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Molecular classification of glioblastoma

PhD thesis

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1. Introduction

Clinical characteristics of glioblastoma

The term glioblastoma first appeared in the scientific literature based on joint works by Bailey and Cushing in 1926. It was assumed that glioblastoma develops from glial cells or their progenitors. Originally, "glioblastoma multiforme" was the official terminology, referring to the extraordinary inter- and intratumor heterogeneity of the tumor. Since the WHO's classification of gliomas in 2007, it is designated as glioblastoma, and abbreviated as "gbm". Symptoms of the disease have a high degree of variability, depending on which brain regions are affected by the cancer, what the biological characteristics are, how advanced the condition is and what physical condition the patient enjoys. Certain symptoms such as epileptic seizures, headaches, nausea, vomiting, nosebleeds, etc. are associated with increased volume of the tumor. The general symptoms may worsen over time and focal neurological signs (speech disturbances, weakness of the arm and leg, cognitive decline, and personality changes) may also develop according to the affected brain area. Glioblastoma may develop in any parts of the central nervous system. It most often affects, however, the hemisferium, the frontal lobes in particular. It is somewhat less likely to develop in the temporal, parietal and occipital lobes, in this order of frequency.

In contrast to the typical appearance of adult glioblastomas, a study by Gusnard et al (1990) highlighted that pediatric glioblastomas are typically localized in the brain stem and cerebellar regions.

Glioblastoma is the most frequently occurring primary malignant brain tumor in adults. The median survival remains just a little over a year, while the five-year survival rate is 5% with complete surgical resection, radiation and chemotherapy. Publication of the Stupp protocol in 2005 was a significant step forward. This protocol recommending surgical removal of the tumor with wide margins, and then combined radiation therapy with temozolomide chemotherapy increased progression-free survival time of patients. The median survival of 12.3 months increased to 14.6 months, and the two-year survival increased from 12 to 26 percent.

While a number of molecular-based experimental therapies are currently being developed or tested in the preclinical setting, the only approved therapy is based on the Stupp protocol world-wide, including Hungary .

The histological characteristics of glioblastoma

The WHO proposed classification of gliomas according to their histological properties into four grades (I-IV, where I is the least, IV is the most malignant) in 2007. Glioblastoma is a grade IV glioma. Glioblastoma is characterized by a high level of inter- and intratumor heterogeneity, that reflects not only cytomorphological heterogeneity, but also a varying mixture of mesenchymal and primitive neuroectodermal elements with tumor cells of glial origin. According to refined histological features, Burger and his colleagues sorted glioblastomas into eight subgroups in 1987.

A histological hallmark of glioblastomas includes invasiveness, meaning that the tumor cells invade into the normal brain tissue, so the actual tumor has often larger size than it appears on MRI. Further, the tumor is characterized by pleomorphism, cellularity, vascularization and necrosis. Because of the angioneogenesis, abnormal blood-vessels grow with impaired blood-brain barrier within the glioblastoma tumors, where immune cells may enter. Berghoff and his collegues (2014), Bottino and his collegues (2014), and Navarro and his his collegues (2014) analyzed in several ways as to how the immune infiltration influences patients' survival, however, without unequivocal results. The anaplastic tumor cells stuck in different stages of differentiation, which can be seen in the histological images.

The tumor also includes glioblastoma stem cells that are likely to contribute to its growth and recidive formation properties as well as its drug- and radiation resistance.

Glioblastoma may be primary *(de novo* transformation of normal glial cells), or secondary in origin (a lower gradient glioma is transformed into glioblastoma). The secondary glioblastoma generally has a better prognosis when patients are typically younger (under 50 years) and have longer survival in conjunction with specific molecular genetic profiles of the tumor.

Metabolic characteristics of glioblastomas

The growth of the tumor leads to hypoxia in the central, less vascularized parts, where consequently necrosis frequently develops. In order to enhance its blood supply, the tumor induces angiogenesis in its microenvironment via the activation specific genes and molecules. Such molecules include the hypoxia inducible factor 1 (HIF-1), tumor necrosis factor alpha (TNF-α), zinc finger protein 39 (ZNF39) the Abelson Interactor family member 3 (ABI3), the ABI3 binding-interacting protein 3 (AIP3) and the Triggering Receptor Expressed on myeloid cells 1 (TREM1). A study by Nagy and his colleagues (2014) surveyed altered expression patterns of regulatory genes associated with hypoxia which greatly contribute to oxidative glycolysis typical of glioblastoma.

However, somatic mutations accumulating in cancer also appear to play important roles in the development of its metabolic disorders. Many of these mutations localized to mitochondrial DNA or nuclear genes of mitochondrial proteins shift the metabolic pathways in the direction of oxidative glycolysis. Several mitochondrial somatic mutations affect the cytochrome C oxidase enzyme. Griguer et al (2013) observed that those who carry such cytochrome C mutations in their tumor may face worse prognosis.

Mutations affecting the isocitrate dehydro-lipoxygenase -1 and 2 (IDH-1, IDH-2) enzymes are particularly characteristic of glioblastomas. The most frequent of IDH-1 mutation is the R132H kodon mutation. The IDH-1 R132H mutant enzyme is functionally impaired, and tthus instead of its normal product, alpha-ketoglutaratean onkometabolite called 2-D-hydroxy glutarate is formed.. Mutations in IDH-2 and 3 also lead to the accumulation of 2-D-

hydroxyglutarate. 2-D-hydroxyglutarate competitively inhibits a number of alpha-ketoglutarate-dependent molecular processes, which leads, among other things, to extensive changes in epigenetic regulation and gene expression patterns.

Other key mutations contributing to metabolic changes in the tumor are those affecting the pyruvate kinase (PK) and pyruvate dehydrogenase kinase (PDK) enzymes. Due to mutations in the PK enzyme, the process of glycolysis considerably slows down, and many intermediate products accumulate. These changes promote proliferation of tumor cells, and alter nucleotide and amino acid synthesis. Mutations in the PDK enzymes are associated with hypoxia, and PDK – hypoxia-activated genes mutually regulate each other. PDK mutations also shoft the metabolic processes in the direction of oxidative glycolysis.

Molecular Genetic Characteristics of Glioblastoma

As all tumors, glioblastoma is characterized by a sequential accumulation of somatic mutations, some of which have particular importance in differentiating its biological subgroups. One of these mutations is the above mentioned IDH-1 1R132H mutation. Since the 2014 revision of the WHO glioma classification , determination of the IDH mutational status is the first (and only recommended) molecular test for glioblastomas . Hierarchical cluster analyses of OMICS data by The Cancer Genome Atlas (TCGA) Network defined four overlapping molecular subgroups of glioblastomas, including the classic, mesenchymal, neural and proneural subgroups. The proneural subgroup is characterized by IDH-1 mutations, most frequently by the the IDH-1 R132H mutation. The classic subgroup is characterized by abnormalities in the Epidermal Growth Factor Receptor (*EGFR*) gene, including *EGFR* amplification and high expression, as well as the presence of the EGFRvIII (from exon II to VII) deletion mutant . EGFRvIII is a constitutively active receptor that transmits signal without binding of its ligand, leading to increased cell proliferation and reduced apoptotic processes. Somatic mutations in tumor suppressors genes also play an important role in defining the molecular profiles and subgroups of glioblastomas. The mesenchymal subgroup is related to somatic mutations in the *NF-1* (neurofibromin) gene or deletions within 17q11.2 encoding this gene. Interestingly, germline mutations within *NF-1* are also well known in neurological literature, which are inherited in an autosomal dominant manner and lead to the development of the complex disease, neurofibromatosis-1. Due to mutations in the *NF-1* gene, the protein product neurofibromin is unable to exert its normal tumor suppressor effects namely the negative regulation of the Ras / MAPK signaling pathway. The lack of or the impairment of this tumor suppressor function lead to enhanced tumor cell proliferation and survival.

2. Hypotheses and Objectives

Taking into consideration results of glioblastoma basic research studies, and their insufficient clinical translation, we generated the following hypotheses and specific aims:

hypotheses
In this study we postulated that:

- The molecular subgroups determined by OMICS approaches in frozen glioblastoma specimens by the TCGA may be reproduced by testing selected markers and by methods available in routine molecular pathology labs in clinical, formalin-fixed paraffin-embedded specimens..

- The molecular glioblastoma subgroups segregated based on the molecular markers reflect biological heterogeneity of the tumor and correlate with clinical outcomes.

- Glioblastoma tumors largely retain their molecular subgroup classification over time, however, signs of further clonal evolution may also be detected.

objectives
In order to test the above assumptions, we specifically aim to

- Investigate the most important subgroup defining TCGA markers by methods of immunohistochemistry (IHC) and pyrosequencing in FFPE glioblastoma specimens that were collected in the Pathology Department at the Markusovszky University Teaching Hsopital (MUTH) and to test the segregation of subgroups based on the marker profiles.
- Correlate the patients' clinical data (age, gender, overall survival) with these molecular subgroups.

- Analyze the changes in molecular profiles of those patients from whom more than one sequential glioblastoma specimens were available.

3. Materials and methods
patients
For our studies, 127 FFPE glioblastoma blocks were selected from 112 patients obtained between 2000 and 2016 in the Pathology Department, MUTH. Based on the quality and

quantity of samples, 114 samples from 104 patients were included in the study. Diagnosis of glioblastoma was definite based on clinical and histological studies.

The patients were divided into two cohorts material. The first cohort included single specimen from each patient. These samples were surgically obtained after the diagnosis was established, but before chemotherapy and radiotherapy. The second cohort included two or more sequential specimens from patients whose tumors were available from the first surgery (before radiation and chemotherapy) and after recurrence (after radiation and chemotherapy). The 96 glioblastoma specimens from 96 patients in the first cohort were examined by our selected molecular markers to test segregation of the glioblastoma subgroups. he 18 glioblastoma specimens from 8 patients in the second cohortwere used to test the temporal evolution of the molecular tumor profiles.. The majority of the studied patients had primary glioblastoma (101), but there were also 3 secondary glioblastoma specimens from patients included in the study.

In the first cohort 46 patients (44.23%) were men and 58 were women (55.76%). The age of patients ranged from 26 to 88 years, their mean age was 61.01 years and their median age was 63.5 years. The age of men ranged from 26 to 77 years, their average age was 58.89 years, their median age was 60 years. The age of women ranged from 32 to 88 years, their average age was 61.69, their median age was 65 years.

immunohistochemistry

Immunohistochemistry tests were optimized for each primary antibody in a preliminary study. Regions of interest were selected on the first slides of specimens in order to examine consistent and comparable regions in the future. For this purpose a hematoxylin-eosin staining and anti-glial fibrillary acidic glycoprotein (GFAP) immunostaining were used. Our criteria were the following: the selected tissue region had to be malignant with increased cellular density, pleomorphism, and mitosis, which showed GFAP positivity. The proportion of necrotic areas was maximized within the evaluation areas at 20%, and the rate of vascularity was no more than 25%. The ROI selection was performed under 1:20 and 1:200 magnification and selected areas were labeled on each slide. Thus, the IHC analyses with 8 primary antibodies were performed on 8 sequential slides, each containing the same ROI (the same ROI could be confirmed within 8x3 μm). The primary antibodies were as follows: anti-GFAP, anti-NF-1, anti-EGFR, anti-EGFRvIII, anti-P53, anti-ATRX, anti-IDH-1R132H, and anti-CD133. The secondary antibody and the Diamino-benzidin-based visualization system were part of the Novolink Polymer Detection System RE-7140-K (Leica Biosystems, Newcastle, United Kingdom). . The IHC tests were evaluated both manually and digitally. Digitalizedquantitation was performed for nuclear and membrane staining (EGFR, EGFRvIII, p53) at 100X magnification. The completed digital photos were saved on a DVD from the central server of the Pathology Department, at the Tatabanya St. Borbala

Hospital. Evaluation of the scanned disks was performed by a 3DHISTECH Pannoramic Viewer 1:15 program, complemented by a QuantCenter module ((3D HisTech Kft. Budapest, Hungary). The QuanCenter module allows to determine at a selected magnification the intensity of cell membrane and / or nuclear staining and percentage of stained cells. By the computer, the staining intensity of cells was rated as 0, +, ++ and +++, while from the stained cell counts the rate of positive cells was determined. The digital evaluation was used to confirm and to refine the results obtained by manual evaluation within the ROI areas in case of the three antibodies . We performed manual evaluations of ROI areas when IHC for EGFR, p53, NF-1, CD133, ATRX, EGFRvIII, and IDH-1 also R132H were carried out. The evaluations were performed by three separate individuals at 1: 200 magnification on an Olympus BX51 microscope (Olympus Corp. Japan). For the manual evaluation of EGFR, p53, CD133 and ATRX IHC, the staining intensity, similar to that of the computer analysis, was manually designated as 0, +, ++ and +++. Three-three fields of view were studied, and altogether 100-100 cells were read. In addition to the intensity evaluation, the percentage of stained cells was determined. The Histoscore value was obtained by multiplying the percentage of positive cells by the staining intensity . The Histoscore value thus had to fall between 0 and 300. In case of NF-1 (typically cytoplasmic, but also nuclear and cytoplasmic staining), a similar procedure was followed, and the Histoscore was determined by multiplying the IHC staining intensity and percentage of stained cells. However, for the final evaluation of NF-1, four staining categories were distinguished: 1. cytoplasmic positivity, 2. cytoplasmic and nuclear positivity, 3. nuclear positivity, and 4. cytoplasmic and nuclear negativity. In case of two mutation-specific antibodies, the antiEGFRvIII and anti-IDH-1 R132H, the presence or absence of staining (mutation) were documented, wherein the positivity was defined as "++" or "+++" IHC staining intensity (see above).

Pyrosequencing

The IHC results with the anti-IDH-1 R132H antibody were confirmed by pyro-sequencing. We planned this validation of the IHC results by pyrosequencing for all samples positive by IDH-1 R132H IHC and by including two negative controls. In addition, we planned to pyrosequence all those samples where the anti-IDH-1 R132H IHC did not give unambiguous result. Slides were prepared from the selected blocks and subjected to deparaffination using the QiaGen Deparaffinization Solution for FFPE samples kit. Subsequently, DNA was purified from the deparaffinated material by the QiaGen® QIAamp DNA FFPE Tissue Kit. The concentration values of the DNA samples were determined by using the Thermo Scientific® NanoDrop 2000 and the NanoDrop2000 / 2000c software package. The sequences of the PCR and sequencing primers were obtained from a publication by Setty et al (2010).. The reverse PCR primer was biotinylated. PCR was performed on a BOECO Thermal Cycler SQ BOE8085240 in 40 cycles. The PCR products

were confirmed by agarose gel electrophoresis. The biotinylated PCR amplicon was purified by immobilization, and the presence of IDH-1 R132H mutation was investigated by pyrosequencing on an automatic device detailed in the preliminary studies .

statistical methods

Once the results of IHC were confirmed and finalized, statistical analyzes were carried out in several steps first in cohort 1, then in cohort 2.. In the 96 samples of cohort 1: 1. we examined manually the separation of patients / samples based on the IHC markers. 2. Hierarchical cluster analyses were performed using SPSS23 to test and confirm the separation of molecular subgroups. Results of NF-1, IDH-1 R132H and EGFRvIII IHC were evaluated in a nominal +/- based assessment, while for the other IHC markers Histoscore values served for the basis of statistical testing. Trees of cluster analyses displayed negative and positive data groups as 0 and 1, along with the p-values showing statistical differences . Finally, we examined the correlation of clinical parameters (gender, age, overall survival) with the molecular subgroups. For gender distribution, we used the Pearson χ^2 - probe. The age of the patients showed no normal distribution, therefore, the Kruskal-Wallis test (multiple groups comparable) was used, while the subgroups were compared to the whole patient population by using the Mann-Whitney test. The correlation between patient survival and molecular subgroups was examined by the Cox regression analysis. In cohort 2, 18 samples of 8 patients were evaluated for molecular classification, and the changes in the molecular profiles were examined in the sequential samples.

4. Results

Preliminary evaluations

Reviewing data in cohort 1, we can draw some conclusions prior to the statistical analyses. Among all markers examined, three antibodies appeared of key importance in segregating patients / samples into into three subgroups of glioblastoma. These three subgroups include the EGFR - EGFRvIII-positive group, the NF-1 completely (nucleus and cytoplasm) negative group and the IDH-1 R132H positive group. These subgroups (based on the marker molecules) likely overlap with the TCGA subgroups: 1) IDH-1 R132H positivity (Verhaak et al, 2010, proneural subgroup), 2) EGFR and EGFRvIII combined positivity (Verhaak et al, 2010, classic sub-group) and 3) NF-1 double negativity (Verhaak et al., 2010, mesenchymal subgroup). According to our results, the largest group carries the EGFRvIII mutation, and includes 34 patients. Twenty patients showed loss of NF-1 expression in their tumors and 10 patients carried IDH-1R132H mutations. Altogether, classification into one of three molecular subgroups could be given for 64 patients / specimens out of our 96 patients / specimens, also paralleling the TCGA subgroup distribution. While the subgroup positive for

the IDH-1 R132H marker completely separated from the other subgroups, the other two subgroups showed a slight overlap: a simultaneous detection of EGFRvIII mutation and the absence of NF-1 expression were seen in two patients / samples in cohort 1. These results indicate in agreement with the TCGA data, that the separation of the glioblastoma subgroups based on molecular markers is cluster-like, but not complete.

Detailed IHC and clinical correlation data of the first cohort

Ten out of 96 patients were positivefor the IDH-1 R132H mutation by IHC. This figure represents approximately 10% of our cohort, in consensus with the TCGA proneural subgroup representation . Comparing the gender of the patients,there are 46 woman and 40 men (86 individuals) in the IDH-1 R132H negative subgroup. In the IDH-1 R132H positive subgroup 7 women and 3 men (10 individuals) can be observed. We could not detect any relationship between gender and the presence of the IDH-1 mutation (χ^2 test, 95% confidence level p= 0.34).

The age of the patients was also compared within the positive and negative groups. The average age of individualsin the IDH-1 positive group (with the R132H mutation)was 56 years, while in the negative group   it was 62 years. Similarly, there appeared to be a difference between median age of the positive and negative subgroups (52.5 years in positive and 56 in negative subgroup). However, when the relationship between age and IDH-1 R132H mutation was testedby the Mann-Whitney test no difference could be detected between the positive and negative groups, due to the small size of the mutation positive cohort (95% confidence level, p = 0.234). Nevertheless, it is worth of noting that a strong trend appeared indicating that patients in the IDH-1 R132H positive group were younger than those in the negative group. In the third round, patients' survival data were correlated with the presence of the IDH-1 R132H mutation. We could access survival data of 9 (90%) patients in the mutation positive group, and 59 (69%) patients of the negative group. Overall survival was defined as the time interval between the date of surgery and the date of death o, given in weeks. In the IDH-1 R132H positive group, the average survival was 82 weeks, while the median survival was 24 weeks. In the IDH-1 R132H negative group, the average and median survivals were 43 weeks and 26 weeks, respectively. Although there is no significant difference here either, the mean values indicate that patients in IDH mutation group were younger than patients in the other subgroups (negative group).

In the second round, expression profiles of NF-1 were correlated with the clinical data. We observed four staining patterns: nuclear and cytoplasmic double positive (+ / +), nuclear and cytoplasmic double negativity (- / -), nuclear positive and cytoplasmic negative (n + / c -) and nuclear negative and cytoplasmic positive (n - / c +). the pattern observed in the latter case (n- / + c) corresponds typically to the   normal physiological condition . We compared age, gender and survival data of the NF-1 double negative group with those of the n- / c+ group.

Altogether, there were IHC and survival data available for forty individuals. The gender breakdown was as follows: 20 women and 20 men. The NF-1 double negative group had 9 women and 11 men. The n- / c+ group had 11 women and 9 men. The gender distribution did not show correlation with the NF-1 expression profile in this comparison (double negative vs. n- / c+) (χ^2 test, 95% confidence level, p = 0.40). The age distributions in the NF-1 subgroups were similar to that in the IDH-1 subgroup. The average age of surgery was 65 years for the NF-1 double negative patients, compared to 61.5 years for the n- / c+ patients , while the median age was 67 years in the NF-1 double negative group while 62.5 years in the NF-1 n- / c+ group (Mann-Whitney test, 95% confidence level p = 0.22). As to the overall survival figures, the average survival was 56 weeks in NF-1 double negative group, and 49 weeks in the n- / c+ group. Similarly, the median survival of the double negative group was 21 weeks, while that of the the n- / c+ group was 11 weeks.

In the third round, the EGFRvIII positive and negative cohorts were compered for clinical outcomes. Of the 96 samples, 34 were positive, and 62 were negative. The EGFRvIII mutants typically also showed increased expression of the EGFR protein. This subgroup with EGFR overexpression and EGFRvIII mutation likely overlaps with the TCGA classic subgroup. There were 19 women and 15 men in the EGFRvIII positive group, and 35 women and t 27 men in the negative group (χ^2 test, 95% confidence level, p= 0.95). The age at glioblastoma surgery in the EGFRvIII positive group was 60 years, median 61 years, in the negative group these numbers were 62 year and 65 years, respectively (Mann-Whitney test, , 95% confidence level, p = 0.74). We could retrieve the patient survival data for 22 individuals in the EGFRvIII positive group, and for 43 patients in the negative group, giving a total of 65 patients (67.70%). As expected, the overall survival data in these subgroups are similar to those of the total cohort, with 50 weeks of mean survival in the positive vs. 45 weeks in the negative group, and 22 weeks of median survival in the positive group vs. 26 weeks of median survival in the negative group.

Finally, we also performed clinical correlation analyzes in the three molecular glioblastoma subgroups.
Out of the 96 patients, we could obtain overall survival data for 68 patients (~ 71%) . The Cox regression analysis was performed to determine whether or not patients in the three molecular subgroups have different overall survivals (95% confidence level, p= 0.386). Based on this nalysis, there is no difference in the survival data of the three outlined molecular subgroups. However, because of the small sample size and the observed trends, some careful conclusions may be drawn. The longest survival was noted in the subgroup carrying the IDH-1 R132H mutation, while the shortest survival was found in subgroup with the loss of NF-1 expression (double negative subgroup).

Cluster analysis

Separation of the glioblastoma molecular subgroups was tested by hierarchical cluster analyses in cohort 1. The results were presented in a tree diagram. Many possible trees were generated, among which we found one that was statistically most significant and biologically most plausible.

In this tree, the presence or absence of IDH-1 R132D mutation separates the glioblastoma subgroups (in agreement with the most recent revision of WHO glioma classification as reported by Louis et al, 2016) (95% confidence level, $p = 0.001$). The IDH-1 mutant group completely separates from the others. In the second step, the IDH-1 R132H negative group was further analyzed based on the EGFRvIII mutation status. In this step, the EGFRvIII positive and negative subgroups also significantly separate from each other (95% confidence level, $p = 0.022$). In the final step, the group EGFRvIII negative subgroup was further tested according to the NF-1 expression status. Here, the separation of the NF-1 double negative (n-/c-) group from the other three subgroups (NF-1 + / +, NF-1 +/- and NF-1 -/+) could be observed (95% confidence level, $p = 0.059$), although did not quite reach statistical significance. Given that the observed p-value is close to the significance limit, it is possible that the diminished subcohort size in the multi-step analysis is responsible for the lack of significance.

The alternative hierarchical trees were statistically weaker and biologically less plausible than the one detailed above.

Evaluation of the second cohort

In the second cohort (18 FFPE blocks from 8 patients; 2 or more surgically removed specimens from each), we investigated changes in the IHC-defined molecular patterns over time. No profound changes were observed in the molecular patterns of the sequential samples, however, certain trends were noted. First, the IDH-1 status was analyzed. None of the tumors from the eight patients in this cohort carried the IDH-1 R132H mutation. In the second round, we analyzed the NF-1 expression status. t Change was observed only in patient number 4, where no anti-NF-1 staining appeared in the first tumor, but the nucleus was positive in the recurrent tumor. Evaluation of the EGFR IHC results was based on the Histoscore values (staining intensity multiplied by the percentage of positive cells). Recurrent tumors either showed about the same or somewhat higher EGFR Histoscore values as the first surgically removed tumors, an observation compatible with those in other reports. Presence of the EGFRvIII mutation was detected in the majority of cohort 2, a likely by chance result due to the small smaple size. The EGFRvIII status has not changed when the first and recurrent tumors were compared. The anti-ATRX antibody staining pattern, however, was just the opposite of the previous staining patterns, as the initially positive ATRX status

became negative in several of the recurrent tumors. In case of the p53 and CD133 IHC, no change could be observed comparing the first and recurrent tumors.

5. Discussion

Based on our results, we conclude that identifying molecular subtypes of glioblastoma is feasible by testing the TCGA-OMICS subgroup-defining markers in clinical FFPE specimens. Based on the shared markers, a close overlap in the molecular subgroups (proneural, classic and mesenchymal) is very likely in our IHC-based and the TCGA OMICS-based studies,.. Our results not only reproduce the separation of molecular subgroups in clinical glioblastoma samples, but the percentage distribution of these molecular subgroups is also similar to that reported by the TCGA network. In our study, the patients' gender did not show a differential distribution in the molecular glioblastoma subgroups, and did not show any relationship with the onset age or the overall survival of the disease. However, it is worth of noting that patients with the IDH-1 R132H mutation in their tumors appeared to be younger and had longer survival than those without this mutation, a strong trend in consensus with the TCGA dataAnalyses of data in the second cohort, though without statistical testing, show that the main subgroup-defining molecular characteristics are retained in sequential glioblastoma samples, although limited changes may be noted even with these few markers which suggest that some clonal evolution occurs over time. Our observations are consistent with the 2016 revision of the WHO glioma classificationthat proposes the integration of the molecular markers into the histopathology-based classification, as well as the separation of glioblastomas based on their IDH mutation status into negative and positive subgroups. Of note, the IDH-1 R132H positive / negative status also significantly (though not entirely) differentiates primary and secondary glioblastoma tumors. While the 2016 revision of the WHO glioma classification did not propose the use of additional markers for further separation of glioblastoma molecular subgroups, our results suggest that using the complementary key markers for separating the major molecular subgroups is not only feasible, but is also useful to support clinical decision-making.

As to the four NF-1 staining patterns, a number of underlying molecular events may be considered. Such events may include altered gene expression patterns, deletion of epitopes recognized by the primary antibody in IHC, or the altered intracellular localization of the protein. Further research is needed to specify these underlying possibilities, although the TCGA data also allow to identify genotype – phenotype correlations.

The strength of this study was that in archived clinical FFPE glioblastoma samples by using methods easily available in routine histopathological labs we were able to identify the

principal molecular subtypes of the tumor. This approach allowed us to sort 2/3rd of our samples into molecular subgroups that likely overlap with those proposed by the TCGA, based on the shared markers. We are in the process of extending the present investigation with a prospective study in which clinical data and specimens will be collected in real time, in order to reproduce our initial findings, and to better define the biological properties of glioblastoma subtypes. The ultimate success of our studies may result in the generation of a clinical diagnostic panel to support prognostication and therapeutic decisions.

Modern therapies often target molecules that also play important roles in the molecular classification of tumors. Such approaches include a broad technical armamentarium targeting cells with the EGFRvIII mutant, and include vaccination and antibody therapies. Although no breakthrough has been achieved with these modalities, it is highly likely that improved survival may result from some of these approaches in the near future for patients in the "classic" subgroup with EGFRvIII mutation. Another new therapeutic approach based on biotechnological engineering includes the CAR T cells. These cytotoxic T cells express chimeric antigen receptors with arbitrary specificity, which are capable of recognizing a selected antigen, e.g. EGFRvIII, and thus to eliminate the expressing glioblastoma cells.

The identification of the IDH-1 mutants as therapeutic targets goes back to the last decades, and successful testing of effective IDH-based therapies has been achieved in experimental systems, but none of these approaches could proceed as yet from the preclinical to a clinical phase. Replacement or correction of the expression and function of the tumor suppressor molecule, NF-1 could be central to the treatment strategies in several cancers, including glioblastoma. Unfortunately, no such treatment has been successfully developed as yet. It is, however, very likely that targeting not only the marker molecules, but also the associated mutated molecules, as well as their combinations, by a new generation of inventive therapeutic modalities will bring success in the treatment of glioblastomas. The currently available research data altogether suggest that an increasingly deepening molecular profiling of glioblastomas in the clinical setting will play important roles in the elimination of this very malignant and aggressive tumor in the near future.

6. New scientific results

1. Using methodologies (IHC) available in routine pathology labs and testing clinical FFPE tumor specimens, we have examined and reproduced the molecular subgroups of glioblastoma that were originally defined by using OMICS methodologies in frozen tumor specimens by the TCGA.
2. We correlated the IHC marker-defined molecular subgroups with the patients' clinical data.

3. We refined further the molecular classification of gliomas most recently recommended by the WHO, as our analyses demonstrated that determination of the EGFRvIII mutation and the NF-1 expression status have clinical relevance in addition to the determination of the IDH-1 mutation status.

4. Confirmation of our observations in a prospective cohort is in progress. If the conclusions of the first study can be proven, development of a clinico-pathological algorithm is expected for patients with glioblastoma to predict prognosis and to support targeted therapies.

5. We have recognized that recurring tumors - even in this small study cohort - largely retain their initial molecular profiles despite some signs of clonal evolution ,.

7. Publications related to the thesis, abstracts and presentations

Publications related to the thesis
Publications in a foreign language

Adam Nagy, Katalin Eder, Mary Selak, Bernadette Kalman: Mitochondrial energy metabolism and regulation of apoptosis in glioblastoma *BRAIN RESEARCH* 1595: pp. 127-142. (2015)

Adam Nagy, Ferenc Garzuly, Gergely Padányi, Iván Szűcs, Adam Feldmann, Balázs Murnyák, Tibor Hortobágyi, Bernadette Kalman: Molecular subgroups of glioblastoma- an assessment byimmunohistochemical markers. *Pathology AND ONCOLOGY RESEARCH* DOI: 10.1007/s12253-017-0311-6 2017. In Press.

Publications in Hungarian

Nagy Ádám MSc, Éder Katalin, PhD, Kálmán Bernadette, MD, PhD, DSc, FAAN: Characteristics of the immune response and possibilities for immune therapy in glioblastoma. *HEALTH ACADEMY* 7: (1) pp. 44-60. (2016)

Nagy Ádám MSc, Garzuly Ferenc, MD PhD, Kálmán Bernadette, MD, PhD, DSc, FAAN: Biology and pathogenic changes in neurofibromin-1 in oncological diseases. *Hungarian Oncology. 2017. December In press.*

The abstract of the thesis in Hungarian

Nagy Ádám, MSc, Garzuly Ferenc, MD, PhD, Padányi Gergely, MD, Szűcs Iván, MD, Kalman Bernadette, MD, PhD, DSc, FAAN: Determination molecular subgroups of glioblastoma: - In: Carpenter K, Bódog M, Mechler M, Meszaros O (eds.) Book of Abstracts. Interdisciplinary Doctoral Conference - Absztraktkötet. Interdisciplinary Doctoral Conference. 188 p. Meeting Place and time: Pécs, Hungary, 2017.05.19-2017.05.21. Pecs: Pecs University PhD Municipality, 2017, p. 173 (ISBN: 978-963-429-113-8)

Table 8. Summary scientometric table

Prepared based on the MTMT publication and citation lists

Types of publications	number	The number of independent citations
I. Scientific journal article	2 (4)	7
Full article, in international journals (foreign language)	1 (2)	7
Full article in Hungarian	1 (2)	-
II. Book	-	-
Scientific book / edited book	-	-
Scientific book edited books, foreign language	-	-
Scientific book/ Edited book, Hungarian language	-	-
III. book chapter	-	-
a) Professional Book / Book	-	-
Book chapter in a foreign language	-	-
Book chapter in Hungarian	-	-
Proceedings *	4	-
In foreign language	-	-
In Hungarian	4	-
Total Publications (I-IV).	6 (8)	7
Other scientific works **	14	-
Total impact factor	**2561**	
Number of citations		?
Hirsch index	?	

Notes:

    * Presentation at conferences in journals or book, without abstract.

    ** Incomplete publication in scientific journal or publication in journal with unknown reviewer evaluation.