# Analysis of oncogene GLI1 protein expression levels between differing grades of astrocytoma

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Aberrant expression of oncogene GLI1 has been linked to malignancies in many types of tissues including brain, pancreas, skin and breast. It has been found that GLI1 expression is important for tumor progression and has been linked to tumor grade. In this study, GLI1 expression has been analyzed in different malignancy grade astrocytomas.

GLI1 protein expression has been evaluated in 87 clinical tumor samples by using Western blot analysis. Associations between GLI1 expression and tumor grade were analyzed by applying Mann-Whitney or Kruskal-Wallis tests. Kaplan-Meier survival analysis was performed to evaluate the prognosis of patients.

Western blot analysis did not show statistically significant correlation between GLI1 protein expression and astrocytoma tumor grade (P = 0.294). Kaplan-Meier survival also did not show correlation between survival rates and GLI1 expression (P = 0.081). Our results show that GLI1 protein expression levels in astrocytomas have higher variability than previously shown.

Our results indicate that GLI1 protein expression is not an absolute requirement for the process of gliomagenesis. Yet, a relatively high (52%) occurrence of GLI1 expression in astrocytomas suggests the need for further analysis on the involvement of GLI1-mediated Hedgehog signaling pathway in glioma tumorogenesis.

**Key words:** astrocytomas, tumor-grade, tumorogenesis, protein expression, GLI1

## INTRODUCTION

Gliomas are a type of CNS tumor that arises from glial cells. Gliomas account for 28% of all brain and CNS tumors, whereas 54% of those tumors are cases of the highly malignant astrocytoma known as glioblastoma (GBM) (Ohgaki and Kleihues, 2005; Ostrom et al., 2013). The WHO classifies astrocytic brain tumors into 4 grades based on their malignancy: astrocytoma grade I (pilocytic astrocytoma), astrocytoma grade II (diffuse astrocytoma), astrocytoma grade III (anaplastic astrocytoma) and astrocytoma grade IV (glioblastoma) (Louis et al.,

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2007). Patients diagnosed with astrocytoma grade III and GBM have a 5-year median survival rate of 26.5% and 4.7%, respectively (Ostrom et al., 2013). The extremely low survival rate of GBM patients is caused by high levels of invasion of tumorogenic cells into the surrounding tissue and high resistance to therapies (Jovčevska et al., 2013). Current median survival rates, when compared with those in 1995–1998, have increased by approximately 4 months (Mazaris et al., 2014). This can be attributed to a greater understanding about the subgroups of patients with differing genotypes. In order to find more effective ways of treating and diagnosing astrocytomas, it is necessary to expand our knowledge about molecular mechanisms that underlie the process of gliomagenesis.

GLI1 was first discovered highly expressed in a human glioblastoma cell line in 1987 (Kinzler et al., 1987). GLI1 is an intracellular protein belonging to the "Zn" family of transcription regulators (Ruppert et al., 1988). GLI1 is known to play a role as being a part of the Hedgehog signal transduction pathway, which is a key mediator of embryonic development in all vertebrates and is responsible for patterning and morphogenesis of many different regions of the body (Ingham and McMahon, 2001). Aberrant expression of GLI1 has been linked to malignancies in many types of tissues including brain, pancreas, skin and breast (Rossi et al., 2011; Nolan-Stevaux et al., 2009; Grachtchouk et al., 2003; Cao et al., 2012). Previous studies have shown that GLI1 expression is important for tumor progression and that increased expression positively correlates with tumor grade (Rossi et al., 2011; Zhu et al.,

2014; Rush et al., 2010; Li et al., 2011). In this study, GLI1 expression has been analyzed in different malignancy grade astrocytomas.

## MATERIALS AND METHODS

### Patients and tissue samples
We investigated 87 WHO grade I–IV astrocytomas. All glioma tumors were surgically resected from patients without prior treatment and histologically diagnosed according to the 2007 WHO criteria (Louis et al., 2007) in the Department of Neurosurgery of Hospital of Lithuanian University of Health Sciences, Kaunas, Lithuania, from 2003 through 2012. The investigation has been performed in accordance with the principles of Declaration of Helsinki and approved by the Ethics Committee for Biomedical Research of the Lithuanian University of Health Sciences. All patients gave a written informed consent. Patient survival has been calculated from the date of operation to the date of analysis or death of the patient. Patient characteristics are shown in Table 1.

### Whole-tissue extract preparation and Western blot analysis
Whole-tissue extracts of the tumor samples have been routinely prepared by resuspending the sample (100–200 μg) in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630 (Sigma-Aldrich), 0.5% sodium deoxycolate, 0.1% SDS) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and homogenizing using an ultrasonic sonifier (500-Watt Ultrasonic Processor, Cole-Parmer). Subsequently, the extracts were cleared by

**Table 1.** Patient characteristics

| Tumor grade | No. patients | Mean age (years) | Gender | |
| --- | --- | --- | --- | --- |
| | | | Male | Female |
| I | 12 | 32.75 | 3 | 9 |
| II | 28 | 39.57 | 15 | 13 |
| III | 21 | 41.05 | 12 | 9 |
| IV (GBM) | 26 | 64.35 | 12 | 14 |
| **Total** | 87 | 46.39 | 42 | 45 |

centrifugation for 30 min at 13.000×g at 4 °C. 80 μg of the total extract protein were fractionated by 7.5% SDS-PAGE and transferred to nitrocellulose membranes. Immobilized proteins were incubated for 4 h at 25 °C with the primary rabbit antibody against GLI1 (Abcam, dilution 1:250) in blocking solution (5% nonfat milk in phosphate-buffered saline (PBS)). After extensive washing in PBS-T buffer (PBS supplemented with 0.5% Tween-20), membranes were incubated with the horseradish peroxidase- (HRP-) conjugated anti-rabbit secondary antibody (Life Technologies, dilution 1:2000) for 1 h at 25 °C. Immunocomplexes were visualized using enhanced chemiluminescence reagents (Life Technologies) and documented by using gel imaging system GelDoc-It2 (Analytika Jena AG). Values of GLI1 signals were calculated by using image analysis program ImageJ (National Institutes of Health, U.S.A.).

**Statistical analysis**

The software of Statistical Package for the Social Sciences (SPSS) version 22 for Windows (SPSS, Inc, IL, USA) was used for statistical analysis. The associations between protein expression and different clinical parameters were evaluated using the non-parametric Mann-Whitney U-test (if there were two independent groups) or the Kruskal-Wallis H-test (for more than two independent groups). To test the significance of protein expression on survival of patient groups, we used the Kaplan-Meier survival test. Correlations were considered statistically significant when P was less than 0.05.

## RESULTS

**Western blot analysis for GLI1 protein expression**

Western blot analysis was carried out on 87 tumor samples. The samples were divided into six groups each having a relatively equal number of different grade astrocytoma tumor samples. An immunoblot image of one of the groups is shown in Fig. 1. The primary antibody against GLI1 recognized a single protein band migrating at the position of 145 kDa, which corresponds to the electrophoretical migration of the full-length GLI1 as well as the slightly shorter version of GLI1 as described in Lo H-W et al. (2009). GLI1 has been detected only in 45 gliomas (52% of total samples), whereas no signal has been detected in 42 gliomas (48% of total samples).

**Association of GLI1 protein expression with astrocytoma tumor grade and other clinical parameters**

We performed the Mann-Whitney U-test and Kruskal-Wallis H-test in order to evaluate whether the intensity of GLI1 protein expression correlated with astrocytoma tumor grade and other clinical features. As shown in Table 2, no statistically significant correlation was found between GLI1 protein expression and the pathological grade of the tumors (P = 0.294). Our results show that GLI1 protein expression levels in astrocytomas have higher variability (Fig. 2) than previously shown.

**Survival rate compared to GLI1 expression**

Next, we decided to examine the relationship between levels of GLI1 protein expression and
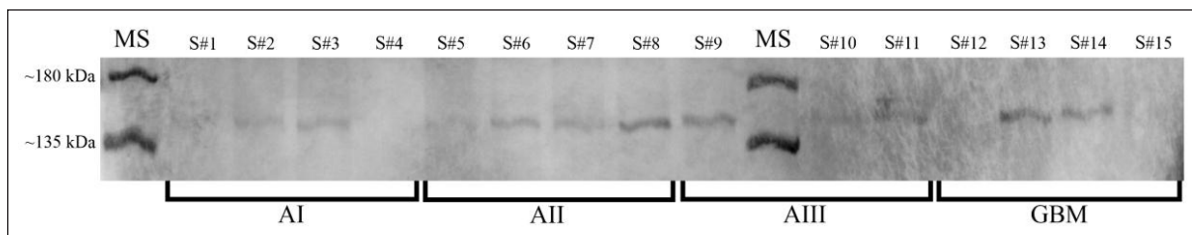


**Fig. 1.** Western blot analysis for GLI1 protein expression. MS – molecular mass standard, AI, AII, AIII and GBM – astrocytoma tumor grades, S#1-15 – patient sample number. The observed molecular mass of GLI1/ tGLI1 was 145 kDa

**Table 2.** GLI1 protein expression in human astrocytoma tissue with different clinicopathological features

| Features | No. patients | GLI1 expression | | | P |
|---|---|---|---|---|---|
| | | Low | Medium | High | |
| WHO grade | | | | | |
| I | 12 | 10 (83.3) | 0 (0.0) | 2 (16.7) | |
| II | 28 | 19 (67.9) | 6 (21.4) | 3 (10.7) | 0.294 |
| III | 21 | 14 (66.7) | 3 (14.3) | 4 (19.0) | |
| IV | 26 | 14 (53.8) | 3 (11.5) | 9 (34.6) | |
| Survival months | | | | | |
| <24 | 30 | 17 (56.7) | 3 (10.0) | 10 (33.3) | 0.115 |
| >=24 | 57 | 40 (70.2) | 9 (15.8) | 8 (14.0) | |
| Age | | | | | |
| <55 | 56 | 40 (71.4) | 11 (19.6) | 5 (8.9) | 0.025 |
| >=55 | 31 | 17 (54.8) | 1 (3.2) | 13 (41.9) | |
| Gender | | | | | |
| Male | 42 | 26 (61.9) | 8 (19.0) | 8 (19.0) | 0.671 |
| Female | 45 | 31 (68.9) | 4 (8.9) | 10 (22.2) | |
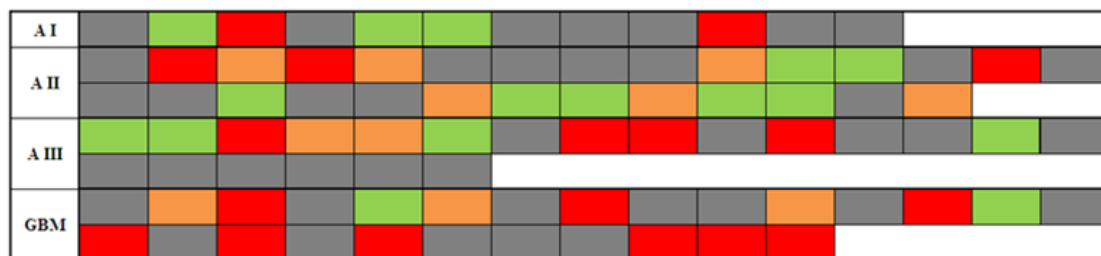


**Fig. 2.** Analysis of GLI1 protein expression in 87 astrocytoma tumor samples. Gray – no expression, green – low expression, orange – medium expression and red – high expression

the overall survival of the patient. For this purpose, GLI1 protein expression data values have been divided into three categories: values that were lower than 0.75 compared to the total average value were ranked as "low" GLI1 protein expression, values ranging between 0.75 and 1.5 were considered as "medium" GLI1 expression, and values that exceeded 1.5 were ranked as "high" GLI1 expression. The association of GLI1 protein expression with the overall survival rate of patients diagnosed with astrocytomas was analyzed by using the Kaplan-Meier survival test (Fig. 3). However, the results did not show a statistically significant association between the patient survival and GLI1 expression (P = 0.081). The plot of the survival test showed that patient groups with low and high GLI1 protein expression had lower survival
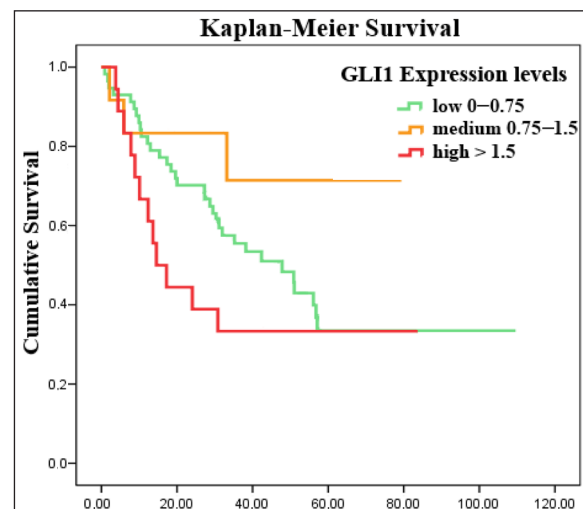


**Fig. 3.** GLI1 Kaplan-Meier survival analysis. Green – low expression, orange – medium expression and red – high GLI1 protein expression

rates compared to the group with medium protein expression levels.

## DISCUSSION

Earlier studies have shown that over-expression of GLI1 promotes tumor progression and invasion in a number of tissues (Zhu et al., 2014; Lo et al., 2009). Based on these and similar results, efforts have been made in search of inhibitors of Hedgehog-GLI1 signaling pathway that could be of value for cancer therapy (Mahindroo et al., 2009). We found that GLI1 protein expression has no significant correlation with the grade of glioma tumor, whereas Kaplan-Meier survival analysis revealed no difference between the patient survival and expression levels of GLI1.

## CONCLUSIONS

Thus, our results demonstrate that GLI1 is not an absolute requirement for glioma tumorogenesis. On the other hand, a relatively high (52%) occurrence of GLI1 expression in astrocytomas suggests the need for further analysis on the involvement of the GLI1-mediated Hedgehog signaling pathway in glioma tumorogenesis.

## ACKNOWLEDGEMENTS

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**ONKOGENO GLI1 RAIŠKOS TYRIMAS SKIRTINGO PIKTYBIŠKUMO LAIPSNIO ASTROCITOMOSE**

*Santrauka*

**Objektas.** GLI1 pirmą kartą atrastas žmogaus glioblastomos ląstelių linijoje, kurioje buvo labai padidėjusi šio baltymo raiška (Kinzler et al., 1987). Vėliau padidėjusi onkogeno GLI1 raiška buvo susieta su kancerogeniniais procesais smegenų, kasos, odos ir širdies audiniuose (Grachtchouk et al., 2003; Nolan-Stevaux et al., 2009; Rossi et al., 2011; Cao et al., 2012). GLI1 raiška taip pat tapatinama su auglio invazyvumu ir auglio piktybiškumo laipsniu (Rush et al., 2010; Li et al., 2011; Rossi et al., 2011; Zhu et al., 2014). Šiame tyrime išanalizavome GLI1 baltymo raiškos lygį skirtingo piktybiškumo laipsnio astrocitomos navikuose.

**Medžiagos ir metodai.** GLI1 raiška analizuota 87 klinikiniuose auglių mėginiuose naudojant Western blot analizės metodą. Asociacijos tarp GLI1 ekspresijos ir auglio piktybiškumo laipsnio bei kitų charakteristikų buvo analizuojamos statistiniais neparametriniais Mann-Whitney ir Kruskal-Wallis testais. Be to, atlikta Kaplan-Meier išgyvenamumo analizė.

**Rezultatai.** Western blot analizės rezultatai neparodė statistiškai patikimo ryšio tarp GLI1 baltymo raiškos lygio ir astrocitomos piktybiškumo laipsnio (P = 0,294). Kaplan-Meier išgyvenamumo analizė irgi neatskleidė koreliacijos tarp pacientų išgyvenamumo ir GLI1 raiškos (P = 0,081). Mūsų rezultatai parodė didesnį GLI1 raiškos heterogeniškumo laipsnį tarp astrocitomos auglių, palyginti su ankstesniais duomenimis.

**Apibendrinimas.** Gauti rezultatai rodo, kad GLI1 baltymo raiška nėra būtina gliomų tumorogenezei. Kadangi pakankamai didelis procentas astrocitomų turi padidėjusią GLI1 baltymo raišką (52 %), papildomi tyrimai yra reikalingi norint nustatyti Hedgehog-Gli signalinio kelio reikšmę gliomagenezei.

**Raktažodžiai:** astrocitomos, piktybiškumo laipsnis, tumorogenezė, baltymų raiška, GLI1