medium with DAPI (Thermo-Fisher Scientific).

Antibody source and dilution

The following antibodies were used: anti-GFP antibody (1:200; Merck Millipore), anti-human nestin (1:200; Merck Millipore), anti-vWF (1:500; Dako), anti-EGFR (1:500; Santa Cruz), anti-pEGFR1068 (1:100; Cell Signaling), anti-olig2 (1:500; Merck Millipore), anti-pMAPK (1:200; Cell Signaling), anti- c-MET (1:200; Abcam), anti-PDGFR-A (1:400; Cell Signaling), anti-pSTAT3 (1:400; Cell Signaling), anti- Ki-67 (1:100; Dako), biotinylated anti-rabbit antibody (1:100; Vector Biolabs), Alexa Fluor 488 anti-Mouse (1:200; Abcam) and Alexa Fluor 647 anti-Rabbit (1:200; Abcam).

Fluorescence in situ hybridization (FISH)

FISH was performed according to protocol published before [3].

Digital analysis of images

All images were analyzed using the Fiji distribution of ImageJ. IHC images using DAB were segmented using color deconvolution as described in [4] and implemented in Fiji by Gabriel Landini. The separation matrix was found by using wavelet decomposition and Independent Component Analysis (ICA) on a representative image from each staining. The brown channel was then either thresholded directly in the case of diffuse membrane stains (EGFR, PDGFR-A, pEGFR), or filtered using a Gaussian function and then thresholded for discrete, particulate stains (Ki67, pSTAT3, vWF). For the diffuse stains, the quantified variable was the fraction of the total area that stained positive; for the discrete stains, the quantified variable was the fraction of the total number of cells that stained positive; and finally, for vWF, the quantified

variable was the fraction of the total area made up of vessel structures, including lumen. For the IF images, background was subtracted using the rolling ball-algorithm implemented in Fiji, images were filtered using a Gaussian function, before finally being thresholded. To evaluate co-localization, the binary channels in question were then AND-combined, and the number of particles in the resulting image was quantified. Quantification of invasive cancer cells was done by automated counting of nestin-positive cells. Areas quantified were a 200 µm band around the outer edge of the solid tumor mass, and about 2.5 mm^2 of white matter tracts and surrounding areas starting from the corpus callosum and into the contralateral hemisphere.

Gene expression analysis

For RNA-seq, TruSeq Stranded mRNA sample preparation kit (Catalogue ID: RS-122-2101) was used to prepare the library. Poly-A containing mRNA molecules were purified from the total RNAs using poly-T oligo attached magnetic beads. The sequencing library was generated by PCR, and sequencing was performed on NovaSeq 6000 System (Illumina, Inc., San Diego). Each sample was sequenced in a separate flow cell lane, producing 29-45 M paired-end reads, with a final length of 101 bases. For the assessment of species-origin of sequence reads, *Bowtie2* was used to align the FASTQ files to the reference human genome, hg38 using default parameters. Aligned BAM files were indexed and co-ordinate sorted by Picard SortSam for downstream convenience. Read counts for gene expression were obtained using *FeatureCounts* function. Resulting read count data were then analysed by pairwise comparisons of the groups in R statistical environment (https://cran.r-project.org) using DESeq package. To find out up- and down-regulated genes padj values of less than 0.05 were considered significant. Positive and negative log2 fold change values were then used to sort the genes. For Gene Set Enrichment Analysis[5] (GSEA) the input data were the following: (1)

a ranked gene list based on the following: -Log10(p.adj) * sign(Fold Change). (2) a list of human gene sets from the Molecular Signature Database (C5: GO Biological process and C2: KEGG pathway). Minimum and maximun gene set size for analysis were set to 10 and 500 respectively. The number of permutation was set to 10 000. Gene sets that shown an adjusted p.value less than 25% were considered significant as recommended by the software manual. The usage of GSEA has been made thanks to the R library "clusterProfiler"[6].

References

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