Identification of Novel Genetic Alterations in Samples of Malignant Glioma Patients

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Abstract

Glioblastoma is the most frequent and malignant human brain tumor. High level of genomic instability detected in glioma cells implies that numerous genetic alterations accumulate during glioma pathogenesis. We investigated alterations in AP-PCR DNA profiles of 30 glioma patients, and detected specific changes in 11 genes not previously associated with this disease: LHFPL3, SGC2, HTR4, ITGB1, CPS1, PROS1, PTEN, PDE4D, KDR3DL3, and INPP5A. Further correlations revealed that 8 genes might play important role in pathogenesis of glial tumors, while changes in PTEN, KCNG2 and KDR3DL3 should be considered as passenger mutations, consequence of high level of genomic instability. Identified genes have a significant role in signal transduction or cell adhesion, which are important processes for cancer development and progression. According to our results, LHFPL3 might be characteristic of primary glioblastoma, SGC2, HTR4, ITGB1, CPS1, PROS1 and INPP5A were detected predominantly in anaplastic astrocytoma, suggesting their role in progression of secondary glioblastoma, while alterations of PDE4D seem to have important role in development of both glioblastoma subtypes. Some of the identified genes showed significant association with p53, p16, and EGFR, but there was no significant correlation between loss of PTEN and any of identified genes. In conclusion our study revealed genetic alterations that were not previously associated with glioma pathogenesis and could be potentially used as molecular markers of different glioblastoma subtypes.

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Introduction

Cancer is a genetic disease characterized by DNA sequence changes, copy number aberrations, chromosomal rearrangements and modification in DNA methylation leading to compromised regulatory mechanisms governing cell proliferation and homeostasis. Studies carried out over the past three decades suggest that malignant gliomas, like other cancers, represent a consequence of the accumulation of genetic alterations, although their nature and exact number required for tumorigenesis remain unclear.

Glioblastoma is the most frequent and aggressive brain tumor. Majority of glioblastomas (GBM, WHO grade IV) develop de novo (primary glioblastomas) without clinical or histological evidence of a less malignant precursor lesion, while others, progressing from low-grade diffuse astrocytoma or anaplastic astrocytoma (AA), represent rare secondary glioblastomas. Despite a similar histological appearance, primary and secondary glioblastomas are distinct tumor entities that originate from different precursor cells containing different genetic alterations [1]. Epidermal growth factor receptor (EGFR) amplification and PTEN mutations are genetic alterations typical of primary glioblastomas, whereas p53 mutations are early and frequent genetic alterations in the pathway leading to secondary glioblastomas. Furthermore, mutations in the active site of isocitrate dehydrogenase 1 (IDH1) were associated with secondary GBMs [2]. On the other hand, LOH 10q and alterations of p16INK4a/RB1 pathway seem to be important in the development of both primary and secondary glioblastomas [3]. Besides these frequently altered pathways, there is evidence of large number of genetic alterations in glioblastoma samples, reported in The Cancer Genome Atlas (TCGA) database. TCGA project enabled the integrated analyses of multi-dimensional genomic data collected from different platforms with the aim to better characterize and understand tumor origin, behavior and treatment [4].

However, further analyses are needed to identify additional potentially useful genetic alterations for the classification and targeted therapy of GBMs. Besides, significant level of genomic instability and heterogeneity detected in glial tumors confirm that they evolve along a multitude of pathways rather than along a single defined pathway [5,6,7]. Even though only a few specific genetic pathways are consistently highlighted, there are undoubt-edly complex interactions among them as well as with additional yet unknown factors. Reliable molecular markers are needed to enable the identification of patients at risk for developing GBM, improve the early detection and appropriate diagnosis, as well as to provide molecular profile for better prediction of patient outcome and response to therapy.
A number of techniques based on PCR, hybridization, and conformation changes, as well as modern high-throughput genome wide techniques can be employed for the detection of specific mutations in cancer cells. AP-PCR DNA fingerprinting method has numerous advantages over conventional methods, mainly because no prior knowledge of the genome under investigation is required and because it allows the screening of the whole genome including non coding DNA regions. Furthermore, AP-PCR is a reliable, inexpensive method that does not require complex equipment, does providing highly reproducible patterns of amplified fragments which faithfully reflect differences in DNA sequences and/or relative abundance of the templates and enable detection of genetic alterations in tumor tissue [8]. Finally, the possibility of further analysis of variant bands by reamplification, cloning, and sequencing enables rapid identification of genes probably linked to the development and progression of malignant tumor [9]. AP-PCR has already proven to be highly informative for analysis of cancer associated somatic mutations since it has been implemented in the analyzes of various cancers, including pancreatic and colorectal carcinomas [10,11], as well as lung [12,13], and breast cancers [14].

We applied AP-PCR for the detection of anonymous multiple genetic alterations in 30 patients with AA and GBM. Following our previous study of genomic instability in glioma patients [15] we analyzed altered sequences in tumor DNA profiles with the aim to identify genes specific for glioma pathogenesis. Furthermore, we examined identified genes in relation to genomic instability, clinicopathological parameters, and patients’ survival. Also, we tested the association of the most frequently present genetic alterations in primary and secondary glioblastomas with newly identified genes with the aim to recognize potential molecular markers of different glioblastoma subtypes.

**Materials and Methods**

**Ethics Statement**

The samples were collected and used after obtaining informed consents and approval from the Ethical Committee of Clinical Center of Serbia (approval number 3672/1), in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki, Laws of Republic of Serbia, as well as GCP guidelines. All participants provided their written informed consent to participate in this study. The form of informed consent was approved by the Ethical Committee of Clinical Center of Serbia.

**Tissue samples**

Paired cancer tissue and blood samples were collected from 30 patients who underwent surgery at Neurosurgery Clinic, Clinical Center of Serbia. The specimens were frozen in liquid nitrogen, where they were kept until DNA extraction. All patients had a histologically confirmed diagnosis of anaplastic astrocytoma (WHO grade III; n = 8) or glioblastoma multiforme (WHO grade IV; n = 22) according to the 2007 WHO classification [16]. All grade IV tumors were considered primary (de novo) because the glioblastoma diagnosis was made at the first biopsy, without clinical or histopathological evidence of a less malignant precursor lesion. The 30 patients included 19 men and 11 women, with a median age of 56.9 years (within the range of 20 to 84 years). The median overall survival was 11 months. Patients received neither radio- nor chemotherapy before surgery.

**Immunohistochemical analysis**

Following routine hematoxylin-eosin method for staining of paraffin tissue sections [15], we performed immunohistochemical staining for p53 to further characterize our samples. Sections of tissue were subsequently heated in phosphate buffered saline (PBS), and stained with the streptavidin-biotin technique using antibody against p53 protein (Dako, Monoclonal Rabbit Anti-Human Antibody, dilution 1:50), according to the manufacturer’s instructions.

**Table 1. Distribution of samples according to the level of genomic instability.**

| Level of genomic instability | Number of patients |
|-----------------------------|--------------------|
| Genomic instability total   | 30                 |
| low                         | 0.3                |
| high                        | 0.3                |
| Microsatellite instability   | 14                 |
| low                         | 0.15               |
| high                        | 0.15               |
| Chromosomal instability     | 13                 |
| low                         | 0.16               |
| high                        | 0.16               |

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Figure 2. AP-PCR fingerprinting analysis of genomic instability in glioma samples. (A) AP-PCR profiles of tumor (T) and normal blood (N) tissues from glioma patients obtained using MDR antisense primer, separated on 6% non-denaturing polyacrylamide (PAA) gel; 18–20 represent patients; M –DNA ladder; arrows indicate altered electrophoretic bands in tumor DNA profiles that were excised, cloned, sequenced and identified as novel genetic alterations in glioma samples.
DNA extraction
DNA was extracted using the phenol/chloroform/isoamyl alcohol method [17]. The quality of the DNA was verified by electrophoresis on 0.8% agarose gel. The DNA concentration was assessed spectrophotometrically.

AP-PCR DNA fingerprinting
AP-PCR DNA fingerprinting, was used to compare DNA profiles of paired tumor and blood samples of the same patient [15]. In short, seven primers were tested for the ability to generate informative fingerprints that distinguish tumor from normal tissue. Optimization of AP-PCR reactions was done for each primer according to Cobb [18] and included the search for conditions that would yield profiles of moderate complexity in order to simplify the analysis [19]. Primer sequences, reaction conditions, and amplification profiles were described previously [15].

AP-PCR products were separated on 6–8% non-denaturing polyacrylamide (PAA) gels and visualized by silver staining. Gel images were acquired with the Multi-Analyser/PC Software Image Analysis System (Bio Rad Gel Doc 1000). Digitized images were loaded into the specialized public software Image J (National Institute of Health, USA, www.rsbl.info.nih.gov/ij) and analyzed by the image enhancement function ‘adapthisteq’ as previously described [15].

Isolation, cloning and sequencing of variant electrophoretic bands
Twenty selected DNA bands with altered mobility were further characterized. The PCR amplicons resolved on the silver stained PAA gels were gently removed with a hypodermic 22-gauge needle pre-wetted with the PCR master mix solution. The needle was dipped in the PCR master mix for 2 min and then discarded. The PCR products were reamplified with the same primers used for AP-PCR reactions at high-stringency conditions specific for each particular primer [20]. The reamplified material was administrated on 1.5% agarose gels, purified using DNA Extraction Kit (Fermentas Life Sciences, Lithuania) and cloned with GeneJet™ PCR Cloning Kit (Fermentas Life Sciences, Lithuania) according to manufacturer’s instructions. Plasmids were purified using GeneJet™ Plasmid Miniprep Kit (Fermentas Life Sciences, Lithuania). Sequences were determined on ABI Prism 3130 Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing was performed in both directions on several clones for each selected DNA band. The obtained sequences were analyzed and identified using BLAST software in the NCBI GenBank and EBI (Sanger Institute) database.

Analysis of alterations in p53, PTEN, and p16 tumor suppressor genes
Alterations of p53 tumor suppressor gene were analyzed in our previous study [15]. Frequently mutated exons of p53 gene (5–9) were amplified and screened for mutations by PCR-SSCP (Single Strand Conformation Polymorphism) analysis according to Orita et al. [21]. Detected mutations were confirmed by sequencing with Applied Biosystems Incorporated dye terminator sequencing kit according to the manufacturer’s specifications on an ABI Prism 3130 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Tumor and blood DNA obtained from all 30 patients was used to study the loss of heterozygosity (LOH) of p53, PTEN and p16 tumor suppressors. As described previously, LOH analysis of p53 was performed by fragment analysis with highly polymorphic microsatellite markers (TP53pentanucleotide, TP53dinucleotide, D17S1537 and D17S876) specific for chromosomal region spanning p53 gene locus [15]. Five polymorphic microsatellite markers lying within or flanking PTEN gene (D10S579, D10S1765, D10S215, AFM086wg9, and D10S541) were selected to cover deletions at the whole PTEN locus on chromosome 10q23. All forward primers were 5′-labeled with Fam, Vic, Ned, Pet, and Fam fluorescent dyes, respectively. The choice of the microsatellite markers and locus-specific PCR conditions was determined from published sources [22,23].

Another set of 3 polymorphic microsatellite markers spanning the INK4a/ARF locus (D9S171, D9S1748 and D9S162) were selected to cover deletions on chromosome 9p21–23 [24]. All forward primers were 5′-labeled with Ned, Pet and Vic fluorescent dyes, respectively. PCR products for all LOH analyses were separated by capillary electrophoresis on an ABI Prism 3130 automated sequencer and sized using GeneScan ~500 LIZ size standard (Applied Biosystems, Foster City, CA, USA). The obtained data were analyzed with the GeneMapper software (Applied Biosystems). DNA extracted from peripheral blood of each patient was used as a reference. A marker was defined as noninformative (homozygote) when only 1 allelic peak was detected in the reference sample. Contrary to this, a marker was considered informative (heterozygote) when two major allelic peaks occurred in a blood specimen. The LOH score was calculated automatically by GeneMapper software according to the following equation: (peak height of normal allele 2)/ (peak height of normal allele 1) divided by (peak height of tumor allele 2)/ (peak height of tumor allele 1). A sample was considered to be a LOH candidate for particular locus if the ratio values were less than 0.67 and higher than 1.35. p16 was also tested for the presence of homozygous deletions by a differential PCR method, according to conditions determined from published sources [12]. Briefly, a 199-bp fragment of INK4a/ARF locus from exon 2 was co-amplified with a 131-bp fragment of the adenine phosphoribosyltransferase (APRT) gene, which was used as internal control. Primer sequences were previously described by Hayashi et al. [25]. Forward primers were 5′-labeled with Fam fluorescent dye. Fluorescent PCR products were separated by capillary electrophoresis on an ABI Prism 3130 automated sequencer and sized using GeneScan ~500 LIZ size standard (Applied Biosystems, Foster City, CA, USA). Obtained data were analyzed by fragment analysis with the GeneMapper software (Applied Biosystems, Foster City, CA, USA). The presence of homozygous deletions was determined according to ratio of peak intensities of INK4a/ARF and APRT in tumor samples relative to the same ratio in normal samples. A sample had homozygous deletion of INK4a/ARF locus and consequently p16 tumor suppressor gene if the value of this proportion was higher than 2.

Amplification status of EGFR gene
For differential PCR analysis, a 110-bp fragment of the EGFR gene on chromosome 7 was co-amplified with a 168-bp fragment.
of β-actin (ACTB) gene on the same chromosome. The primer sequences were as follows: 5′-AGC CAT GCC CGC ATT AGC
TC-3′ (sense) and 5′-AAA GGA ATG CAA CTT CCC AA-3′ (antisense) for EGFR, and 5′-CTC TTT TCT TTC CCG ATA
GTT-3′ (sense) and 5′-CTG GGA TGC TCT TCG ACC TC-3′ (antisense) for the ACTB. Genomic DNA (100 ng) was amplified
with 0.8 µM of each primer, 1.6 KCl Buffer, 1.5 mM of MgCl2, 0.25 mM of dNTPs, and 1 U Taq polymerase (Fermentas,
Thermo Scientific) in reaction volume of 25 µl. The PCR reaction
was performed on the GeneAmp® PCR System 9700 (Applied
Bioscience) under the following conditions: initial denaturation at
95°C for 10 minutes was followed by 30 cycles at 95°C for 1
minute, at 58°C for 1 minute and at 72°C for 1 minute, with final
elongation at 72°C for 10 minutes. The PCR products were
loaded on 2% agarose gels and stained with ethidium bromide.
Multi-Analyst/PC Software Image Analysis System (Bio-Rad
GelDoc 1000) was employed for densitometric analysis.
Ratio of the EGFR/ACTB score from tumor and blood tissue of
each patient was calculated and values higher than 2 indicated
presence of EGFR gene amplification.

Statistical analysis
Significant differences between the data sets were determined by
STATISTICA 6.0 software (StatSoft, Inc., Tulsa, USA). The
correlations between identified genes and genomic instability,
clinicopathological parameters and alterations of p53, PTEN, p16,
and EGFR were evaluated using Fisher exact test. Survival analyses
were performed using Kaplan and Meier product-limit method. The
log rank test was used to assess the significance of the
difference between pairs of survival probabilities. Overall survival
was calculated from the day after surgery to the last follow-up
examination or death of the patient. Statistical differences were
considered significant when p was ≤0.05 (*), p≤0.01 (**), and
p≤0.005 (***).

Results
Histopathological classification of tumor samples
After staining of surgically removed tissues by routine hema-
toxylin-eosin method and immunohistochemical testing for p53
positivity (Fig. 1) we confirmed diagnosis of 8 anaplastic
astrocytoma (WHO grade III) and 22 glioblastoma multiforme
samples (WHO grade IV) according to WHO criteria (2007).

Analysis of variant DNA fragments
Seven AP-PCR primers were used to discriminate normal from
tumor tissue. Four of them produced informative AP-PCR DNA
profiles containing explicit and countable differences between
tumor and blood in all 30 patients that were analyzed. Observed
differences were further classified as qualitative (mobility shifts in
the banding pattern due to mutations at the primer-template
interaction sites) and quantitative (altered band intensities repre-
senting amplifications or deletions of existing chromosomal
material) and were used as a measurement of the level of total,
microsatellite and chromosomal instability, as described in our
previous paper [15]. Significant level of genomic instability was
present in all samples, and based on the distribution of the
Novel Genetic Alterations in Glioma Samples

Table 2. Association between the frequency of altered genes, clinicopathological parameters and genomic instability.

| Parameter                     | Total NP | NP | %   | p  | NP | %   | p  | NP | %   | p  |
|-------------------------------|----------|----|-----|----|----|-----|----|----|-----|----|
| **LHFL3**                     |          |    |     |    |    |     |    |    |     |    |
| Total                         | 30       | 10 | 33.3| 0  | 8  | 26.6| 0  | 9  | 30  | 0  |
| **SGCG**                      |          |    |     |    |    |     |    |    |     |    |
| Total                         | 10       | 2  | 20  | 0  | 1  | 10  | 0  | 7  | 70  | 0  |
| **PDE4D**                     |          |    |     |    |    |     |    |    |     |    |
| Total                         | 14       | 2  | 14.3| 0  | 2  | 14.3| 0  | 8  | 57.1| 0  |
| **HTR4**                      |          |    |     |    |    |     |    |    |     |    |
| Total                         | 13       | 2  | 15.4| 0  | 2  | 15.4| 0  | 9  | 69.2| 0  |
| **Microsatellite instability**|          |    |     |    |    |     |    |    |     |    |
| Low                           | 14       | 4  | 28.6| 0  | 3  | 21.4| 0  | 6  | 42.8| 0  |
| High                          | 16       | 6  | 37.5| 0  | 5  | 31.2| 0  | 8  | 50.0| 0  |
| **Chromosomal instability**   |          |    |     |    |    |     |    |    |     |    |
| Low                           | 13       | 2  | 15.4| 0  | 2  | 15.4| 0  | 9  | 69.2| 0  |
| High                          | 17       | 8  | 47.1| 0  | 5  | 29  | 0  | 8  | 47.1| 0  |

*AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; *Bold indicates statistically significant values; ^NP, number of patients per group; LHFL3, lipoma HMGIC fusion partner-like 3; SGCG, sarcoglycan, gamma; PDE4D, cAMP-specific 3',5'-cyclic phosphodiesterase 4D; HTR4, 5-hydroxytryptamine (serotonin) receptor 4.

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frequency of DNA alterations, patients were assorted into two groups, with low or high level of genomic instability (Table 1).

The next step in our work was to identify some of the aberrant bands in DNA AP-PCR profiles common to more than 5 patients. Hence, twenty aberrant bands were retrieved from the PAA gels and cloned (Fig. 2A). Bands (amplicons) with the same electrophoretic mobility were isolated and characterized from at least two patients in order to confirm that they represent the same DNA sequence (Fig. 2B). Two clones of each band were sequenced. Obtained sequences were submitted for homology or identity search in NCBI GenBank and EBI (Sanger Institute) databases. Some of the sequences matched known genes and were easily identified, while others represented parts of certain contigs mapped on chromosomes 1, 2, 8q21–q23, 9, and 12, and their identification is yet to be determined (Fig. 2C).

The following 11 genes were identified: lipoma HMGIC fusion partner-like 3 (LHFL3); sarcoglycan, gamma (SGCG); 5-hydroxytryptamine (serotonin) receptor 4 (HTR4); integrin beta 1 (ITGB1); mitochondrial carboxamyl-phosphate synthetase 1 (CPS1); protein S (alpha) (PROS1); glycoprotein 2 (zymogen granule membrane) (GP2); potassium voltage-gated channel, subfamily G, member 2 ( KCNG2); cAMP-specific 3',5'-cyclic phosphodiesterase 4D (PDE4D); killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 3 ( KIR3DL3); inositol polyphosphate-5-phosphatase (INPP5A); we were also able to identify types of mutations in these genes. Namely, we identified nucleotide substitutions in KIR3DL3, INPP5A and KCNG2; multiple nucleotide substitutions in SGCG, PDE4D and LHFL3; while HTR4, ITGB1, CPS1, PROS1 and GP2 were carrying both multiple substitutions and 2 nucleotide deletions (Fig. 3). We then analyzed eight out of 11 identified genes regarding the expression profile of these genes in glial cells as a basic criterion.

Association of identified DNA alterations with genomic instability and clinicopathological parameters

Alterations of eight out of eleven identified genes were further examined in relation to the level of total, microsatellite and chromosomal genomic instability, tumor grade (grade III AA or grade IV GBM), age and sex (Tables 2 and 3). There was no statistical significance in correlation of identified DNA alterations with age and sex of the patients.

LHFL3 was altered in 10 out of 30 patients (33.3%), predominantly in grade IV glioblastoma samples (36.4% vs. 25% in grade III anaplastic astrocytoma). It was detected in significantly higher percentage in samples with high level of total genomic instability (52.9% vs. 7.7% of samples with low level of total instability, p = 0.005). The same trend was observed for microsatellite (37.5% of samples with high vs. 28.6% of samples with low level) and chromosomal instability (47.1% of samples with high vs. 15.4% of samples with low level), but without statistical significance.

Alterations of SGCG, HTR4, ITGB1, CPS1, PROS1 and GP2 were detected in 26.6% of samples (8 out of 30 patients) and were slightly increased in patients with anaplastic astrocytoma (37.5%) compared to GBM (22.7%), as well as in the samples with low level of total genomic instability (38.3% vs. 17.64% with high...
Table 3. Association between the frequency of altered genes, clinicopathological parameters and genomic instability.

| Parameter                      | ITGB1 | INPP5A | CPS1 | PROS1 |
|-------------------------------|-------|--------|------|-------|
|                               | Total | NP     | %    | p     | Total | NP     | %    | p     | Total | NP     | %    | p     |
| Total                         | 30    | 8      | 26.6 |       | 13    | 43.3   |       |       | 8     |       | 26.6  |       |
| Glioma subtype                |       |        |      |       |       |        |      |       |       |        |      |       |
| AA                            | 8     | 3      | 37.5 | 0.36  | 5     | 62.5   | 0.19 |       | 3     | 37.5   | 0.36  |
| GBM                           | 22    | 5      | 22.7 |       | 8     | 36.4   | 5     |       | 22.7  |       |
| Sex                           |       |        |      |       |       |        |      |       |       |        |      |       |
| Male                          | 19    | 5      | 26.3 | 0.64  | 10    | 52.6   | 0.17 |       | 5     | 26.3   | 0.64  |
| Female                        | 11    | 3      | 27.3 |       | 3     | 27.3   |       |       | 3     | 27.3   |       |
| Age                           |       |        |      |       |       |        |      |       |       |        |      |       |
| ≥50y                          | 24    | 5      | 20.8 | 0.17  | 12    | 50.0   | 0.16 |       | 5     | 20.8   | 0.17  |
| <50y                          | 6     | 3      | 50.0 |       | 1     | 16.7   | 3     |       | 50.0  |       |
| Genomic instability total     |       |        |      |       |       |        |      |       |       |        |      |       |
| Low                           | 13    | 5      | 38.5 | 0.19  | 5     | 38.5   | 0.46 |       | 5     | 38.5   | 0.19  |
| High                          | 17    | 3      | 17.6 |       | 8     | 47.0   | 3     |       | 17.6  |       |
| Microsatellite instability    |       |        |      |       |       |        |      |       |       |        |      |       |
| Low                           | 14    | 3      | 21.4 | 0.42  | 5     | 35.7   | 0.34 |       | 3     | 21.4   | 0.42  |
| High                          | 16    | 5      | 31.2 |       | 8     | 50.0   | 5     |       | 31.2  |       |
| Chromosomal instability       |       |        |      |       |       |        |      |       |       |        |      |       |
| Low                           | 13    | 7      | 53.8 | 0.005 | 4     | 30.8   | 0.20 |       | 7     | 53.8   | 0.005 |
| High                          | 17    | 1      | 5.9  |       | 9     | 52.9   | 1     |       | 5.9   |       |

AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; bold indicates statistically significant values; NP, number of patients per group; ITGB1- integrin, beta 1; INPP5A- inositol polyphosphate-5-phosphatase; CPS1 - carbamoyl-phosphate synthetase 1, mitochondrial; PROS1 - protein S (alpha).

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Table 4. Association of the frequency of identified genes with p53, PTEN, p16 and EGFR alterations.

| Parameter | LHFLPL3 | SGCGL | PDE4D | HTR4 |
|-----------|---------|-------|-------|------|
|           | Total   | NP    | %     | p    | Total | NP    | %     | p    | Total | NP    | %     | p    | Total | NP    | %     | p    |
| p53       |         |       |       |      |       |       |       |      |       |       |       |      |      |       |       |      |      |
| YES       | 12      | 5     | 41.7  | 0.34 | 4     | 33.3  | 0.40  | 1     | 8.3   | 0.04* | 4     | 33.3  | 0.40 |       |       |      |
| NO        | 18      | 5     | 27.8  | 0.34 | 4     | 22.2  | 0     | 8     | 44.4  | 4     | 22.2  |       |       |       |      |
| PTEN      |         |       |       |      |       |       |       |      |       |       |       |      |      |       |      |
| YES       | 20      | 7     | 35.0  | 0.56 | 4     | 20.0  | 0.23  | 6     | 30.0  | 0.67  | 4     | 20.0  | 0.23 |       |       |      |
| NO        | 10      | 3     | 30.0  | 0     | 4     | 40.0  | 3     | 30.0  | 4     | 40.0  |       |      |       |       |      |
| p16       |         |       |       |      |       |       |       |      |       |       |       |      |      |       |      |
| YES       | 18      | 5     | 27.8  | 0.34 | 6     | 33.3  | 0.28  | 3     | 16.7  | 0.05  | 6     | 33.3  | 0.28 |       |       |      |
| NO        | 12      | 5     | 41.7  | 0.34 | 2     | 16.7  | 6     | 50.0  | 2     | 16.7  |       |      |       |       |      |
| EGFR      |         |       |       |      |       |       |       |      |       |       |       |      |      |       |      |
| YES       | 13      | 5     | 38.4  | 0.45 | 5     | 27.8  | 0.19  | 3     | 23.1  | 0.38  | 5     | 27.8  | 0.19 |       |       |      |
| NO        | 17      | 5     | 29.4  | 0     | 3     | 25.0  | 6     | 35.3  | 3     | 25.0  |       |      |       |       |      |

*Bold indicates statistically significant values; NP, number of patients per group; LHFLPL3- lipoma HMGIC fusion partner-like 3; SGCGL- sarcoglycan, gamma; PDE4D- cAMP-specific 3’,5’-cyclic phosphodiesterase 4D; HTR4-5-hydroxytryptamine (serotonin) receptor 4.

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instability). Statistical significance was observed in case of chromosomal instability, were 53.8% of patients with low level of CIN had alterations in these genes compared to only 5.9% of patients with high level of CIN (p = 0.005).

**PDE4D** was changed in 9 out of 30 patients (30%) and distributed almost equally in grade III and grade IV glioma (25% vs. 31.8% respectively). Altered **PDE4D** is associated with high level of chromosomal instability (p = 0.02) because it was detected in 47% of patients with high level of CIN, compared to 7.7% of patients with low level of this type of instability (Table 2).

The most frequently present alteration was in **INPP5A** gene, detected in 43.3% of patients, predominately in patients with anaplastic astrocytoma (62.5% vs. 36.4% of GBM patients), and patients with high level of genomic instability (Table 3).

**Correlation analysis of identified DNA alterations and p53, PTEN, p16 and EGFR alterations**

We focused on four most frequently altered genes (**p53**, **PTEN**, **p16** and **EGFR**) in the genetic pathways of primary and secondary glioma [3] and analyzed their alterations in our set of samples. Alterations of **p53** were detected in 12 samples (40%), preferentially anaplastic astrocytoma (p = 0.03), as reported in our previous paper [15]. LOH analyses of **PTEN** revealed that all 30 examined tumor specimens were heterozygous for at least one of the examined loci and 66.7% (20/30) of them demonstrated LOH. **p16** was analyzed for the loss of heterozygosity and homozygous deletions, two most common mechanisms for the inactivation of this tumor suppressor. LOH was detected in 14 out of 30 patients (46.7%). Homozygous deletions were studied by combination of differential PCR and fragment analysis and were detected in 11 out of 30 patients (36.7%). Overall, alterations of **p16** were present in 18 samples (60%).

**Amplification of EGFR** gene, assessed by differential PCR, was detected in 13 samples (43.3%). The Fisher exact test revealed that the frequency of **PTEN** and **EGFR** alterations was significantly higher in higher grade tumors (GBM) in comparison to anaplastic astrocytoma (82% vs 25%, p = 0.007 for **PTEN** and 54.5% vs. 12.5%, p = 0.047 for **EGFR**). Alterations of **p16** were almost equally present in both histological subtypes (data not shown).

Next, we correlated obtained results with the presence of newly identified DNA alterations from AP-PCR DNA profiles of all patients (Tables 4 and 5). Remarkably, alterations of **PDE4D** were more frequently present in patients with wild-type **p53** and **p16** (p = 0.04 and p = 0.05, respectively). On the other hand, altered **INPP5A** was significantly associated with wild-type **EGFR** (p = 0.05). Interestingly, there was no significant correlation between loss of **PTEN** and any of the identified genes.

**Identified DNA alterations in relation to follow-up**

The overall survival of 30 patients was evaluated in relation to presence or absence of alterations of newly detected genes. In all cases patients were divided into two groups, those with and those without altered gene. Although there was no statistically significant difference among patients’ survival rate, it was noticed that patients with alterations of **SGCG**, **HTR4**, **ITGB1**, **CPS1** and **PROS1** had shorter survival rate (Fig. 4A). The survival of patients with alterations of **LHFPL3** was significantly shorter than the survival of patients without these alterations (p = 0.04, Fig. 4B), while alterations of **PDE4D** and **INPP5A** seem to have no impact on patients’ survival (Fig. 4C,D).

**Discussion**

The purpose of this study was to identify altered genes in 30 human glioma samples specifically associated with progression and outcome of this type of tumor. Our study revealed that AP-PCR fingerprinting is very useful in the identification and characterization of the regions of the genome of human glial tumors that have undergone alterations. The high level of genomic instability (median 34%) observed in all grade III and grade IV tumor samples analyzed in this study indicates the possibility of involvement of many more genes than those currently known to be of importance for development of these tumors. Our results indicate that alterations of eight out of eleven identified genes

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### Table 5. Association of the frequency of identified genes with p53, PTEN, p16 and EGFR alterations.

| Parameter | ITGB1 Total | NP | % | p | INPP5A NP | % | p | CPS1 NP | % | p | PROS1 NP | % | p |
|-----------|-------------|----|----|---|--------|----|---|--------|----|---|---------|----|---|
| p53       |             |    |    |   |        |    |   |        |    |   |         |    |   |
| YES       | 12          | 4  | 33.3 | 0.40 | 7      | 58.3 | 0.16 | 4      | 33.3 | 0.40 | 4      | 33.3 | 0.40 |
| NO        | 18          | 4  | 22.2 |  | 6      | 33.3 |  | 4      | 22.2 |  | 4      | 22.2 |  |
| PTEN      |             |    |    |   |        |    |   |        |    |   |         |    |   |
| YES       | 20          | 4  | 20.0 | 0.23 | 7      | 35.0 | 0.18 | 4      | 20.0 | 0.23 | 4      | 20.0 | 0.23 |
| NO        | 10          | 4  | 40.0 |  | 6      | 60.0 |  | 4      | 40.0 |  | 4      | 40.0 |  |
| p16       |             |    |    |   |        |    |   |        |    |   |         |    |   |
| YES       | 18          | 6  | 33.3 | 0.28 | 8      | 44.4 | 0.59 | 6      | 33.3 | 0.28 | 6      | 33.3 | 0.28 |
| NO        | 12          | 2  | 16.7 |  | 5      | 41.7 |  | 2      | 16.7 |  | 2      | 16.7 |  |
| EGFR      |             |    |    |   |        |    |   |        |    |   |         |    |   |
| YES       | 13          | 5  | 27.8 | 0.19 | 3      | 23.1 | 0.05* | 5      | 27.8 | 0.19 | 5      | 27.8 | 0.19 |
| NO        | 17          | 3  | 25.0 |  | 10     | 58.8 |  | 3      | 25.0 |  | 3      | 25.0 |  |

*Bold indicates statistically significant values; NP, number of patients per group; ITGB1- integrin, beta 1; INPP5A- inositol polyphosphate-5-phosphatase; CPS1 - carbamoyl-phosphate synthetase 1, mitochondrial; PROS1 - protein 5 (alpha). doi:10.1371/journal.pone.0082108.t005
might have an important role in pathogenesis of glial tumors, while detected changes in \( GP2, \) \( KCNG2 \) and \( KIR3DL3 \) should be considered as passenger mutations, since these genes are not expressed in glial cells. We assume that alterations of these three genes occur during clonal expansion of the genetically unstable tumor cells, and represent a consequence of detected significant level of genomic instability [15]. Genetic alterations identified in our study are infrequent in TCGA database samples (http://tcga-portal.nci.nih.gov), and represent an addition to other multi-institutional studies reports.

Alterations of lipoma \( HMGIC \) fusion partner (\( LHFPL3 \)) were more frequently detected in grade IV GBM, as well as in older patients and samples with high level of genomic instability which was shown to be present in \textit{de novo} glial tumors [15]. This gene, located at the long arm of chromosome 13, acts as a translocation partner of \( HMGIC \) in lipoma with \( t(12;13) \) [26] and the latest work of Nagaishi et al. [27] showed association of its amplifications with mesenchymal differentiation in gliosarcoma. According to our results, multiple nucleotide substitutions detected in this gene could also have impact on glioma invasiveness, especially considering significantly shortened survival rate of the patients carrying these alterations.

On the other hand, alterations of \( SGCG, HTR4, ITGB1, CPS1, PROS1 \) and \( INPP5A \) were detected predominantly in anaplastic astrocytoma, while alterations of \( PDE4D \) were present in both glioblastoma subtypes. Most importantly, the majority of identified genes have a significant role in signal transduction or cell adhesion, the important processes for cancer development and progression, but were not previously associated with glioma pathogenesis. Namely, \( HTR4, \) and \( PDE4A \) are among key players in cAMP signaling [28,29], while \( INPP5A \) regulates the level of another two secondary messengers, phosphatidylinositol (1,4,5)-trisphosphate and inositol-1,3,4,5-tetrakisphosphate [30].

Inverse relation between cAMP levels and the degree of malignancy was already shown in several types of brain tumors [31], and overexpression of \( PDE4A \) was considered to be one of the main mechanisms for reduction of its intracellular level [32]. In our study, alterations of \( PDE4D \) were more frequently found in patients carrying wild-type \( p53 \) and \( p16 \) genes, indirectly suggesting that decrease of cAMP in glial tumors might be one

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**Figure 4. Kaplan–Meier survival curves.** (A) Patients without alterations in \( SGCG, HTR4, ITGB1, CPS1 \) and \( PROS1 \) had tendency for better survival; (B) patients with alterations in \( LHFPL3 \) lived significantly shorter (\( p = 0.04 \)); (C) alterations of \( PDE4D \) had no impact on patients’ survival (D) alterations of \( INPP5A \) had no impact on patients’ survival.

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of the mechanisms for regulation of the activity of these tumor suppressors.

Serotonin (5-hydroxytryptamine; 5HT) and its receptors also regulate cAMP production [33]. Alterations of HTR4 gene, frequently present in our set of samples, were not previously detected in glioma. On the other hand, there are several reports on different tumor types (breast cancer, small cell lung cancer, prostate cancer and adenocortical adenoma) indicating that tumor cells acquire alterations in the 3-HT signaling that favor tumor-promoting actions and have a fundamental role in tumor growth, differentiation and gene expression [34,35].

As already mentioned, InsP3 5-phosphatase (INPP5A) functions mostly as a signal-terminating enzyme with implication for several cellular processes, including proliferation [36]. It was shown that loss of INPP5A was an early event in development of cutaneous squamous cell carcinoma [37]. Speed et al. [38] showed that absence or loss of this enzyme activity was also associated with transformation of NRK cells, while Mengubas et al. [39] connected decrease of inositol polyphosphate 5-phosphatase activity with several human leukemias. INPP5A is located in the short arm of chromosome 10 (10q26.3), and LOH of this region represent the most frequent genetic abnormality in both primary and secondary GBM, due to the presence of multiple tumor suppressor genes in this chromosomal region [3,40].

Our study also showed that alterations of INPP5A were significantly more frequent in samples without EGFR amplification. This is in accordance with finding that chronic elevation of inositol phosphate in unstimulated human fibroblast cells leads to mitosis. This is in accordance with finding that chronic elevation of inositol phosphate in unstimulated human fibroblast cells leads to mitosis. This is in accordance with finding that chronic elevation of inositol phosphate in unstimulated human fibroblast cells leads to mitosis. This is in accordance with finding that chronic elevation of inositol phosphate in unstimulated human fibroblast cells leads to mitosis. This is in accordance with finding that chronic elevation of inositol phosphate in unstimulated human fibroblast cells leads to mitosis. This is in accordance with finding that chronic elevation of inositol phosphate in unstimulated human fibroblast cells leads to mitosis.

Next, our results suggest that CPS1, one of the five key enzymes of the urea cycle [41] might also be aberrant in gliomas. Numerous point mutations and polymorphisms in this gene diversely affecting its biological function have been previously identified [42]. Altered expression of CPS1 was demonstrated in gastric cancer [43], hepatocellular carcinoma [44] and small-intestinal adenocarcinoma [45], but there are no data on glial tumors published so far. In our set of samples alterations of CPS1 were mainly found in grade III anaplastic astrocytoma with low level of genomic instability, suggesting potential role of this enzyme in neoplastic progression of secondary glioblastoma.

Protein products of SGCG and PROS1 are glycoproteins involved in cell adhesion [46,47], while transmembrane receptor ITGB1 has additional important role in cell signaling and regulation of cell cycle [48]. Data about their role in cancer progression are scarce, consisting of a few reports on different types of cancer. Downregulation of SGCG was detected in NSCLC [49] and breast cancer [50], while Sarason et al. [51] revealed elevation of PROS1 in high grade aggressive prostate cancer. Contrary to this, PROS1 seems to be downregulated in anaplastic meningiomas [52]. To the best of our knowledge, this is the first study that shows association between alterations of these two genes and glioma progression.

Integrin family members, due to their biological features, have numerous functions in cancer progression, especially in invasion and metastasis formation. The action of integrins has been studied in malignant melanoma, breast, prostate and pancreatic cancer [53,54,55,56]. There are also reports suggesting important roles of ITGB1 in glioma invasiveness and resistance to temozolomide treatment [57], which are in accordance with our findings.

Our results indicate that alterations of SGCG, PROS1 and ITGB1 genes appeared prevalently in grade III astrocytomas and resulted in shorter survival of patients, implying more aggressive nature of tumors with this genetic signature.

In conclusion, our study revealed that AP-PCR fingerprinting was extremely useful in the identification and characterization of the regions of glial tumor genome that have undergone alterations in comparison to their corresponding controls. Most importantly, we were able to identify genes associated with glioma pathogenesis, previously not related to this type of cancer. In particular, majority of identified genes play an important role in cell signaling, regulation of cell cycle, and cell adhesion, therefore participating in processes severely affected during malignant transformation. Further investigations of the detected genes on larger sample size, as well as in vitro studies, are under way, with special emphasize on clarifying the mechanisms of their action in progression of malignant glioma.

Author Contributions
Conceived and designed the experiments: VM NT. Performed the experiments: VM JB TS. Analyzed the data: VM NT. Contributed reagents/materials/analysis tools: MR MSG SR. Wrote the paper: VM NT.

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