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SHORT COMMUNICATIONS

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HIGH EXPRESSION OF *GLI1* IS ASSOCIATED WITH BETTER SURVIVAL IN ADVANCED SCLC

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Aim: **Aberrant Sonic hedgehog (Shh) pathway signaling has been described in small cell lung cancer (SCLC), as well discrepancies, when analyzing expression of pathway components in SCLC cell lines *vs* tumor biopsies. Shh key component *GLI1* was evaluated in advanced SCLC and data correlated with patient survival.** ***Materials and Methods: GLI1*** **expression was analyzed by quantitative real-time polymerase chain reaction in pre-treatment fresh frozen tumor biopsies of 12 advanced SCLC patients and mRNA level of *GLI1* was compared in short-term *vs* long-term survivor's samples (stratified by median survival, independent samples t-test).** ***Results:*** **Expression of *GLI1 mRNA* was significantly higher in long-term (> 9.6 months, n = 6) survivor's biopsies than in short-term (⩽ 9.6 months, n = 6) survivors ($p = 0.0196$, 95% CI: 0.000016 to 0.000147, two-tailed independent samples t-test).** ***Conclusion:*** **High *GLI1* mRNA expression in SCLC was found to be positive prognostic marker associated with longer survival. Further research is needed for validation of these results due to the small number of patients in the study.**

Key Words: **small cell lung cancer, Sonic hedgehog pathway, *GLI1*, survival, prognosis.**

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Aberrant Sonic hedgehog (Shh) pathway signaling has been described in small cell lung cancer (SCLC), however, controversial results have been obtained, when analyzing expression of pathway components in SCLC cell lines *vs* tumor biopsies [1]. There is an increasing evidence that the embryonic signaling pathways are essential for the maintenance and chemoresistance of SCLC cancer stem cells [2]. These chemoresistant cells may be the cause for non-successful treatment of some SCLC patients, who do not respond well to therapy and survive much shorter than other patients.

The aim of this study was to evaluate the prognostic role of Shh pathway activity in SCLC. We evaluated mRNA expression of Shh pathway key component *GLI1* in advanced stage SCLC samples and correlated data with patient survival.

MATERIALS AND METHODS

Patients and tissue samples. Study tissue samples were obtained along with a standard biopsies by fibrobronchoscopy from patients, diagnosed with advanced (stage III and IV) SCLC at Pauls Stradins Clinical University Hospital between October 2010 and January 2014. The Ethics Committee of the Institute of Experimental and Clinical Medicine of University of Latvia has approved this study. Written informed consent was obtained from the patients before inclusion in the study.

The tissue specimens were submerged in RNAlater solution (Thermo Fisher Scientific, USA) and stored at –20°C. Board-certified cytologist confirmed the presence of tumor cells in the sample by cytological evaluation of the smear, which was obtained from study specimen before placing it to the RNAlater. All diagnoses were histologically confirmed in standard biopsies as SCLC by board-certified pathologists. Pre-treatment biopsies from 20 SCLC patients were obtained and they were followed during treatment.

Clinical information, treatment and survival data were collected. 7 patients were excluded from the analysis due to the lack of cytological confirmation of tumor cell presence in a study biopsy. 1 patient died early during treatment from non-cancer related cause and therefore was also excluded from the analysis.

12 SCLC patients were stratified in a short-term and long-term survivors by median overall survival (OS) time (9.6 months) to correlate Shh related genes expression with patient survival. Demographics and clinical characteristics of 12 patients with qualitative RNA and survival data are shown in Tables 1 and 2.

Preparation of tissue mRNA. Biopsy homogenization was done by FastPrep®-24 Instrument and Lysing Matrix D (MP Biomedicals, USA) at 0.4 m/s for 40 s. Total RNA was isolated using MirVana total RNA Isolation Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. RNA was treated with DNAse I (Thermo Fisher Scientific, USA) and RNA concentration and purity was quantified by Nanodrop ND-100 spectrophotometer. cDNA was synthesized by random hexamer priming from 1 μg of total RNA by using Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction. Quantitative real-time polymerase chain reaction was performed using 2 μl 1:10 diluted cDNA reaction mixtures, ABSolute Blue ™ SYBR green Low ROX (Thermo Fisher Scientific, USA) and ViiA 7 real — time polymerase chain reaction system (Applied Biosystems, Life Technologies, USA). Sequences of primers used in this study are available on request. To normalize the expression data, normalization factor was calculated

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Abbreviations used: OS – overall survival; SCLC – small cell lung cancer; Shh – Sonic hedgehog.

for each cDNA from the expression values of three most stable references genes (*ACTB, LRP10, YWHAZ*) selected among seven frequently used housekeeping genes by using geNorm software. All quantitative real-time polymerase chain reaction experiments were performed in duplicates and the data were presented in graphs as means ± standard deviation.

Statistical analysis. *GLI1* mRNA level was compared by calculating two-tailed independent samples t-test in short-term *vs* long-term survivor's samples, stratified by median survival. Data were statistically interpreted using SPSS Statistics 21. *P*-values of < 0.05 were considered significant.

RESULTS AND DISCUSSION

A small cohort of 12 patients with qualitative RNA and survival data was analysed:

- 6 SCLC patients in long-term survivors group (OS > 9.6 months) had mean *GLI1* mRNA level of 0.000141757 ± 0.000038223 in their pre-treatment samples.
- 6 SCLC patients in short-term survivors group (OS ⩽ 9.6 months) accordingly had mean *GLI1* mRNA level of 0.000060198 ± 0.000016324.

Significantly higher *GLI1* mRNA levels were found by two-tailed independent samples t-test in pre-treatment biopsies of long-term SCLC survivors when comparing with short-term survivors ($p = 0.0196$, 95% CI: 0.000016 to 0.000147) (Figure).

Negative prognostic role of *GLI1* has been drawn from numerous studies and meta-analyses for breast [3] and gastric cancer [4], lung adenocarcinoma and squamous cell carcinoma [5, 6], gallbladder and liver cancer, cervical cancer, rhabdomyosarcoma, colon cancer, ovarian cancer, bladder cancer, esophageal cancer, head and neck squamous cell carcinoma, pancreatic cancer [7]. Over-expression of *GLI1* tends to progressive stages and is related to unfavourable prognosis. Only in intracranial tumors GLI1 positivity was not correlated to poorer survival. Further study has confirmed the adverse effect of low nuclear *GLI1* expression in glioblastomas, which is in contrast with the negative prognostic effect of high *GLI1* expression reported in non-cranial malignancies [8]. Concerning *GLI1* as a prognostic factor in lung adenocarcinoma there is also study that has reported longer survival in *GLI1* positive tumors [9].

SCLC is a specific malignancy with a few common critical genetic and epigenetic alterations, leading to frequent *GLI1* overexpression and ligand-independent *GLI1* activation. *GLI1* activation could be caused for example by bi-allelic inactivation of *TP53* genes and TP53 deficiency observed nearly in all SCLC cases [10, 11]. Another frequently observed *GLI1* activating alteration in SCLC is downregulation of Notch pathway and downregulation of PI3K-AKT oncogenic signaling. Notch pathway signaling was found to be downregulated in 77% of 110 SCLC clinical tumor specimens, while Notch family genomic alterations were found here in 25% of cases [11]. Loss of function of tumor suppressor PTEN, an endogenous inhibitor of AKT, which could be found in 10–18% of SCLC tumors [12], will also lead to increased transcriptional activity of *GLI1*.

Our finding with higher *GLI1* expression in long-term SCLC survivors might reflect in this case lower mutational burden and only few epigenetic changes with retained *GLI1* upregulation characteristic for SCLC. *GLI1* downregulation and increased chemoresistance in short-term SCLC survivors may be explained by the higher number of genetic and epigenetic changes in tumor cells.

The main objects for SCLC molecular research still are cell lines and xenografts due to the clinical features

Table 1. Characteristics of 12 patients qualified for gene expression and survival analysis. The comparison between groups was carried out by the Student's *t*-test or the Chi Square test, according to the type of variable

Patient characteristics	Long-term survivors (OS > 9.6 months, n = 6)	Short-term survivors (OS ⩽ 9.6 months, n = 6)	*P*-value
Gender			
Male	5	5	$p = 1.0$
Female	1	1	
Age			
Median (range)	61.5 (54–77)	51.5 (49–70)	$p = 0.078$
ECOG			
0	1	1	
1	0	2	$p = 0.446$
2	4	2	
3	1	1	
4	0	0	
Stage			
III A	1	0	$p = 0.565$
III B	2	2	
IV	3	4	
Deviations of treatment dosing/timing due toxicity			$p = 0.079$
None	2	5	
Present	4	1	
Best response			
CR	0	0	
PR	6	4	$p = 0.301$
SD	0	1	
PD	0	1	
Disease progression type			
Local	2	1	$p = 0.601$
Distant	2	3	
Combined	1	2	
Not progressed	1	0	

Note: CR – complete response; PR – partial response; SD – stable disease; PD – progressive disease.

Table 2. Survival and treatment data of 12 study patients

Patient ID Nr.	Survival in months (days)	Survival in months (days)	Received treatment
Patient LC145		4 (140)	4 CE
Patient LC137		6 (191)	6 PE → RT (57.6/50.4/45 Gy)
Patient LC146		8 (250)	4 PE → 3 CAV
Patient LC102		8 (252)	5 PE
Patient LC171		8 (267)	1 PE → 5 CAV
Patient LC135		8 (269)	4 PE → RT (54/50.4 Gy) → 37.5 Gy WBRT
Patient LC130	10 (305)		5 PE → 3 CAV
Patient LC127	10 (318)		5 PE → 30 Gy WBRT
Patient LC134	12 (383)		5 PE → RT (54/54/45 Gy) → 36 Gy WBRT
Patient LC139	13 (396)		6 PE → RT (54/50.4 Gy) → PCI (30 Gy)
Patient LC121	15 (462)		6 CE → RT (54/50.4/50.4 Gy) → PCI (36 Gy) → 1 CAV
Patient LC126	82 (2499)		5 PE → RT (56/50.4 Gy) → PCI (36 Gy)

Note: CE – carboplatin/etoposide; PE – cisplatin/etoposide; RT – radiation therapy; CAV – cyclophosphamide/adriamycin/vincristine; WBRT – whole brain radiation therapy; PCI – prophylactic cranial irradiation.

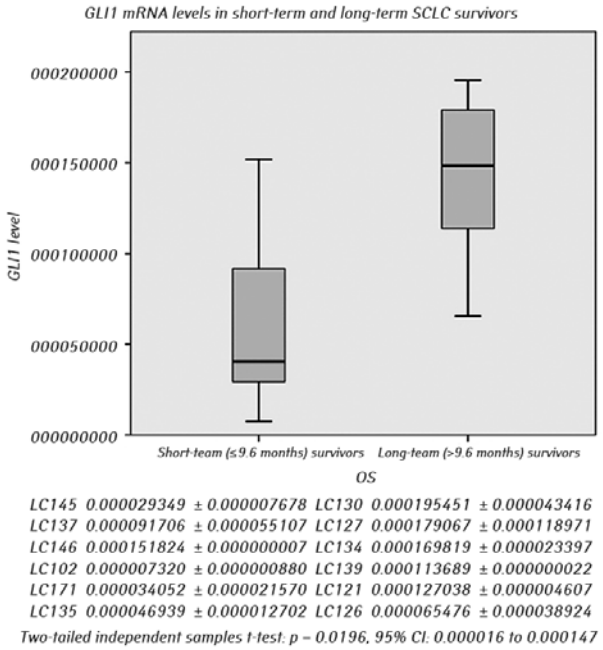

Figure. Dot plots showing normalized *GLI1* gene expression levels in pre-treatment SCLC biopsies of long- *vs* short-term survivors cohorts. The median gene expression level for each group is indicated by a line inside the box. Data presented in graphs are means obtained from experiments in duplicates ± standard deviation

of disease. Aberrant Shh pathway signaling has been described in SCLC, as well as discrepancies, when analyzing expression of pathway components in SCLC cell lines/xenografts *vs* tumor biopsies. Only small percentage of SCLC cell lines express transcriptional regulator *GLI1*, which can be used as an indicator of Shh signalling activity [13]. Contrary, when GLI1 immunohistochemical staining was performed on SCLC biopsies to investigate human tumors for *GLI1* expression, 34 (85%) out of 40 SCLC tumors demonstrated *GLI1* expression and 26 of these displayed medium or strong GLI1 reactivity [14]. Thus, caution should be taken when working with tumor derived cell lines, as the expression and signaling may not reflect the *in vivo* situation. These findings were supported also by Affymetrix oligonucleotide microarray analysis of human SCLC tumors, where significantly higher mean *GLI1* expression in SCLC tissues was found when compared to that of the 20 SCLC cell lines investigated [15].

So advantage of this study was that mRNA expression of key Shh pathway member *GLI1* was evaluated in SCLC fresh biopsies and survival data of patients were available. Unfortunately, small size of biopsy samples was an issue, which lead to exclusion of notable part of study patients from analysis due to the lack of cancer cells in a study specimen.

In conclusion, at our best knowledge this represents the first study investigating the prognostic role of *GLI1* in SCLC patients. Our findings suggest that the Shh pathway activation might be a positive prognostic marker in SCLC contrary to its negative role observed in other malignancies. Further research is needed for validation of these results due to the small number of patients in the study.

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DISCLOSURE OF INTEREST

The authors declare no conflict of interest.

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