Mapping of molecular pathways involved in the pathogenesis and progression of glioblastoma by epigenomic approach

Doctoral (PhD) – thesis

Zoltán Krabóth

University of Pécs, School of Medicine

Doctoral School of Clinical Neurosciences (D221)

Supervisor:

Prof. Bernadette Kálmán, MD, PhD, DSc, FAAN

Leader of the Doctoral Program:

Prof. Sámuel Komoly, MD, PhD, DSc

Head of the Doctoral School:

Prof. Sámuel Komoly, MD, PhD, DSc

Pécs, 2021

Table of contents

1. Introduction 4

2. Hypotheses and Aims.. 6

3. Materials and methods 7

3.1 Subjects of the studies in general 7

3.2 Genome-wide analysis of DNA CpG methylation in controls and sequential GBM sample pairs 8

3.3 Quantification of CpG methylation levels in promoter + gene regions and protein expression levels of four selected catecholamine markers in individual control samples and sequential GBM sample pairs 8

3.4 DNA isolation 9

3.5 Library preparation 9

3.6 Bioinformatics 10

3.7 Immunhistochemistry (IHC) 11

3.8 Statistics 12

4. Results.. 12

4.1 DNA CpG methylation in sequential FFPE GBM samples and controls 12

4.1.1 Comparison of primary tumors (GBM1) with normal brain control (CG2) samples 13

4.1.2 Comparison of recurrent tumors (GBM2) with normal brain control (CG2) samples 14

4.1.3 Comparison of recurrent tumors (GBM2) with primary tumors (GBM1)... 14

4.1.4 Locus Overlap Analysis (LOLA) 15
4.1.5 Clinicopathological correlations................... 16
4.2 Correlation analyses of promoter + gene CpG methylation and protein expression levels of the selected catecholamine pathway markers.................................... 17
4.2.1 Quantitative IHC assessments of protein expression for the selected catecholamine pathway markers in GBM sample pairs and normal controls 17
4.2.2 Promoter + gene methylation levels of selected catecholamine pathway markers in individual GBM and control samples.. 18
4.2.3 Promoter + gene methylation levels of the four selected catecholamine pathway markers in the validation database GBM cohort 19
5. Discussion.. 20
6. New findings 25
7. References: 27
8. Acknowledgements.. 31

1. Introduction

Glioblastoma (GBM) is the most common and malignant primary tumor of the central nervous system, a grade IV glioma based on The World Health Organization (WHO) classification. Of all GBM, 90% is primary tumor that develops *de novo* with an older age of onset (median 62 years). In contrast, the remaining 5-10% of GBM is secondary tumor that evolves from grade II diffuse astrocytomas or grade III anaplastic astrocytomas (5-10%), with a lower age of onset (median 45 years) [1, 2]. The standard of care for GBM includes a temozolomide (TMZ)-based chemotherapy and radiotherapy, which is preceded by resection of the tumor in operable cases. Even with the standard Stupp protocol and experimental molecular treatment strategies, GBM has an extremely poor prognosis. The median survival time of the disease is around 15 months [3; 4]. GBM is characterized by high degree of histological and molecular heterogeneity. This heterogeneity is related to the clonal heterogeneity of accumulating somatic mutations and epigenetic changes within the tumor. The first comprehensive identification of GBM mutations and signaling pathways was described by the "The Cancer Genome Atlas" (TCGA) consortium [5]. A subsequent analysis of integrated genomic and transcriptomic data by TCGA also revealed separation of GBM into molecular subgroups named classical (CL), mesenchymal (MES) and proneural (PN) [6]. The CL

subgroup is characterized by EGFR (epidermal growth factor receptor) amplification and EGFRvIII mutation, while in the MES subgroup deletions/mutations in NF-1 (neurofibromin 1) gene are the most common. In the PN subgroup, the p53 and IDH-1/2 (isocitrate dehydrogenase 1/2) genes are frequently mutated. The IDH gene mutations result in a malfunctioning enzyme that produces an oncometabolite affecting DNA CpG methylation genome-wide, and thus influences transcriptional profiles [7].

It is now well established that epigenetic mechanisms, such as DNA CpG methylation, play prominent roles in the development, progression, treatment resistance and recurrence of GBM. Tumors are generally characterized by a shift in the direction of genomic CpG hypomethylation that can enable the transcription of normally inactive oncogenes through increased access of transcription factors to enhancers and promoters [8]. Sparse data suggest that epigenomic modifications of genes in the catecholamine pathway including certain monoamines (dopamine, epinephrine, norepinephrine etc.) and their receptors may contribute to tumorigenesis in gliomas [9, 10]. Neurotransmitters and their receptors are not only key mediators of neuronal communication, but also play important roles in shaping the microenvironment, and influencing tumorigenesis and development.

Considering the available information, first we focused our studies on the identification of genome-wide CpG methylation profiles of sequential GBM samples. Based on the results, we subsequently analyzed the involvement of the catecholamine pathway in tumorgenesis and progression. A better understanding as to how monoamines and the associated pathways work in gliomagenesis and recurrence could be of importance, and its elements may also be potential treatment targets in the future.

2. Hypotheses and Aims

Hypotheses

- DNA CpG methylation is an important mechanism regulating the formation and development of GBM
- Patterns of DNA CpG methylation differ between primary and recurrent GBM tumor samples
- Promoter and gene methylation levels show an inverse correlation with protein expression levels in primary and recurrent GBM

Aims

- Determination genome-wide the differentially methylated DNA CpG sites, genes, regions and

pathways in sequential GBM samples and normal controls

- Comparison of the methylation profiles of DNA CpG sites, genes, regions and pathways in primary and recurrent GBM sample pairs
- Correlation analyses between the degree of promoter and gene CpG methylation and the levels of protein expression of selected catecholamine pathway markers in sequential GBM sample pairs.

3. Materials and methods

3.1 Subjects of the studies in general

Primary and recurrent GBM samples were obtained between 1997 and 2017 at the Department of Pathology, Faculty of Medicine, University of Pécs (UP). All primary samples were surgically obtained prior to radiotherapy and chemotherapy, while recurrent samples were surgically obtained after treatments. All tumors were formalin-fixed and paraffin-embedded (FFPE) samples left over from histopathological work up. Sample selection was preceded by histological quality control evaluating HE (hematoxylin-eosin) stained sections and by IDH-1 R132H mutation analysis. The studies were approved by the Regional Scientific Ethics Committee

(RSEC) of the UP. Documentation of RSEC approvals: PTE RSEC 7517-2018 and -2019.

3.2 Genome-wide analysis of DNA CpG methylation in controls and sequential GBM sample pairs

We had 48 FFPE blocks (24 pairs of primary and recurrent tumors) from 24 patients. After reviewing the clinical data, samples from 2 patients were excluded from the study due to the patients' young age. Therefore, 44 samples from 22 patients were finally included in the study. For methylation control (control group 2 – CG2), we used reduced representation bisulfite sequencing (RRBS) data of 5 patients who underwent epilepsy surgery, and whose data were deposited in a publicly available database. RRBS data of 112 primary and recurrent GBM sample pairs from the same database were used to validate our methylation results (https://www.ebi.ac.uk/ena, access number: EGAS00001002538) [11].

3.3 Quantification of CpG methylation levels in promoter + gene regions and protein expression levels of four selected catecholamine markers in individual control samples and sequential GBM sample pairs

Expression analyzes of four selected catecholamine markers (ADRA1D - alpha-1D adrenergic receptor,

ADRBK1 - adrenergic beta-receptor kinase 1, DRD2 - dopamine-D2 receptor, SLC18A2 - synaptic vesicle monoamine transporter) were performed from the same GBM FFPE blocks as the epigenomic analyzes. However, due to the limited availability of samples, one patient's material had to be excluded from this analysis. Six FFPE post-mortem control brain samples were used in the protein expression studies of catecholamine markers as for ethical reasons no surgically removed normal brain tissue was available.

3.4 DNA isolation

Four-five 5 µm sections were prepared from the FFPE tissue blocks. After deparaffinization and ethanol wash, DNA was isolated by using the QIAamp DNA FFPE Tissue Kit (Qiagen®). As a quality assessment of the isolated DNA, a fragment analysis was performed using the Agilent Genomic DNA ScreenTape Assay kit on an Agilent 4200 TapeStation System.

3.5 Library preparation

Bisulfite converted libraries were prepared from the isolated DNAs using the RRBS kit 24x (Diagenode), according to the manufacturer's instructions. To compensate for an overt DNA degradation, we increased the amount of input DNA from the recommended 200ng to 350-400ng.

Library preparation in brief: DNA was digested by MspI restriction endonuclease that recognizes CCGG sites and generates fragments ending with CpGs. The next steps included the end preparation, adapter ligation and size selection followed by sample pooling to undergo bisulfite conversion. Converted libraries were PCR amplified, and after the last size selection step, were checked for quality and quantity before sequencing on a NextSeq 550 device. Raw sequencing data were uploaded to the European Nucleotide Archive (https://www.ebi.ac.uk/ena, Primary Accession: PRJEB38380, Secondary Accession: ERP121800).

3.6 Bioinformatics

The quality of the raw sequencing data generated by NextSeq 550 was checked with FastQC, and the low-quality sequences and adapters were filtered out with TrimGalore software. Our RRBS sequences were aligned with the hg19 (GRCh37) reference genome. Bisulfite-converted reads and methylated sites were determined by using the Bismark program. The R software, supplemented by its RnBeads plugin, was used to identify differentially methylated CpG sites, promoters, regions, and genes at the cohort level, and Gene Ontology (GO) analysis was used to examine differentially methylated signaling pathways in controls and in primary and recurrent GBM tumors. The methylation levels and distributions in promoters and genes of the four selected

catecholamine markers (ADRA1D, ADRBK1, DRD2, SLC18A2) were examined in individual samples using a script created for the study by our bioinformatician colleague. The Script was also run with the BioMethyl package in the R software. The methylation level in the promoter + gene region of a given sample was determined by first identifying all methylated sites in all individual samples to obtain the total number of CpGs in the region. The degree of methylation of these CpGs was then assessed individually and scored between 0 and 100%, as the number of methylated sites in a sample was divided by the number of all possible (detected) CpGs and multiplied by 100.

3.7 Immunhistochemistry (IHC)

The primary antibodies for the four selected catecholamine markers (ADRA1D, ADRBK1, DRD2, SLC18A2) were optimized in a pilot IHC study. The specificity of the antibodies was checked in tumors of various histological origins, for which the expressions of the given antigens were known (positive control) or for which the selected markers were known to be negative (negative control). Primary antibodies were detected using the NovoLink Polymer Detection Systems RE-7150-K kit (Leica). A "region of interest" (ROI) was determined in each sample in HE stained section of the specimen to include the most malignant looking regions lacking necrosis or overt vascularity. The percentage of

stained cells and the staining intensity within the ROI were determined, and multiplied with each other to generate the "Complex Score" (CS) for each marker.

3.8 Statistics

The pathological characteristics of the tumors, the clinical data of the patients, and the median and interquartile ranges of the quantitative IHC results (CS values) of the catecholamine markers were compared among subgroups in substudies using various statistical tests of the SSPS v.26.0 software package. Our data followed a non-parametric distribution in all cases, so we used non-parametric tests. The Mann-Whitney U test was used to compare two independent samples, while the Wilcoxon signed rank test was used to compare dependent samples. When comparing several samples simultaneously, the Kruskal-Wallis test was applied.

4. Results

4.1 DNA CpG methylation in sequential FFPE GBM samples and controls

As a first step, the technical characteristics of bisulfite conversion and sequencing were examined. The conversion rate was 98.48%, reflecting high efficiency. A trend-like decrease in the number of informative CpGs was observed for lower quality DNAs. In addition to the

differentially methylated CpG sites, the analysis program also defined the differentially methylated CpG islands, tiles, genes and promoters. In the study, we focused primarily on the differentially methylated promoters in the GBM1-CG2, GBM2-CG2 and GBM1-GBM2 comparisons. Methylation levels of CpG sites, islands and tiles showed no differences among the three groups. However, the gene ontology (GO) analysis revealed a number of differentially methylated gene pathways in the three comparisons.

4.1.1 Comparison of primary tumors (GBM1) with normal brain control (CG2) samples

In primary tumors compared to the CG2 controls, hypermethylation was observed in signaling pathways for neuronal differentiation and morphogenesis as well as for transcriptional and metabolic processes. The most significantly hypermethylated pathways based on gene promoter methylation status were found in the regulation of gastrulation (OTX2) and cellular responses to fibroblast growth factor (PTBP1; POLR2D; NOG). In addition, promoters of 17 different genes involved in nucleic acid template transcription processes, 18 promoters involved in nucleic-based complex metabolic processes and 19 promoters involved in morphogenesis and neuron differentiation showed higher levels of methylation in GBM1. Compared to CG2, in GBM1 we found hypomethylation at the promoters of genes

involved in synapse organization (GHSR; HSPA8; FZD9; SEMA3F) and formation (AMIGO1; NTRK1; THBS2), proliferation of endothelial cells (HIF1A; EGFL) and myelination of neurons (NKX-6; KCNJ10; NCSTN; TENM4).

4.1.2 Comparison of recurrent tumors (GBM2) with normal brain control (CG2) samples

Pathways were identified based on significantly hypermethylated gene promoters for signaling pathways involved in the regulation of transcription (e.g., CEBPB; ENY2), cell adhesion processes (e.g., ASTN1-2; NLGN1), and embryonic development (ALX3; HOXD10; NOG). SALL4) when GBM2 tumors were compared to CG2. Pathways involving significantly hypomethylated gene promoters were found in signaling pathways of purine and pyrimidine bases (SLC28A1), Golgi transport (SGSM2; GCC2) and allantoin catabolism (ALLC) in GBM2 samples compared to CG2.

4.1.3 Comparison of recurrent tumors (GBM2) with primary tumors (GBM1)

Based on the methylation status of gene promoters, GO analysis identified hypermethylated pathways involved in various functions of the canonical Wnt signaling and the regulation of catecholamine secretion (SYT15; SYT17; PINK1; OXTR) and transport (SLC18A2; TORA1). In

addition, significant hypermethylation was observed in biologically important pathways of receptor signaling (CACNG8; TSG101; DLG1), generation of cellular responses (NDUFA13; DROSHA; FMR1) and other signaling processes (PTP4A3; FRMPF1; PRKD2; MBIP; RNF6; NOD1). In contrast, compared to primary tumors, recurrent tumors were found to have significantly lower methylation in gene promoters within signaling pathways involved in the regulation of immune responses. Pathways were also identified with hypomethylated promoters in the regulation of lymphocyte-mediated immunity (TFRC; FOXJ1; ILR4; ILR6), natural killer (NK) cells (HAVCR2; SERPINB9; LAMP1; CADM1), leukocyte-mediated (ICAM1), and T cell-mediated cytotoxicity (MICA; DUSP22).

4.1.4 Locus Overlap Analysis (LOLA)

LOLA analysis was used to identify genomic regions and regulatory elements that are particularly relevant for functional interpretation of epigenomic data. We focused on the 1000 most hypomethylated and hypermethylated tiling regions in the three (GBM1, GBM2, CG2) datasets.

When comparing the control group with primary and recurrent tumors, the enrichment analysis showed similar results. In both GBM1 and GBM2, we observed enrichment of hypomethylated regions for transcription factor (e.g., RUNX1; ESR1; ESR2; CTCF) binding sites

and for histone proteins (e.g., H3K4me1; H3K4me2; H3K4me3; H3K9me3; H3K27me3) essential for the differentiation of normal embryonic stem cells and the maintenance of a differentiated lineage.

Comparing GBM2 vs. GBM1, enrichment of hypomethylated regions were noted also for binding sites of different transcription factors (FOXA2; ESR1; ESR2; RXR) and histone proteins (H3K27me3; H3K9me3; H3K4me1; H3K4m2) in the recurrent tumors.

4.1.5 Clinicopathological correlations

In these analyses, the time periods elapsed between the diagnosis of the primary (T1) and recurrent tumors (T2) were compared with the sex and age of patients, and the morphological subgroups of tumors, mitotic rates, measures of microvascular proliferation, the numbers of infiltrating lymphocytes and the degrees of necrosis. We did not find any significant correlation among these parameters. However, a trend-like relationship was found between T1-T2 and the amount of lymphocytes infiltrating the tumor in GBM1 ($p = 0.08$).

4.2 Correlation analyses of promoter + gene CpG methylation and protein expression levels of the selected catecholamine pathway markers

In this substudy, we opted to assess the promoter+gene methylation levels of the four selected catecholamine pathway markers in individual GBM1, GBM2 and CG2 samples, and to correlate these measures with the protein expression of these markers in the same samples. Among the selected markers, we had two receptors (ADRA1D; DRD2), one receptor kinase (ADRBK1) and one transporter (SLC18A2), thereby providing information for several points of the signaling pathway.

4.2.1 Quantitative IHC assessments of protein expression for the selected catecholamine pathway markers in GBM sample pairs and normal controls

In the expression studies, the control group (HC) was represented by 6 postmortem FFPE brain samples (from individuals who died of non-neurological disease). After performing quantitative IHC, the CS (complex score) values were compared between the HC, GBM1 and GBM2 sample groups.

The median and IQR [interquartile range] CS values of the ADRA1D marker were significantly lower in GBM2

(5 [15-5]) (p = 0.005) and tendentially lower in GBM1 (15 [25-5]) than in HC (30 [59 - 13]). In contrast, ADRBK1 expression was significantly higher in both GBM1 (75 [85-30]) (p = 0.004) and GBM2 (40 [75-20]) (p = 0.012) compared to HC (9 [25-4]). Although no significant difference was found for DRD2, a trend for higher expression was seen in GBM1 (70 [80-40]) compared to HC (45 [54-40]). In the GBM1 – GBM2 comparisons, quantitative IHC studies showed significantly higher expression levels in GBM1 for ADRBK1 (75 [85–30] vs. 40 [75–20]) (p = 0.011) and DRD2 (70 [80-40] vs. 40 [60-20]) (p = 0.026). CS values of ADRA1D (15 [25-5] vs. 5 [15-5]) and SLC18A2 (10 [20-5] vs. 5 [10-3]) were only tendentially higher in GBM1 than in GBM2.

4.2.2 Promoter + gene methylation levels of selected catecholamine pathway markers in individual GBM and control samples

Promoter + gene CpG methylation levels of the four catecholamine markers were compared among the methylation control group, CG2 and GBM1 and GBM2. Similar ADRA1D (median 2.56 [3.04-2.08]) and SLC18A2 (median 1.08 [2.30-0.95]) promoter + gene methylation was found in the CG2 and GBM1 (0 [1.44-0] vs. 0 [1.22-0]) and the CG2 and GBM2 (0 [1.44-0] vs. 0.54 [1.08-0]) comparisons. In contrast, the promoters and genes of ADRBK1 and DRD2 showed significantly lower methylation levels in GBM1 (ADRBK1: 0.76 [1.33-0]; p

= 0.006; DRD2: 0 [0.46-0]; $p = 0.041$) and GBM2 (ADRBK1: 0.76 [1.71-0]; $p = 0.01$; DRD2: 0 [0.15-0]; $p = 0.019$) than in CG2 (ADRBK1: 5.31 [7.97-4.56]; DRD2: 1.22 [2.06-1.22]), which inversely correlated with the protein expression levels of these markers found in the GBM1 and HC groups. No significant differences were found between promoter + gene methylation levels of the four markers in the GBM1 vs. GBM2 comparisons, indicating that the significant (ADRBK1, DRD2) and trend-like (ADRA1D, SLC18A2) decreases of protein expression levels in GBM2 vs. GBM1 is not exclusively related to epigenetic regulation by CpG methylation.

4.2.3 Promoter + gene methylation levels of the four selected catecholamine pathway markers in the validation database GBM cohort

To confirm the methylation results of the selected catecholamine markers observed in our own cohort, we determined the DNA CpG methylation levels within the same regions of the four markers in a database cohort containing similar RRBS data from 112 FFPE GBM sample pairs [11; https://www.ebi.ac.uk/ena, access number: EGAS00001002538]. To avoid statistical bias due to differences in cohort size, only the methylation results of the 112 GBM1 and GBM2 pairs were compared. Similar to our own cohort, we found no statistically significant differences in the promoter + gene methylation levels when GBM1 and GBM2 groups of this validation

cohort were compared, though a slight trend-like increase in the methylation of some markers (ADRA1D: 8.33 [16.66-3.84] vs. 8.01 [19.38-3.84] ; ADRBK1: 17.46 [28.85-8.35] vs. 18.98 [33.40-9.87]; DRD2: 4.59 [7.95-2.14] vs. 5.96 [9.17-2.75]; SLC18A2: 5.96 [11.37-3.25] vs. 7.58 [13.00-3.25]]) was noted in the recurrent samples.

5. Discussion

Our first, CpG methylation study on sequential FFPE tumor samples revealed several important differentially methylated signaling pathways that may play a role in the development and progression of GBM. Based on lower methylation levels, we hypothesize higher gene expression and thus, activity, of the identified pathway.

In the comparisons of GBM1vs.CG2 and GBM2vs.CG2, pathways involved in synapse organization, endothelial cell proliferation and myelination or those involved in intracellular biological processes and metabolisms were identified with lower methylation in GBM1 or GBM2, respectively. In contrast, pathways involved in gastrulation, trasncription and metabolic processes, and neuronal differentiation, or those involved in cell adhesion and embryonic development were more methylated in GBM1 or GBM2 compared to CG2, respectively. Altogether, these observations reflected a

disordered process of normal cellbiology during gliomagenesis.

In the comparison of GBM2vs.GBM1, we detected lower methylation in pathways of more specific cellular functions in GBM1. For example, regulatory elements, ligands (e.g. Wnt11) and receptors (e.g. Wnt11) of the canonical Wnt signaling pathway involved in endothelial cell migration and cell adhesion appeared to be more active in GBM1. In contrast, the promoters of some Wnt ligands (e.g., Wnt6; Wnt7b) and receptors (e.g., Fzd1; Fzd3) were less methylated in GBM2, and thus, likely more active. Another notable result of GO analyzes is the lower methylation (presumably higher activity) of promoters in GBM1 for genes involved in catecholamine secretion and transport. In glioma initiator cells, monoamine signaling is involved in the diversion of normal developmental mechanisms and promotes tumor formation. Synaptic monoamines in the GBM microenvironment affect angiogenesis and tumor growth [10]. The lower methylation of these pathways in GBM1 than in GBM2 may be a valuable molecular indicator of early- and late-stage tumor characteristics that merit further studies. Using different methodological approaches, there have been sparse reports in the cancer literature regarding other differentially methylated catecholamine genes (e.g. ADRA2c; ADRA1a; DRD5) and pathways (e.g. ADRA1b, ADRA2a; DRD1) [10, 12; 13; 14]. However, analyses of catecholamine pathway

changes in sequential FFPE GBM samples has not been described to date. In contrast to elements of the catecholamine pathway, immune pathways, including leukocyte regulation, and lymphocyte and NK cell mediated immunity, showed lower methylation in GBM2 than in GBM1. However, there were immunoregulatory processes such as macrophage inflammatory protein production and proliferation of CD8 + T cells that appeared to be more active in GBM1 based on lower methylation of gene promoters. These data are consistent with the correlation we found between the amount of lymphocytes (TIL) infiltrating GBM1 tumors (as determined by IHC) and the time between occurrence and recurrence of GBM (T1 – T2).

In our second study, to obtain more accurate information about catecholamine markers and to test the putative inverse relationship between CpG methylation and protein expression levels, we first performed quantitative IHC on sections from the same FFPE sample blocks of the GBM1 and GBM2 cohorts. Protein expression levels were significantly higher for ADRBK1 and DRD2, but only tendentially higher for ADRA1D and SLC18A2 in GBM1 than in GBM2. Compared with the HC group, ADRA1D protein expression levels were lower and ADRBK1 levels were higher in the tumors. DRD2 and SLC18A2 showed no significant differences in these HCvs.GBM1/2 comparisons.

Subsequently, the promoter + gene CpG methylation levels of the 4 markers were analyzed in individual samples and compared with the methylation control group (CG2). These evaluations showed significantly or slightly higher methylation levels for the 4 markers in CG2 compared to GBM1 and GBM2. In case of ADRBK1 and DRD2, significantly higher methylation levels were detected in the CG2 samples compared to both GBM1 and GBM2, which were inversely correlated with the protein expression levels of these markers in the HC and GBM1 comparisons. We expected that consistently lower promoter + gene methylation levels would be detected, when the protein expression levels were high, however, no such inverse relationship was found in the GBM2vs.GBM1 comparisons, where no methylation differences accompanied the differences in protein expression levels.

As a final step, we validated our promoter + gene methylation results in GBM1/GBM2 by analyzing RRBS data from 112 FFPE GBM pairs in a database [11]. Comparing theses primary and recurrent GBMs, we found no significantly different methylation levels for the 4 markers, similar to our own data. Altogether, the lack of consistently lower promoter + gene methylation along with the observed significantly or slightly higher protein expression of the 4 markers in GBM1 compared to GBM2, may suggest mechanisms other than CpG methylation involved in gene expression regulation

during GBM progression. An inverse correlation between protein expression and promoter + gene methylation was also observed only for ADRBK1 and DRD2 in the GBM1vs.CG2 comparisons, and for ADRBK1 in the GBM2vs.CG2 comparisons, further reinforcing the role of other factors influencing protein expression even during gliomagenesis. Changes in methylation may affect not only a particular gene and promoter region, but also a number of other molecular elements involved in the regulation of gene expression. Such elements include gene enhancer / silencer regions, transcription factor binding sites, splice sites, or regions encoding microRNAs and siRNAs [15]. These mechanisms, may at least partly explain, why we did not see the expected inverse correlation between marker protein expression and promoter + gene methylation levels in every comparison.

In summary, our studies identified several differentially methylated signaling pathways in longitudinal GBM, using clinical FFPE specimens and a novel library preparation method, RRBS. This is the first human GBM study demonstrating in depth the involvement of neurotransmitters, namely elements of the catecholamine pathway, in gliomagenesis. Although our data are still exploratory, it is likely that neurotransmitters, their receptors and mediators may play an important modulatory role in gliomagenesis. Despite the technical limitations, these results are consistent with, and

complementary to, the hitherto available sporadic data in the literature, and provide a ground for further focused research.

6. New findings

We infer from our epigenomic results that

-Biochemical pathways involved in synapse formation, myelination and endothelial cell proliferation are less methylated, and thus, transcriptionally likely more active in primary GBM samples compared to normal brain controls.

- Pathways responsible for essential cellular responses, signaling and communication, and catecholamine signaling, secretion, and transport are more active in primary than recurrent GBM tumors.

- Several immunoregulatory pathways (leukocyte, lymphocyte and NK cell mediated immunity) have lower activity in primary GBM compared to recurrent tumors

- Analyzing promoter + gene methylation and protein expression levels in individual GBM and control samples we observed that

- Protein expression levels of targeted catecholamine markers are significantly (ADRKB1; DRD2) or tendentiously (ADRA1D; SLC18A2) higher in primary than in recurrent GBM.

- Promoter + gene CpG methylation levels of the 4 markers are significantly or tendentiously higher in the control samples (CG2) than in primary or recurrent GBM, but do not differ between the two tumor groups.

- Similar promoter + gene methylation levels of the 4 markers could be also confirmed in the validation database cohort of primary and recurrent GBM.

- Apart from the methylation status of the promoter + gene regions, other processes also play important roles in the regulation of catecholamine gene expression.

- The studied neurotransmitters and their receptors play an important, thus far unexplored, modulatory role in the molecular pathogenesis of gliomas, and their further studies may reveal new potential therapeutic targets.

7. References:

1. Louis, D. N., Perry, A., Wesseling, P., Brat, D. J., Cree, I. A., Figarella-Branger, D., ... & Ellison, D. W. (2021). The 2021 WHO classification of tumors of the central nervous system: a summary. *Neuro-oncology*, *23*(8), 1231-1251. https://doi.org/10.1093/neuonc/noab106

2. Ohgaki H, Kleihues P (2012). The definition of primary and secondary glioblastoma. *Clin Cancer Res*. 9:764-772.https://doi.org/10.1158/1078-0432.CCR-12-3002.

3. Stupp R, Mason WP, Van Den Bent MJ, Weller M, Fisher B, Taphoorn MJ et al. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *New Engl J Med.* 352:987–996. https://doi.org/10.1056/NEJMoa043330.

4. Weller M, Cloughesy T, Perry JR, Wick W (2012) Standards of care for treatment of recurrent glioblastoma—are we there yet? NeuroOncol 15(1):4–27. https://doi.org/10.1093/neuonc/nos273

5. The Cancer Genome Atlas (TCGA) Research Network (2008). Comprehensive genomic characterization defines human glioblastoma

genes and core pathways. *Nature*. 455:1061–8. https://doi.org/10.1038/nature07385.

6. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD et al. (2010). Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell*. 17:98–110. https://doi.org/10.1016/j.ccr.2009.12.020.

7. Noushmehr H, Weisenberger DJ, Diefes K, Phillips HS, Pujara K, Berman BP, Pan F, Pelloski CE, Sulman EP, Bhat KP, Verhaak RG, Hoadley KA, Hayes DN, Perou CM, Schmidt HK, Ding L, Wilson RK, van den Berg D, Shen H, Bengtsson H, Neuvial P, Cope LM, Buckley J, Herman JG, Baylin SB, Laird PW, Aldape K, Cancer Genome Atlas Research Network (2010) Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. Cancer Cell 17(5):510–522. https://doi.org/10.1016/j.ccr.2010.03.017

8. Horgan RP, Kenny LC (2011) ‘Omic’technologies: genomics, transcriptomics, proteomics and metabolomics. Obstet Gynecol 13(3): 189–195. https://doi.org/10.1576/toag.13.3.189.27672

9. Sarkar C, Chakroborty D, Basu S. Neurotransmitters as regulators of tumor angiogenesis and immunity: the role of catecholamines. J NeuroImmune Pharmacol. 2013;8:7–14. https://doi.org/10.1007/s11481-012-9395-7

10. Caragher, S. P., Hall, R. R., Ahsan, R., & Ahmed, A. U. (2018). Monoamines in glioblastoma: complex biology with therapeutic potential. *Neuro-oncology*, *20*(8), 1014-1025. https://doi.org/10.1093/neuonc/nox210

11. Klughammer J, Kiesel B, Roetzer T, Fortelny N, Nemc A, Nenning KH, Furtner J, Sheffield NC, Datlinger P, Peter N, Nowosielski M, Augustin M, Mischkulnig M, Ströbel T, Alpar D, Ergüner B, Senekowitsch M, Moser P, Freyschlag CF, Kerschbaumer J, Thomé C, Grams AE, Stockhammer G, Kitzwoegerer M, Oberndorfer S, Marhold F, Weis S, Trenkler J, Buchroithner J, Pichler J, Haybaeck J, Krassnig S, Mahdy Ali K, von Campe G, Payer F, Sherif C, Preiser J, Hauser T, Winkler PA, Kleindienst W, Würtz F, Brandner-Kokalj T, Stultschnig M, Schweiger S, Dieckmann K, Preusser M, Langs G, Baumann B, Knosp E, Widhalm G, Marosi C, Hainfellner JA, Woehrer A, Bock C (2018) The DNA methylation landscape of glioblastoma disease progression

shows extensive heterogeneity in time and space. Nat Med 24(10):1611–1624. https://doi.org/10.1038/s41591-018- 0156-x

12. Etcheverry, A., Aubry, M., De Tayrac, M., Vauleon, E., Boniface, R., Guenot, F., ... & Mosser, J. (2010). DNA methylation in glioblastoma: impact on gene expression and clinical outcome. *BMC genomics*, *11*(1), 1-11. https://doi.org/10.1186/1471-2164-11-701

13. Nagarajan, R. P., Zhang, B., Bell, R. J., Johnson, B. E., Olshen, A. B., Sundaram, V., ... & Costello, J. F. (2014). Recurrent epimutations activate gene body promoters in primary glioblastoma. *Genome research*, *24*(5), 761-774. doi:10.1101/gr.164707.113

14. Caragher, S. P., Shireman, J. M., Huang, M., Miska, J., Atashi, F., Baisiwala, S., ... & Ahmed, A. U. (2019). Activation of dopamine receptor 2 prompts transcriptomic and metabolic plasticity in glioblastoma. *Journal of Neuroscience*, *39*(11), 1982-1993. DOI: https://doi.org/10.1523/JNEUROSCI.1589-18.2018

15. Li, Z., & Rana, T. M. (2012). Molecular mechanisms of RNA-triggered gene silencing machineries. *Accounts of chemical*

research, *45*(7), 1122-1131. https://doi.org/10.1021/ar200253u

8. Acknowledgements

I would like to express my gratitude to my supervisor, who over the past three years, with endless patience and support, has paved the way for me as a professional leader and allowed me to get this far.

I am grateful to the staff of the Institute of Pathology of the University of Pécs, who were always ready to help us with our work.

I would like to thank for the collaborators at the Laboratory Medicine Institute of the University of Pécs and the Genomics and Bioinformatics Core Facility of the János Szentágothai Research Center for their joint work, especially Péter Urbán, to whom we could turn with our questions and problems at any time.

I am grateful to all those unfortunate patients whose samples lef over from routine clinical trials we were able to use in our work.

Last but not least, I am grateful to my family, friends, and partner for their perseverance, encouragement, and assistance throughout my studies and work.