The role of the Wnt pathway in the biology of glioblastoma

Doctoral (PhD) – thesis

Márton Tompa

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

Abbreviations

APC – Adenomatous Polyposis Coli Protein
AXIN2 – Axis inhibiton protein 2
CS – „Complex Score”
DAAM1– Dishevelled-associated activator of morphogenesis 1
DNA – Deoxyribonucleic acid
DVL1 – Dishevelled segment polarity protein 1
EGFR – Epidermal growth factor receptor
EMT – Epithelial–mesenchymal transition
FFPE – Formalin-Fixed Paraffin-Embedded
Fzd – Frizzled
GBM – Glioblastoma
GO – Gene ontology
GSK-3β – Glycogen synthase kinase 3β
IDH1/2 – Isocitrate dehydrogenase 1 and 2
IHC – Immunohistochemistry
LEF1 – Lymphoid enhancer factor 1
LRP6 – Low density lipoprotein receptor-related protein 6
MUTH – Markusovszky University Teaching Hospital
NF-1 – Neurofibromin 1
NGS – Next generation sequencing
NLK – Nemo Like Kinase
PCR – Polymerase chain reaction
UP –University of Pécs
ROI – „Region of Interest”
ROR2 – Receptor tyrosine kinase-like orphan receptor 2
Ryk – Receptor-like tyrosine kinase
RRBS – Reduced representation bisulfite sequencing
TCGA – The Cancer Genome Atlas

1. Introduction

Glioblastoma (GBM) is the the most severe (grade IV) glioma and the most common primary brain tumor with a median overall survival of 14–20 months [1,2]. The currently used therapeutic intervention is described by Stupp et al. [1]. The majority of GBMs (90-95%) develop *de novo*. These primary GBM tumors show aggressive and highly invasive properties, and typically occur in older adults (median age 62 years). In contrast, secondary GBMs are less frequent (5–10%) and develop at a younger age (median age 45 years) as a result of progression from lower-grade gliomas [3]. Molecular properties of the primary and secondary GBMs differ as they accumulate different mutations and activate different pathways during their formation.

"The Cancer Genome Atlas" (TCGA) published the first comprehensive identification of the most common somatic mutations accumulated in GBM, and also separated the classical, mesenchymal and proneural molecular subgroups based on integrated analyses of genomic and transcriptomic data [4,5]. The classical subgroup is characterized by EGFR (epidermal growth factor receptor) amplification, and EGFRvIII mutation. Deletions/mutations in the NF-1 (neurofibromin 1) gene are common in the mesenchymal subgroup. Finally, the p53 and IDH-1/2 (isocitrate dehydrogenase 1/2) genes are frequently mutated in the proneural subgroup. The mutant IDH enzyme produces an oncometabolite that profoundly affect DNA CpG methylation genome-wide and consequenty influences transcriptional profiles [6]. Overall, a genome-wide shift

towards hypomethylation of CpGs is characteristic of cancers, which, however, may occur with locus-specific hypermethylation [7-9].

We focused our studies on the activity and expression regulation of Wnt pathway in GBM [10-13]. The involvement of the Wnt pathway is well defined in many tumors, but relatively little information is available in gliomas. Both the canonical and the non-canonical Wnt subpathways are highly conserved, and play key roles in the early stages of embryogenesis as well as in pathological conditions such as cancer development [11-13]. Reactivation of the Wnt pathway in tumors is predominantly caused by epigenetic changes rather than by mutations. The activation or inhibition of the pathway is not only based on ligand-receptor binding, but is also influenced by negative and positive regulators. A few studies suggest that expressions of most Wnt ligand and frizzled (Fzd) receptor proteins are significantly higher in GBM than in normal brain [14, 15], while the highest in the invasive zone [16,17]. Wnt mRNA molecules are also overexpressed in GBM as well as in other, lower grade gliomas [5, 17, 18,]. Though the relationship between Wnt pathway markers and IDH 1/2 mutations is of major interest, it has not been unequivocally defined [13, 19, 20].

Taken together, both the canonical and non-canonical Wnt pathways play prominent roles in the development, spread, chemo- and radiation therapy resistance and recurrence of GBM. Therefore, a better understanding as to how the pathway works may be key to the management of GBM, and some of its elements may also be potential treatment targets.

2. Hypotheses and Aims

Hypotheses

The Wnt canonical and non-canonical pathway elements:

1. Play important roles in the development of gliomas depending on grade, histological subtype and molecular characteristics;
2. Are differentially expressed in molecular subgroups and during the progression of GBM; and
3. Are expressed at least under partial control of promoter and gene CpG methylation during tumor progression.

Aims

1. Quantitative IHC (immunohistochemistry) analyses of Wnt pathway marker expression patterns according to glioma grade, histological origin and molecular characteristics;
2. Investigation of the Wnt pathway marker expression patterns by quantitative IHC in classical, mesenchymal and proneural subgroups of GBM;
3. Analyses of the relationship between protein expression and promoter+gene CpG methylation in elements of Wnt pathway markers by RRBS (reduced representation bisulfite sequencing), NGS (next generation sequencing) and quantitative IHC in sequential GBMs.

3. Materials and methods

3.1. Subjects of the studies in general

Glioma samples were collected at the Department of Pathology, Markusovszky University Teaching Hospital (MUTH), and at the Institute of Pathology, University of Pécs (UP). The studies were carried out in the laboratories of MUTH and the Szentágothai János Research Center. Glioma samples of grade II, III and IV were obtained before radiotherapy and/or chemotherapy. All primary samples of GBM pairs were obtained by surgery before chemo- and radiotherapy, while recurrent samples were obtained after treatment. Local and regional ethical committee (EC) approvals include MUTH EC 56/2012, 21/2014, 28/2014, 025782-003/2014 and UP EC 7517- 2018 and -2019.

3.2. Expressions of Wnt pathway markers according to glioma grade, lineage and molecular determinants

A total of 72 grade IV FFPE (formalin-fixed paraffin-embedded) glioma samples from 69 patients and 19 grade II and III glioma samples from 19 patients were analyzed.

3.3. Expressions of Wnt pathway markers in GBM molecular subgroups

Three cohorts were studied. In cohort 1, 81 cross-sectional FFPE samples from a total of 78 patients were included. Patients' age, sex and overall survival (weeks between surgery and death) were used for correlations with molecular subgroups. Cohort 2 consisted of 19 sequential FFPE GBM samples from 8 patients. Cohort 3 included 8 FFPE post-mortem sections each including a normal brain region, invasion zone and GBM.

3.4. Expressions of Wnt pathway markers in correlation with their promoter and gene CpG methylation profiles in sequential GBM

This study was conducted in two subcohorts. In cohort 1, 21 pairs of primary (GBM-P) and recurrent (GBM-R) FFPE GBM samples were included from the Pathology Department, UP. In Cohort 2, we used the publicly available methylome data of sequential FFPE GBMs by Klughammer et al. [21].(https://www.ebi.ac.uk/ena: EGAS00001002538). In the IHC analyses, 6 postmortem normal brain tissues, while in the epigenomic analyses, 5 epilepsy surgery samples from the European Nucleotide Archive database were used as a controls [21].

3.5. Immunohistochemistry

Primary antibodies were optimized in a pilot study. Tissue samples were retrieved by citrate buffer, or by proteinase K treatment. Binding of primary antibodies was detected by using the Novolink Polymer Detection System. For each section, a pathologist selected the "region of interest" (ROI). The percentage of stained cells and the staining intensity within the ROI were determined, and multiplied by each other to generate the "Complex Score" (CS). Grade II and III astroglial and oligodendroglial tumors were distinguished by histological and molecular studies following the 2016 revision of the WHO guideline for brain tumor classification [22]. For IDH-1 R132H and EGFRvIII, the presence or absence of the mutant molecule was recorded. For Wnt3a, beta-catenin, Fzd10, Wnt5a, Wnt7b and Fzd2, as well as for EGFR and NF-1, the CS values were calculated.

3.6. DNA isolation

Four-five sections were made from the FFPE tissue blocks. After deparaffinization, DNA was isolated by using the a QIAamp DNA FFPE Tissue Kit. DNA fragment analysis for the epigenomic study was performed by using the Genomic DNA ScreenTape Assay kit on an 4200 TapeStation System.

3.7. Pyrosequencing

The IHC detected IDH-1 R132H mutations were validated and further refined by pyrosequencing. PCR (polymerase chain reaction) and sequencing primers were synthesized based published sequences by the BioScience Kft. The reverse PCR primer was biotin conjugated.

3.8. Epigenomics

We used the reduced representation bisulfite sequencing (RRBS) kit 24x for library preparation following the manufacturer's instructions (Diagenode). To compensate for DNA quality variations in the samples, we increased the amount of input DNA from the recommended 200ng to higher amounts.

DNA was digested by MspI that recognized CCGG sites, thus samples were enriched for fragments ending with CpG. After end preparation, adapter ligation and size selection, samples were pooled to undergo bisulfite conversion. Converted libraries were PCR amplified and checked for quality and quantity, before sequencing on a NextSeq 550 sequencer. Raw sequencing data were uploaded to the European Nucleotide Archive (https://www.ebi.ac.uk/ena, Primary Accession: PRJEB38380, Secondary Accession: ERP121800).

3.9. Bioinformatics

The raw sequence data was filtered by the FastQC program and adapters were removed. Bisulfite-converted reads were compared to the hg19 (GRCh37) reference genome. The RnBeads software was used to determine differentially methylated CpG sites, genes, regions, and gene ontology (GO) pathways comparing controls and primary and recurrent GBM samples at cohort level. DNA methylation levels in individual promoter+gene regions of selected Wnt pathway markers were determined by using an in-house script prepared in the BioMethyl R package. Methylation levels were given as percentages calculated from the identified numbers of methylated sites with the relative degree of methylation at each site also computed in the numerator that was divided by the number of all possible methyltated sites within the selected genomic region of interest, and then multiplied by 100. We also assessed methylation levels within enhancers of the selected six Wnt markers using the GeneCards Genhancer program for the database GBM samples.

3.10. Statistics

The Mann-Whitney U test was used to compare two independent samples, while the Wilcoxon signed rank test was used for dependent sample pairs. When comparing several samples simultaneously, the Kruskal-Wallis test was used. The relationships between Wnt markers and subgroup determinants, or markers and overall survival data were examined by the Kendall's τ correlation analysis. Kaplan-Meier curves were used for comparing the overall survival data in different subgroups of GBM.

4. Results

4.1. Expressions of Wnt pathway markers according to glioma grade, lineage and molecular determinants

Significantly higher expression levels were observed for Wnt5a (grade III vs. grade IV $p<0.001$) and Wnt3a (grade II vs. grade IV $p=0.036$; and grade III vs. grade IV $p=0.001$) in 72 GBM samples compared to 9 grade II and 10 III gliomas. We detected a gradual increase in the expression levels of Wnt markers with increasing tumor grades. In the case where the merged 19 grade II-III gliomas were compared to 72 grade IV GBM, the expression of Wnt5a ($p<0.001$), Wnt3a ($p<0.001$) and cytoplasmic beta-catenin ($p=0.039$) were elevated in GBM. When GBM samples were compared to 8 normal brain region samples, all markers (except Wnt3a) showed elevated expression (Wnt5a $p=0.004$; Fzd2 $p=0.015$; cytoplasmic beta-catenin $p<0.001$; nuclear beta-catenin $p=0.001$) in the tumors. Only the citoplasmic ($p=0.016$) and nuclear ($p=0.030$) expression of beta-catenin differed between the normal brain regions and the grade II-III tumors.

In the 8 astroglial and 11 oligodendroglial sample comparisons, only the expression of Wnt5a was significantly higher in the astroglial group ($p=0.02$).

Of the 19 grade II-III gliomas, 15 were IDH-1 mutant and 4 wild-type. No significant differences were found in the expression levels of Wnt markers in the mutant vs. wild-type samples. When we compared CS values of Wnt markers in all 91 gliomas (grade II–IV), only Wnt3a showed significantly higher expression ($p=0.006$) in the IDH-1 wild-type, which consisted of 69 samples.

4.2. Expressions of Wnt pathway markers in GBM molecular subgroups

In the cohort 1 cross-sectional studies, 61.7% (50/81) of tumors from 78 patients segregated into one of the GBM subgroups (**Figure 1**).

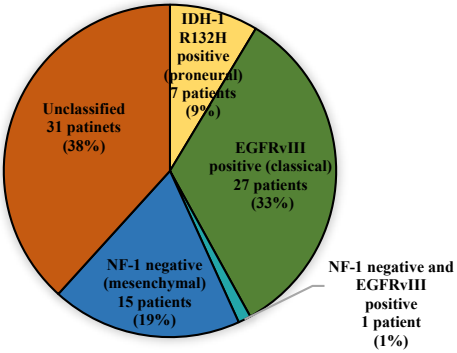

Figure 1: *Segregation of GBM specimens based on key markers.*

The 7 patients with IDH-1 R132H mutant GBM (proneural subgroup) were younger (p=0.05) than the 74 patients with IDH-1 wild type GBM, while the 15 patients with double NF-1 negative (cytoplasm-/nucleus- or c-/n-, mesenchymal subgroup) GBM were older than the 66 patients with NF-1 protein expressing GBM (p=0.039). There was no age difference between the 27 patients with EGFRvIII mutant (classical subgroup) and the 54 patients with EGFR wild type tumors. When the patients' overall survival figures were compared based on GBM subgroups, no significant differences were observed, except for the longer survival of patients with IDH-1 R132H mutant tumors (p=0.052) compared to those with IDH1 wild-type tumors.

When we compared Wnt expression levels among the molecular subgroups, no significant differences were found. Nevertheless, we detected significant differences in the expression distribution levels of the canonical and non-canonical pathway markers within subgroups. In the proneural subgroup which consisted of 7 patients, significant difference was only observed between Wnt5a and Wnt3a ($p=0.012$). More striking differences were seen in the mesenchymal subgroup with 15 patients: Wnt5a expression was significantly higher (Wnt5a vs. Wnt3a $p<0.001$; Wnt5a vs. cytoplasmic beta-catenin $p=0.002$; Wnt5a vs. nuclear beta-catenin $p<0.001$) than any of the canonical pathway markers. Also, Fzd2 showed increased expression compared to Wnt3a ($p=0.009$) and nuclear beta-catenin ($p=0.04$). In the classical subgroup of 27 patients, Wnt5a had higher expression than Wnt3a ($p=0.021$) and nuclear beta-catenin ($p<0.001$), and Fzd2 also had higher CS values than nuclear beta-catenin ($p=0.046$). Finally in the unclassified subgroup with 31 patients, Wnt5a levels were significantly higher than Wnt3a ($p<0.001$) or nuclear beta-catenin levels ($p<0.001$), while Fzd2 also had higher CS values than nuclear beta-catenin ($p=0.01$). Only in this subgroup did we find significantly higher expression levels of cytoplasmic that nuclear beta-catenin ($p=0.005$). Regarding the IDH-1 mutation status, only Wnt5a alone showed a tendency for increased expression ($p=0.071$) in mutant GBM group.

Both NF-1 and beta-catenin are known to localize to the cytoplasm under normal circustances, but translocate to the nucleus or lost in tumors. In our cohort, the expression of nuclear beta-catenin was higher in the subgroup with nuclear NF-1 expression or NF-1 lost phenotype compared to those

with cytoplasmic NF-1 expression (p=0.021 and p=0.03, respectively).

When analyzing the relationship between molecular subgroup and Wnt pathway markers, we only detected correlation between the cytoplasmic (p=0.01) or membrane EGFRvIII (p=0.035) expression levels and Fzd2 levels.

In the combined cohort 1 analysis, Wnt5a significantly correlated with Fzd2 (p=0.036).

In the sequential cohort 2 study, we detected no significant differences in the expression levels of any of the Wnt markers when comparing the primary and recurrent GBM pairs of eight patients.

In the post-mortem cohort 3, a gradual increase in the expression levels of Wnt markers was noted when regions of normal tissue - invasion zone - tumor were compared. The expression of Wnt5a (p=0.003) and Wnt3a (p=0.029) was the lowest in normal brain tissue and the highest in the tumor central zone.

4.3. Expressions of Wnt pathway markers and DNA methylation patterns in sequential glioblastoma

In our cohort level genome-wide methylation analyses, Wnt7b, Wnt11 were hypermethylated, while Wnt6 was hypomethylated in GBM-P compared to the GBM-R group. For receptors and co-receptors, Fzd1, Fzd3, Fzd10, LRP6 (Low density lipoprotein receptor-related protein 6), and ROR2 (Receptor tyrosine kinase-like orphan receptor 2) were hypermethylated, and Ryk (Receptor-like tyrosine kinase) was hypomethylated in GBM-P and GBM-R comparison. Among the intermediate molecules of the canonical pathway, AXIN2 (Axis inhibiton protein 2) was

hypermethylated, while APC (Adenomatous Polyposis Coli Protein), DVL1 (Dishevelled segment polarity protein 1), GSK-3β (Glycogen synthase kinase 3β), LEF1 (Lymphoid enhancer factor 1) were hypomethylated in GBM-P vs. GBM-R. Among the intermediate molecules of the non-canonical pathway, DAAM1 (Dishevelled-associated activator of morphogenesis 1) was hypermethylated and NLK (Nemo Like Kinase) was hypomethylated in GBM-P vs. GBM-R comparison.

These cohort level observations prompted us to simultaneously investigate protein expression and DNA CpG methylation levels for selected Wnt pathway markers in individual control (C-PM in IHC, C-DB in epigenomics), and GBM-P and GBM-R samples.

Compared to C-PM controls, significantly higher protein expressions were observed for Wnt5a ($p=0.003$) in GBM-P and for Wnt3a ($p=0.003$) in GBM-R. The expression levels of Fzd10 were uniformly very low in all of GBM-P, GBM-R and C-PM samples. Further, a significant decrease was noted for Fzd2 in GBM-P compared to C-PM ($p<0.001$). The Wnt7b protein expression was high in C-PM, with a small decrease in GBM-P and significant drop in GBM-R ($p=0.005$). When we compared the expressions of Wnt markers at individual level in the GBM-P and GBM-R sample pairs, Wnt3a ($p=0.009$) and Fzd2 ($p=0.016$) showed a significant increase during progression. In case of Wnt7b ($p=0.019$), we observed an inverse pattern.

Comparing the Wnt marker methylation patterns in promoter+gene regions of individual GBM-P and GBM-R samples, no significant differences were detected. However

in the C-DB, GBM-P and GBM-R comparisons, significantly higher methylation levels were noted for Wnt3a ($p=0.028$) and Wnt7b ($p=0.015$) in C-DB compared to GBM-P, and for Wnt3a ($p=0.012$) and Wnt7b ($p=0.034$) in C-DB compared to GBM-R. The methylation levels of Fzd10 were markedly higher then those of other Wnt pathway markers in samples of C-DB, GMB-P and GBM-R.

We found no differences in the methylation levels of the six Wnt markers in the 21 GBM-P and GBM-R sample pairs. To validate these observations, we also assessed the methylation levels within the promoter+gene regions of the six markers in a sequential database cohort of 112 patients with GBM [21]. Similar to our own results, we found no changes in methylation levels of the selected Wnt promoters+genes during tumor progression.

We only found partialy negative correlation when comparing the protein expression and promoter+gene CpG methylation levels in the controls and our sequential GBM samples. Therefore, we also assessed the methylation levels within enhancer regions of the six Wnt markers in both our smaller and the database larger GBM cohorts. The enhancers with GeneHancer ID of Wnt7b GH22J045611, Fzd10 GH12J130124 and Fzd10 GH12J130138 had markedly higher methylation than other enhancers in the database GBM samples, and the methylation levels of these three enhancers were also higher in GBM-R than in GBM-P.

5. Discussion

Our studies underscored the involvements of Wnt pathway markers in GBM, and thereby provided further support for considering these pathway elements as therapeutic targets.

In our first substudy, we analyzed the relationship between Wnt marker expression and glioma grade, lineage and molecular profile. Pu et al. [23] and Denysenko et al. [24] reported smiliar results to our own showing correlations between Wnt marker expression levels and glioma grade, and very low levels of these markers in normal brains. [23, 24]. Wnt markers showed no expression differences according to IDH-1 mutation status in grade II, III or IV glioma subgroups, but Wnt3a appeard significantly higher in the IDH-1 wild type vs. mutant combined grade (II-III-IV) comparions. The literature on the relationship between IDH mutation and Wnt pathway marker expression is divided. Some researchers found that IDH-1 mutation causes a significant decrease in the proliferative and invasive potential of gliomas due to repression of beta-catenin signaling [19]. Others, however, found that in patients with tumors carrying IDH-1 mutation have high activity of the canonical Wnt pathway [20, 25]. It is highly likely that there is an association between the Wnt pathway activity and the IDH mutation status, however, this relationship remains to be clarified. Thus far, we and others detected only suggestive relationship between Wnt marker expression and glioma lineage, prompting further efforts to gain final conclusions.

In the second substudy, consistent with previous observations, patients in the proneural subgroup with the IDH-1 R132H mutation were the youngest and had the

longest overall survival, while patients in the mesenchymal subgroup, who lost both cytoplasmic and nuclear NF-1 expression, were the oldest and with the shortest survival [5, 12, 22].

We detected no significant differences in the expression levels of Wnt markers among the GBM molecular subgroups. However, significant differences were noted in the distribution patterns of these markers within subgroups, particularly in the non-canonical vs. canonical comparisons in the mesenchymal subgroup. Reports from TCGA and several other publications support our findings regarding Wnt5a overexpression in the mesenchymal subgroup [5, 15]. In the classical subgroup, correlations were observed between EGFR expression and both the canonical and non-canonical Wnt markers. EGFR and beta-catenin are mutually able to activate or inactivate each other's regulatory elements [26], while elements of the non-canonical pathway play an important role in the EGFRvIII-mediated tumor formation [27]. Similar to our study, data from the TCGA show that the expression patterns of Wnt markers in the unclassified GBM subgroup is similar to those of the classical subgroup, presumably due to EGFR (over) expression [5].

Investigating the connection between nuclear translocations of beta-catenin and NF-1, we found a strong correlation between them, not quite surprisingly, since these translocations in both cases are associated with tumor development. The loss or functional alteration of NF-1, a tumor suppressor, increases the aggressiveness and invasiveness of tumors through EMT (epithelial–

mesenchymal transition) [28]. We hypothesize that GBM tumors with cytoplasmic expression of NF-1 and beta-catenin have a lower probability of EMT, and thus, such tumors will be less aggressive, while GBMs with high levels of these molecules translocated to the nucleus may show the opposite properties. Overall, however, we found no correlation between expression levels of GBM subgroup markers and Wnt markers, with the exeption of Fzd2 and EGFRvIII in the classical subgroup. Furthermore, the expression levels of Wnt5a and Fzd2 correlated tendentiously with each other in this subgroup, indicating an association between the EGFRvIII mutation and the non-canonical Wnt pathway markers. In the combined cohort 1, (regardless of subgroup classification) Wnt5a significantly correlated with Fzd2, in consensus with previous GBM publications [17, 14].

Based on the literature, we expected increases in the expressions of Wnt markers in the recurrent compared to the primary GBMs in cohort 2, but our data suggested that Wnt ligands and receptors follow complex patterns of expression during tumor progression.

In cohort 3, our findings are consistent with the results of Kahlert et al. [29] and Binda et al. [16] showing that both the canonical and non-canonical pathway markers are highly expressed in the invasion and tumor zones compared to normal brain regions [29, 16].

In our third substudy, first we analyzed the differential methylation patterns in promoters+genes of six selected Wnt markers at cohort levels comparing groups of C-DB, GBM-P and GBM-R [10]. The observations from this preliminary

study prompted us to zoom into promoters and genes of selected Wnt markers, and analyze simultaneously the methylation patterns in correlation with protein expression levels in individual controls and GBM sample pairs. We found no different methylation levels in the promoter+gene regions of the six Wnt markers when comparing GBM-P and GBM-R, either in our own or in the database sequential GBM samples [21]. However, as expected based on the known shift towards hypomethylation in tumors compared to controls [30, 31], we observed a shift towards hypomethylation for most markers in GBMs compared to those in controls (with the exception of Fzd2 and Fzd10).

The promoter+gene hypomethylations were associated with a tendency or significant increase in protein expression levels of Wnt5a, Wnt3a, and beta-catenin in GBM-P and GBM-R compared to C-PM. The high promoter+gene methylation of Fzd10 consistently resulted in low levels of membrane protein expression in both control and tumor samples.

Based on the above observations showing that increasing Wnt marker expression may not be always accompanied by inverse changes in promoter and gene methylation, we can conclude that CpG methylation in these regions is not the only regulator of gene expression. Therefore, in a corollary study, we assessed the methylation levels within enhancers (which were mostly mapped outside the gene+promoter region) of the six Wnt markers in our and in the database samples. One Wnt7b and two Fzd10 enhancers showed increased methylation status compared to other enhancers, and all three of these enhancers had higher levels of methylation in the recurrent compared to primary tumor

samples of the database cohort. The very low protein expression of Fzd10 in all samples can be explained by the high level of methylation in both the gene+promoter and the enhancer regions. In case of Wnt7b, the gradual decrease of protein expression from control to GBM-P and GBM-R might be caused by the increasing DNA methylation during tumor progression. As the Wnt7b ligand binds to the Fzd10 receptor, the simultaneous hypermethylation of their promoters+genes and their low or decreasing protein expression are likely to rule out their involvement in progressive GBM.

Methylation changes may not only affect promoters, enhancers and open reading frames, but also regions of regulatory RNA molecules, splicing sites, or binding sites of topological domains of insulators, which may enhance or repress mRNA expression, translation or protein isoforms [32, 33, 34]. The complexity of regulatory mechanisms may partly explain the discrepancy between the expected and observed protein expression and promoter+gene methylation levels both in our study and the literature.

6. New findings

- In the progression of astocytic gliomas, the non-canonical pathway plays a greater role than the canonical.

- Correlations exist between the IDH-1 mutation status and the expression of some Wnt pathway elements, a finding that is more comprehensively being analyzed in our new, ongoing studies.

- The non-canonical pathway markers are more highly expressed than the canonical markers in all molecular subgroups of GBM.

- Nuclear appearance of beta-catenin and NF-1 positively correlate with each other, and likely contribute to a more aggressive behavior of GBM.

- Expression levels of our selected Wnt markers increase, but are not accompanied by inverse promoter+gene methylation patterns in primary and recurrent GBM.

- The methylation levels not only within promoter+gene regions control gene expression regulation, but those within enhancers often outside of promoters also play important roles.

- The Wnt7b and Fzd10 ligand – receptor pair does not contribute to the growth and progression of GBM.

- Wn7b and Fzd10 may have tumor suppressor potential in GBM, an unexplored possibility that merits further studies.

7. List of publications

7.1. Publications related to the PhD dissertation

Tompa M, Nagy A, Komoly S, Kalman B (2019). Wnt pathway markers in molecular subgroups of glioblastoma. *Brain Res.* 1718:114-125. https://doi.org/10.1016/j.brainres.2019.05.008. IF (2019): 2.733

Nagy A*, **Tompa M***, Kraboth Z, Garzuly F, Maraczi A, Kalman B (2021). Wnt pathway markers in low-grade and high-grade gliomas. *Ideggyogy Sz.* 74(9-10):000–000. (in press). https://doi.org/10.18071/isz.74.0000. IF (2020): 0.427

(*Shared first authors)

Tompa M, Kajtar B, Galik B, Gyenesei A, Kalman B (2021). Gene promoter methylation and protein expression of Wnt pathway markers in progressive glioblastoma. *Pathol Res Pract.* 222 (2021):153429. https://doi.org/10.1016/j.prp.2021. IF (2020): 3.250

Kraboth Z, Galik B, **Tompa M**, Kajtar B, Urban P, Gyenesei A, Miseta A, Kalman B (2020). DNA CpG methylation in sequential glioblastoma specimens. *J Cancer Res Clin* Oncol.146:2885–2896. https://doi.org/10.1007/s00432-020-03349-w. IF (2020): 4.553

The total impact factor of the publications related to the PhD dissertation: **10.963 (2021.08.08)**

7.2. Publications not related to the PhD dissertation

Maraczi A, Nagy A, Eder LK, Gyurjan I, **Tompa M**, Kovacs G, Csejtei A, Kovalszky I, Kalman B (2020). Daganatos betegek molekuláris vizsgálatai a nyugat-dunántúli régióban 2015 és 2018 között. *Egészség-akadémia.* 9(3):1-8. IF (2019):0

Kalovits F, **Tompa M**, Nagy A, Kalman B (2018). Isocitrate dehydrogenase mutations in defining the biology of and supporting clinical decision making in glioblastoma. *Ideggyogy Sz.* 71(7-08):237-247. https://doi.org/10.18071/isz.72.0141. IF (2018):0,113

Tompa M, Kalovits F, Nagy A, Kalman B (2018). Contribution of the Wnt pathway to defining biology of glioblastoma. *Neuromolecular Med. 20(4):437-451. https://doi.org/10.1007/s12017-018-8514-x.* IF (2018):2.576

Kalovits F, Takats L, Somogyi K, Garzuly F, **Tompa M**, Kalman B (2019). Secretory meningioma with bone infiltration and orbital spreading. *Ideggyogy Sz.* 72(3-4):141-144. https://doi.org/10.18071/isz.72.0141. IF (2019):0,337

7.3. Scientific presentations and posters not releated to the PhD dissertation

Tompa M, Kalman B. A glioblastoma őssejtek molekuláris genetikai jellegzetességei és szerepük a tumorgenezisben. Magyar Neurológiai Társaság konferenciája: *Magyar Klinikai Neurogenetika Szekció.* 2018. June 7-10. Debrecen

Tompa M, Kalman B. A Wnt útvonal aktivitása glioblastomában. Magyar Neurológiai Társaság konferenciája: *Magyar Klinikai Neurogenetika Szekció*. 2019. December 6-7. Debrecen

Tompa M, Kalovits F, Nagy A, Kalman B. A Wnt markerek expressziós mintázata a glioblastoma alcsoportokban. *Pécsi Tudományegyetem Idegtudományi Centrum III. PhD és TDK Konferencia* 2018. November. 22-23. Pécs

Tompa M, Kalovits F, Nagy A, Kalman B. The importance of the Wnt pathway in glioblastoma. *Medical Conference for PhD Students and Experts of Clinical Sciences* (MedPécs) 2018.Oktober.27. Pécs

Tompa M, Kalman B. Expression patterns of Wnt pathway markers in glioblastoma. *5th Congress of the European Academy of Neurology* 26:917-917; Paper: EPO3301. 2019. June 29-July 2. Oslo, Norway

8. Acknowledgements

First of all, I would like to express my gratitude to my supervisor, Prof. Dr. Bernadette Kálmán for her endless support, patience and professional leadership over the past four years.

I am grateful to the staff of the Department of Pathology and Molecular Pathology of the Markusovszky University Teaching Hospital and to the Department of Pathology of the University of Pécs.

I would like to thank for the collaborations and support by the staff of the Genomics and Bioinformatics Core Facility, the János Szentágothai Research Center and the Institute of Laboratory Medicine, University of Pécs.

I express my thanks to Dr. Ádám Nagy for his professional support and advice given to me during my works.

I am grateful to all the unfortunate patients with GBM whose sample left over from routine clinical evaluations I was able to use in my works.

Last, but not least, I thank my Family and Friends for the patience and support they provided to me during my studies and works.

9. References

1. 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.
2. Weller M, Butowski N, Tran DD, Recht LD, Lim M, Hirte H et al. (2017). Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial. *Lancet Oncol*. 18:1373–1385. https://doi.org/10.1016/S1470-2045(17)30517–X.
3. 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.
4. The Cancer Genome Atlas (TCGA) Research Network (2008). Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 455:1061. https://doi.org/10.1038/nature07385.
5. 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.
6. Noushmehr H, Weisenberger DJ, Diefes K, Phillips HS, Pujara K, Berman BP et al. (2010). Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell*. 17:510–522. https://doi.org/10.1016/j.ccr.2010.03.017.
7. Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, Herman JG et al. (2001). Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. *Hum Mol Genet*. 10:687–92. https://doi.org/10.1093/hmg/10.7.687.
8. Laird PW (2005). Cancer epigenetics. *Hum Mol Genet*. 14:65–76. https://doi.org/10.1093/hmg/ddi113.
9. Zhang Z, Tang H, Wang Z, Zhang B, Liu W, Lu H, et al. (2011). MiR-185 targets the DNA methyltransferases 1 and regulates global DNA methylation in human glioma. *Mol Cancer*. 10:124. https://doi.org/10.1186/1476-4598-10-124.
10. Kraboth Z, Galik B, Tompa M, Kajtar B, Urban P, Gyenesei A, Miseta A, Kalman B (2020). DNA CpG methylation in sequential glioblastoma specimens. *J Cancer Res Clin Oncol*. 146:2885–2896.https://doi.org/10.1007/s00432-020-03349-w.
11. Tompa M, Kalovits F, Nagy A, Kalman B (2018). Contribution of the Wnt pathway to defining biology of glioblastoma. *Neuromolecular Med*. 20:437-451. https://doi.org/10.1007/s12017-018-8514-x.

12. Nagy A, Tompa M, Kraboth A, Garzuly F, Maraczi A, Kalman B (2021). Wnt pathway markers in low-grade and high-grade gliomas. *Ideggyogy Sz.* 74:1-10. (in press).
13. Tompa M, Nagy A, Komoly S, Kalman B (2019). Wnt pathway markers in molecular subgroups of glioblastoma. *Brain Res*. 1718:114-125. https://doi.org/10.1016/j.brainres.2019.05.008.
14. Zhang H, Qi Y, Geng D, Shi Y, Wang X, Yu R, Zhou X (2018). Expression profile and clinical significance of Wnt signaling in human gliomas. *Oncol Lett*. 15:610–617. https://doi.org/10.3892/ol.2017.7315.
15. Liu C, Tu Y, Sun X, Jiang J, Jin X, Bo X (2011a). Wnt/beta-Catenin pathway in human glioma: expression pattern and clinical/prognostic correlations. *Clin Exp Med*. 11:105–112. https://doi.org/10.1007/s10238-010-0110-9.
16. Binda E, Visioli A, Giani F, Trivieri N, Palumbo O, Restelli S et al. (2017). Wnt5a drives an invasive phenotype in human glioblastoma stem-like cells. *Cancer Res*. 77:996–1007. https://doi.org/10.1158/0008-5472.CAN-16-1693.
17. Kamino M, Kishida M, Kibe T, Ikoma K, Iijima M, Hirano H et al. (2011). Wnt-5a signaling is correlated with infiltrative activity in human glioma by inducing cellular migration and MMP-2. *Cancer Sci*. 102:540–548. https://doi.org/10.1111/j.1349-7006.2010.01815.x.
18. Kaur N, Chettiar S, Rathod S, Rath P, Muzumdar D, Shaikh ML, Shiras A (2013). Wnt3a mediated activation of Wnt/β- catenin signaling promotes tumor progression in glioblastoma. *Mol Cell Neurosci*. 54:44–57. https://doi.org/10.1016/j.mcn.2013.01.001.
19. Cui D, Ren J, Shi J, Feng L, Wang K, Zeng T et al. (2016). R132H mutation in IDH1 gene reduces proliferation, cell survival and invasion of human glioma by downregulating Wnt/β- catenin signaling. Int. J. Biochem. *Cell Biol*. 73:72–81. https://doi.org/10.1016/j.biocel.2016.02.007.
20. Huang T, Alvarez AA, Pangeni RP, Horbinski CM, Lu S, Kim SH et al. (2016). A regulatory circuit of miR-125b/miR-20b and Wnt signalling controls glioblastoma phenotypes through FZD6-modulated pathways. *Nat. Commun*. 7:12885. https://doi.org/10.1038/ncomms12885
21. Klughammer J, Kiesel B, Roetzer T, Fortelny N, Nemc A, Nenning KH et al. (2018). The DNA methylation landscape of glioblastoma disease progression shows extensive heterogeneity in time and space. *Nat Med*. 24:1611-1624. https://doi.org/10.1038/s4159 1-018-0156-x.
22. Louis DN, Perry A, Reifenberger G, Von Deimling A, Figarella-Branger D, Cavenee WK et al. (2016). The 2016 World Health Organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol*. 131: 803–820. https://doi.org/10.1007/s0040 1-016-1545-1.
23. Pu P, Zhang Z, Kang C et al. (2009). Downregulation of Wnt2 and β-catenin by siRNA suppresses malignant glioma cell growth. *Cancer Gene Ther*. 16:351. https://doi.org/10.1038/cgt.2008.78.

24. Denysenko T, Annovazzi L, Cassoni P, Melcarne A, Mellai M, Schiffer D. (2016) WNT/β-catenin signaling pathway and downstream modulators in low-and high-grade glioma. *Cancer Genom Proteom*. 13:31–45.
25. Yao Q, Cai G, Yu Q, Shen J, Gu Z, Chen J et al. (2018). IDH1 mutation diminishes aggressive phenotype in glioma stem cells. *Int J Oncol*. 52:270–278. https://doi.org/10.3892/ijo.2017.4186.
26. Hu T, Li C (2010). Convergence between Wnt-β-catenin and EGFR signaling in cancer. *Mol. Cancer*. 9:236. https://doi.org/10.1186/1476-4598-9-236.
27. Puliyappadamba VT, Chakraborty S, Chauncey SS, Li L, Hatanpaa KJ, Mickey B et al. (2013). Opposing effect of EGFRWT on EGFRvIII-mediated NF-κB activation with RIP1 as a cell death switch. *Cell Rep*. 4:764–775. https://doi.org/10.1016/j.celrep.2013.07.025.
28. Gong A, Huang S (2012). FoxM1 and Wnt/β-catenin signaling in glioma stem cells. *Cancer Res*. 72:5658–5662. https://doi.org/10.1158/0008-5472.CAN-12-0953.
29. Kahlert UD, Maciaczyk D, Doostkam S, Orr BA, Simons B, Bogiel T et al. (2012). Activation of canonical WNT/β-catenin signaling enhances in vitro motility of glioblastoma cells by activation of ZEB1 and other activators of epithelial-to-mesenchymal transition. *Cancer Lett*. 325:42–53. https://doi.org/10.1016/j.canle t.2012. 05.024.
30. Mohtat D, Susztak K (2010). Fine tuning gene expression: the epigenome. *Semin Nephrol*. 30:468-476. https://doi.org/10.1016/j.semnephrol.2010.07.004.
31. Rauluseviciute I, Drabløs F, Rye MB (2020). DNA hypermethylation associated with upregulated gene expression in prostate cancer demonstrates the diversity of epigenetic regulation. *BMC Med Genomics*. 13:6. https://doi.org/10.1186/s12920-020-0657-6.
32. Behm-Ansmant I, Rehwinkel J, Izaurralde E (2006). MicroRNAs silence gene expression by repressing protein expression and/or by promoting mRNA decay. *Cold Spring Harb Symp Quant Biol*. 71:523-530. https://doi.org/10.1101/sqb.2006.71.013
33. Li Z, Rana TM (2012). Molecular mechanisms of RNA-triggered gene silencing machineries. *Acc Chem Res*. 45:1122-1131. https://doi.org/10.1021/ar200253u.
34. Maor GL, Yearim A, Ast G (2015). The alternative role of DNA methylation in splicing regulation. *Trends Genet*. 31:274-280. https://doi.org/10.1016/j.tig.2015.03.002.