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Glutaminase enzyme (GLS1) in small cell lung cancer

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Abstract Cover Page

Glutaminase enzyme (GLS1) in small cell lung cancer

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Abstract

[Glutaminase enzyme (GLS1) in small cell lung cancer]

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Small cell lung cancer (SCLC) is a fatal disease with limited treatment options. A better understanding of the biology of this disease is needed to guide new treatment options that will result in improved outcomes. Glutaminase enzyme (GLS1) catalyzes the conversion of glutamine to glutamic acid. Preclinical work suggests that this enzyme impacts efficacy of chemotherapeutics used for the treatment of SCLC. The current work was therefore conducted to characterize the expression of GLS1 in SCLC and to assess the association between expression and survival in these patients.

The study employed tissues samples of patients diagnosed and treated for SCLC at Emory University. Archival surgical resection samples were employed to determine expression using a standard immunohistochemistry approach. Clinical characteristics and patient outcomes, overall survival (OS) and progression free survival (PFS), were obtained by review of the electronic medical record and summarized using descriptive statistics. Differences in GLS1 expression across race, gender and ethnicity were assessed using t-test, chi-square test or Kruskal-Wallis test based on the distribution of the data. Differences in survival between patient groups defined by GLS1 expression level were tested using Log-rank test and potential covariates were adjusted with Cox proportional hazards model. Statistical significance was set at 0.1 for all analyses.

Among the 48 patients for which tissue sample and clinical data was available, median age was 60 years, 58% were females and the majority (79%) were Caucasians. GLS1 expression was significantly higher in normal lung ($p= 0.054$) compared to cancer cells

but did not vary significantly by age, gender or race. There was no significant correlation between GLS1 and its other isoform, GLS2, or with the counteracting enzyme, glutamine synthetase (GS). GLS1 expression impacted both progression free survival (PFS) and overall survival (OS) in an age-dependent manner. In conclusion, this study successfully characterized the expression pattern of GLS1 and showed a higher expression in normal lung tissue in comparison to SCLC. It also showed that high GLS1 expression associates significantly with better OS and PFS.

Cover Page

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INTRODUCTION

Small cell lung cancer (SCLC) is diagnosed in approximately 30,000 new patients annually in the United States.(1) Initial treatment approach for this disease is currently based primarily on anatomic staging by which patients are classified as having limited stage (limited to the chest and able to undergo curative radiation therapy) or extensive stage disease (widespread within or outside the chest and therefore unable to undergo curative radiation and chemotherapy). This classification has great utility and has been used for more than 4 decades in selection of therapy.(2) Currently, the most widely employed classifiers of SCLC patients rely either on staging information (limited vs. extensive stage) or on the duration or degree of response to frontline therapy (platinum sensitive vs. platinum resistant/refractory). An important drawback of the current classification algorithms is that the underlying tumor biology responsible for the differing extent of tumor spread and response to therapy is poorly understood. These algorithms are therefore insufficient for optimal decision-making for patient management since it is of limited benefit to a large proportion of patients treated with ineffective chemotherapy. Approximately 40% of patients with extensive stage SCLC do not respond to empiric frontline platinum-based chemotherapy and therefore fail to obtain significant survival benefit from therapy.(3, 4) This is a major drawback in the current era of personalized therapy where the use of the deeper understanding of tumor biology and mechanism-based biomarkers for selecting patient for treatment has led to impressive clinical benefit in carefully defined subsets such as EGFR mutant and ALK-EML4 translocated non-small cell lung cancer.(5, 6)

A biologically meaningful biomarker capable of classifying SCLC will therefore be of great clinical value. Specifically, such a biomarker might allow for the identification of 40% of patients unlikely to benefit at all from empiric frontline chemotherapy. In a gene expression profile analysis conducted with a panel of nine SCLC cell lines, we identified increased expression of glutaminase (GLS1) gene as the dominant change in a 5-gene profile (submitted manuscript) that classifies the tested cell lines as sensitive or insensitive to platinum-based combination therapy following cell exposure to platinum in vitro.(7) GLS1 and its product, glutamate, have been shown in breast cancer to determine cell polarity response to standard chemotherapy agents.(8, 9) Previous work in lymphoma and prostate cancer cells also showed that this gene is modulated by *c-myc* gene amplification. Interestingly, *c-myc* amplification is associated with poor response to chemotherapy and adverse prognosis in SCLC.(10-14) Based on these preclinical observations, we predict that SCLC with high GLS1 expression will have poor sensitivity to chemotherapy due to the high levels of glutamate, a product of GLS1 enzyme activity, leading to poorer outcome. The first step in our systematic attempt to establish this multi-step hypothesis is to demonstrate that there is a measurable difference in the level of expression of GLS1 in tumor tissue samples from defined SCLC patient subgroups.

BACKGROUND

In an attempt to identify potential determinants of response to chemotherapy for SCLC, gene expression profiling was performed comparing SCLC cell lines that were sensitive and insensitive to chemotherapy agents. This effort led to the identification of 5 genes and pseudogenes (*GLS, UBE2C, MSI2, HACL1* and *LOC100129585*) that were differentially expressed between the sensitive and insensitive SCLC cell lines.(7) Glutaminase (GLS) is an intracellular enzyme that catalyzes the generation of glutamate from glutamine.(15, 16)

Tissue-specific isoenzymes of glutaminase (GLS1 and GLS2) are expressed in periportal hepatocytes and renal tubular epithelium.(15) The action of GLS leads to the release of glutamate, which is incorporated into GSH for free radical scavenging and tissue repair. Because standard chemotherapeutic agents for the treatment of SCLC and other cancers rely on the generation of reactive free radicals, we postulate that high GLS1 will impair the efficacy of cytotoxic chemotherapy and ionizing radiation.

C-myc gene amplification portends poor prognosis in SCLC.(10, 11, 13) Prior work showed that amplification of *c-myc* leads to GLS1 overexpression in lymphomas and leukemia.(14) This observation along with mutation in other metabolizing enzymes in part provided the justification for the ongoing clinical testing of inhibitors of GLS1 in lymphoma patients. This class of agents will provide new therapeutic options for SCLC if a biologically relevant role of GLS1 in SCLC is established.

Our preclinical work described above also suggested that platinum sensitive and insensitive SCLC cell lines differ in their levels of GLS1 expression. We therefore decided to characterize GLS1 expression in SCLC tissue samples as an important step for establishing GLS as a validated predictor of chemosensitivity, patient survival and a potential therapeutic target.

METHODS

Hypothesis

H_0: There is no association between high GLS1 expression and poor survival in SCLC

H_A: High GLS1 expression in tissue sample is associated with poor survival in SCLC

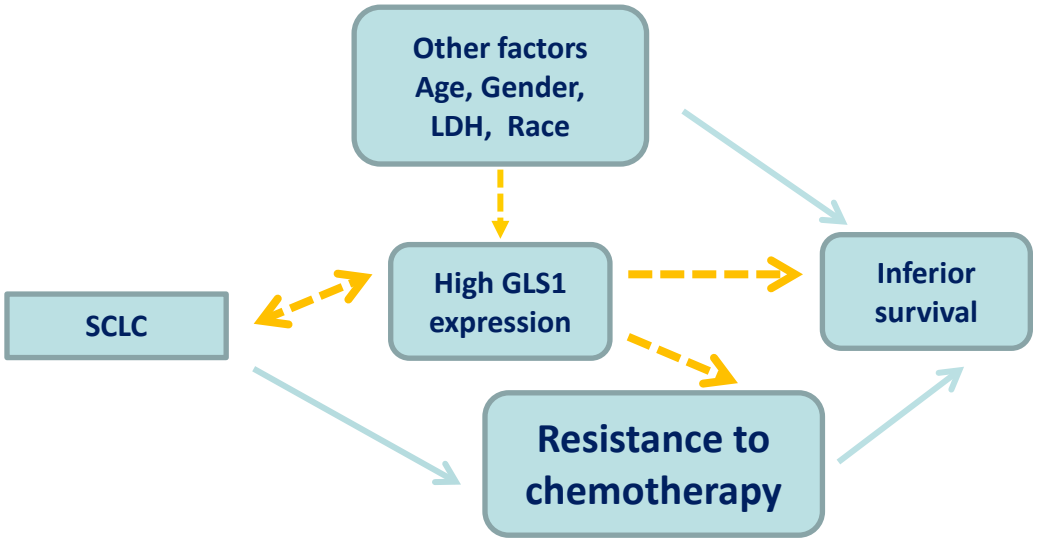

Figure 1: Causal Diagram

The causal diagram above summarizes the possible pathways mediating the *a priori* hypothesis that GLS1 expression is associated with patient survival. We anticipate that SCLC patients with high GLS1 expression will have inferior survival. This could be a true and direct consequence of GLS1 enzyme activity. Alternatively, it could also be a true but indirect effect of GLS1 e.g. through reduced sensitivity of cancer cells with high GLS1 to chemotherapy and radiation. A false association of GLS1 expression and survival might result from the confounding effects of known prognostic factors in SCLC such as age, gender, and possibly race (potential confounders).

Objectives:

1. To characterize the expression of GLS1 in SCLC tissue and in normal lung tissue

1.1 Expression will be detected in resected tissue samples using standard immunohistochemistry approach

1.2 Degree of expression will be quantified using a categorical staining intensity scoring (0, 1+, 2+, 3+) and a continuous scoring method “immunoscore” - product of intensity and percent cell staining

2. To determine whether GLS1 expression is associated with reduced survival of SCLC patients

2.1 Overall survival (OS) measured as time interval from diagnosis to death

2.2 Progression free survival (PFS) measured as time interval from surgery to first documented progression of disease or death

Study design

A retrospective, single institution, cohort study was selected. There was no direct patient contact but human tissue samples and clinical data were employed for this analysis. The study protocol was previously approved by the Emory University IRB (protocol IRB00018386). The study population was open to any patient who underwent surgical resection for small cell lung cancer within the Emory Healthcare organization between 1989 and 2011. Using a convenient sampling method, all consecutive cases starting from the oldest to the most recently diagnosed were evaluated for inclusion. All patients meeting the defined inclusion/exclusion criteria up to the required sample size were included in this study.

Inclusion/exclusion criteria

- Confirmed pathologic diagnosis of SCLC treated at Emory University Hospital between 1989 and 2011
- Adequate tissue sample available in the pathology archives for the planned IHC analysis
- Surgical resection specimen available for proper characterization of protein expression
- Clinical record must be available for accurate estimation of the endpoints of progression free survival (PFS) and overall (OS)
- Patients younger than 18 years at time of diagnosis are excluded because the biology of SCLC in the pediatric age group is likely to be different than the tobacco-associated SCLC in adults
- Other cancer diagnosis within 5 years of SCLC diagnosis
- Diagnosis established by fine needle aspiration
- Insufficient tissue samples for the planned IHC

Measurements

The following variables were measured for each subject.

- Protein expression measured using immunohistochemistry (IHC). This is a well established and validated technique for research and clinical use.(17) The level of expression was quantified jointly by 2 observers including an attending pulmonary pathologist.

- Overall survival
- Progression free survival

Sources of data

Demographic (age, gender, and race) and survival data were obtained from the electronic medical record and publically available databases for vital status.

Definition of outcome, predictor, and covariates - The primary outcomes of interest are PFS and OS as defined above. The primary predictor of interest is GLS1 expression. The following variables are covariates of interest based either on clinical relevance or potential biologic relationship with GLS1: Age, gender, race, receipt of chemotherapy/radiation.

Patients were excluded for specific analysis where they have missing values.

Sample-size and power considerations/calculations

The median OS for patients with advanced SCLC is approximately 10-12 months based on data from randomized phase III trials. This survival outcome has not changed in more than 2 decades. Likewise, PFS following frontline therapy is 4-6 months. We assumed that patients with good prognosis SCLC based on GLS1 expression will have a median OS of 10 months and PFS of 6 months in comparison to 5 and 3 months respectively for poor prognostic patient group, for a HR of 0.5 for either OS or PFS comparison. We expect to demonstrate this difference with 58 patients under the assumption that the 2-sided alpha error rate is less than 10% and beta error rate of 20% i.e. power of 80%.

Analysis plan

The following specific analyses were performed using demographic, survival and protein expression data from the eligible patients.

We characterized GLS1 (GLS2 and GS) expression in cancer tissue and adjacent normal lung. Expression was quantified by light microscopy jointly by 2 investigators. The intensity of staining was graded on an ordinal scale ranging from 0 (no staining) to 3 (maximal staining intensity).

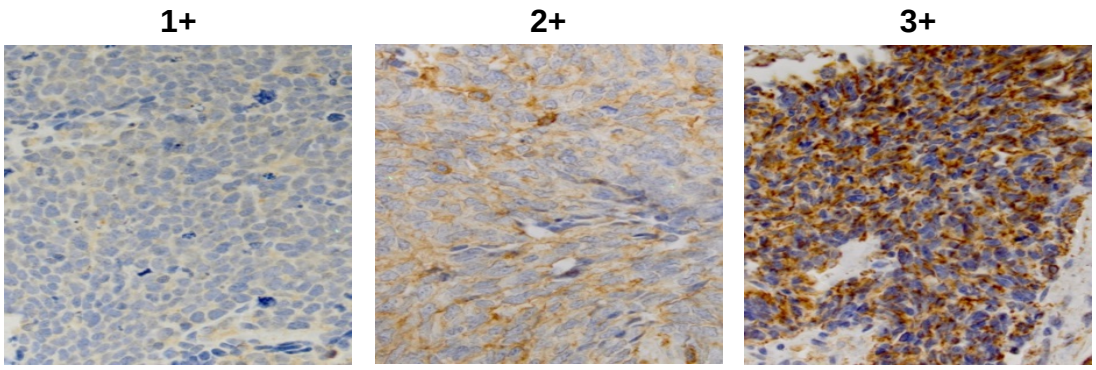

Figure 2: Staining intensity scoring algorithm

Representative sections demonstrating the semi quantitative assessment of staining intensity on IHC slides 0, 1, 2 or 3.

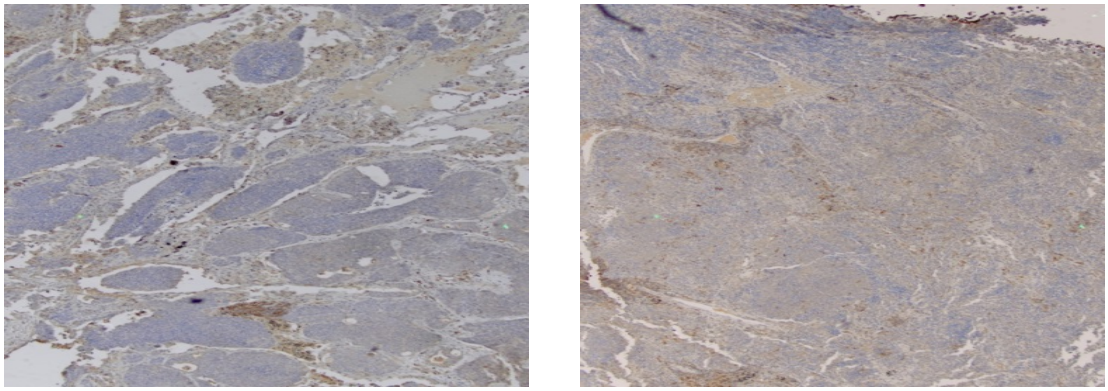

Figure 3: Immunoscore determination
Representative sections showing 100% of cells staining at an intensity of 1+ (right) and 0% of cancer cells staining at an intensity of 0 (left) for an immunoscore of 100 and 0 respectively.

A derivative score “immunoscore” representing the product of intensity of staining (0, 1, 2 or 3) and the percentage of cells staining (0-100%) was also generated for each case to derive a score ranging between 0 and 300 on a continuous scale.

The derived variables were subsequently employed to compare protein expression (GLS1, GLS2 and GS) in cancer tissue to expression in adjacent normal lung. Descriptive analyses using summary statistics of frequencies, mean and median were performed for the demographic variables. Point estimates along with confidence intervals were calculated for protein expression according to demographic groups.

Association between protein expression and specific patient demographic or treatment status were tested by univariate, bivariate and multivariate models. Potential differences in GLS1 expression based on established and possible prognostic factors in SCLC (age, gender, and race) were also evaluated. Finally, outcome variables (OS and PFS) were compared between patient categories of GLS1 expression [median immunoscore and by

various categories of staining intensity (0, 1+) vs. (2+, 3+); (0) vs. (1+, 2+, 3+); (0) vs. (1+) vs. (2+) vs. (3+)]. Exploratory sensitivity analyses using other definitions of expression categories were also performed in order to identify the optimal cut point for GLS1 expression to define different prognostic groups of SCLC.

Survival curves were generated using the method of Kaplan and Meier and differences in survival between defined patient subgroups were tested by the log-rank test. In order to test the additional impact of known prognostic factors in SCLC as well as the impact of other variants of Glutaminase enzyme (GLS2) and the counteracting enzyme, glutamine synthetase (GS), a multivariable analysis was performed for outcome variables (PFS and OS) along with these predictor variables GLS1, GLS2, GS, age, gender, chemotherapy and radiation using Cox proportional hazards model. To assess for effect modification, the Cox proportional hazards model was reformulated to include interaction terms between GLS1 expression and prognostic clinical variables such as age, gender, race and treatment.

Exploratory analyses:

The initial analyses employed a dichotomous definition of GLS1 expression (< vs. >median immunoscore and 0/1+ intensity vs. 2+/3+ intensity). Because these categories were arbitrary with no biological rationale, we decided to explore other categorical definitions of GLS1 expression in order to fully explore whether GLS1 impact patient survival.

RESULTS

Case selection for the analysis: We identified 48 patients meeting the specified eligibility criteria for the study.

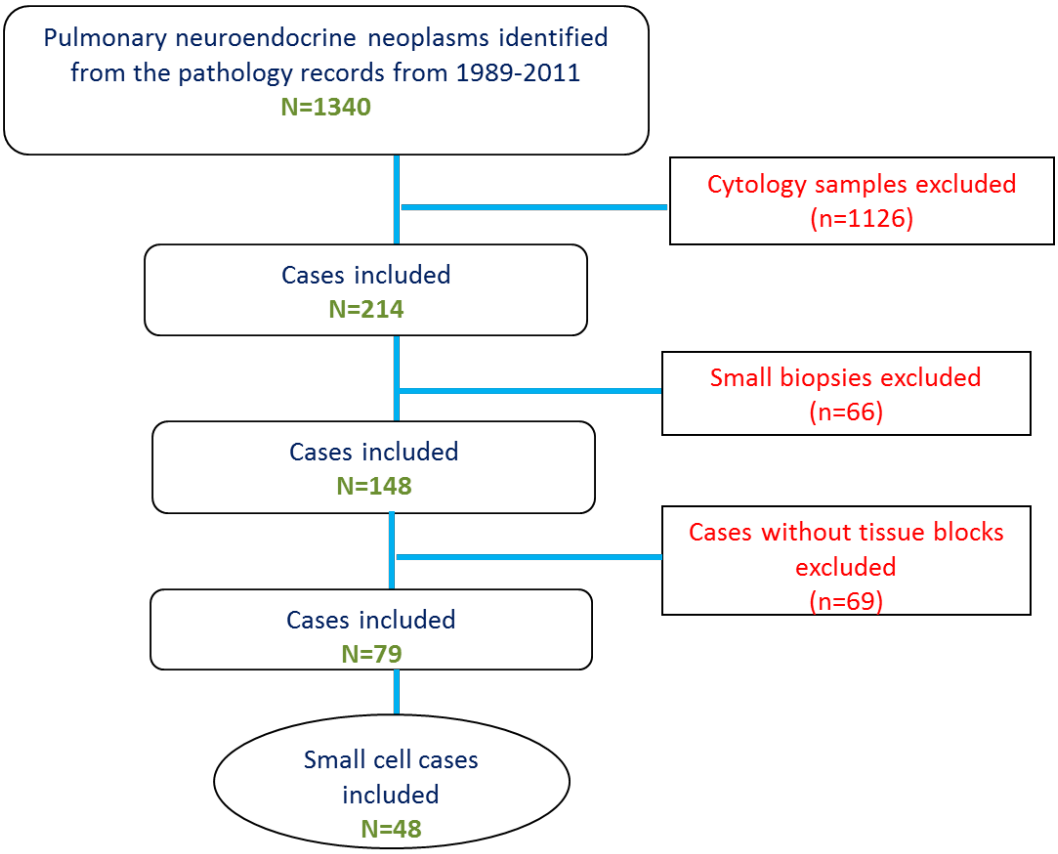

Figure 4: Consort diagram

Detailed steps employed to identify eligible patients included in this study. Electronic health record and archival tumor samples of these selected patients were employed for this analysis.

Patients: Details of the eligible patients are provided in Table 1. A total of 48 patients met the inclusion criteria for the study. The median age was 60.3 (±12) years; there were 28 females (58.3%) and the majority of the patients were Caucasians (79.1%).

Prior to detailed analysis, normality assumption for the variables of interest was assessed specifically for patient age and GLS1 expression. Age was normally distributed as assessed graphically and by Shapiro-Wilk test, GLS1 expression was normally distributed within the adjacent lung but not in the cancer tissues (Figure 5).

Univariate analysis performed using the median or mean expression as a cut-point to dichotomize the patients revealed no significant differences in the expression of GLS1, GLS2 or GS by age, gender, race and treatment with chemotherapy or radiation (Table 2). There was also no correlation between GLS1 and GLS2 expression (R^2: 0.034, p=0.835) or between GLS1 and GS (R^2:0.125; p=0.510) but GS and GLS2 showed a modest statistically significant positive correlation (R^2:0.551; p<0.002); Table 3. GLS1 expression was significantly higher in normal lung relative to SCLC tissue; p=0.054 (Fig 6).

The proportional hazards assumption for PFS and OS when conditioned on GLS1 expression was established to be valid using graphical log(-log) plot (Figure 7) and statistical approaches (Kolmogorov-type p-values of 0.638 and 0.924 for PFS and OS respectively) prior to conducting the survival analysis. Cox proportional hazards analysis based on median immunoscore without adjustment for any of the specified covariates of interest did not demonstrate a significant association between GLS1 expression and PFS (Figure 8) as well as OS (Figure 9).

Likewise, a multivariable Cox proportional hazards model with PFS and OS as outcome variables and GLS1 along with age, gender, race and receipt of other treatment as predictor variables also failed to demonstrate any significant difference in survival between patient groups dichotomized by GLS1 median immunoscore (HR: 1.07; 95% CI: 0.5 - 2.29; p=0.867). Tables 4 & 5 showed the result of the multivariable Cox proportional hazards model when conditioning on other covariates. Female gender was associated with a superior PFS (HR: 0.30; 95% CI: 0.09-1.02; p<0.06) and OS (HR: 0.24; 95% CI: 0.06-0.98; p<0.047) when compared to male patients.

Data analysis using staining intensity of GLS1 expression (0, 1+ vs. 2+, 3+) to categorize patients showed comparable results to that obtained with median immunoscore of no significant differences in PFS or OS based on GLS1 expression (data not shown). Finally, univariate analysis limited to the 22 patients treated with chemotherapy showed an outcome similar to the entire study population with no significant difference in PFS or OS based on GLS1 expression (HR: 1.096; 95% CI:0.432-2.783; p=0.846). However, multivariable analysis in this subset of patients revealed a trend towards improved OS in patients with high GLS1 immunoscore (HR: 0.693; 95%CI: 0.263-1.828; p=0.459).

Exploratory analyses:

We subsequently explored other categories of GLS1 expression in order to fully explore the potential impact of GLS1 expression on patient survival. This exploratory analysis also included a reformulation of the Cox proportional hazards model to include interaction terms between GLS1 expression and other clinical variables including age, gender, race and treatment.

Analysis using other definitions of staining intensity (0 vs. 1+, 2+, 3+; 0, 1+ vs. 2+, 3+; 0, 1+, 2+, vs. 3+) to categorize GLS1 expression did not significantly alter the prior results obtained using immunoscore that GLS1 expression of no significant association between GLS1 and PFS or OS in SCLC (Appendix Tables A1 & A2 and Appendix Figures A1 & A2). Notably however, the comparison of high expression defined as an intensity of "2+" vs. no expression, defined as "1+", demonstrated a significant impact of GLS1 expression on OS (HR:122.2, 95%CI:1.03-144; p=0.049; Appendix Table A2C).

The addition of interaction terms in the model also revealed a significant association between GLS1 expression and PFS with GLS1 immunoscore handled as a dichotomous (HR:265.6; 95%CI:2.98-23675.9; p=0.0148) variable with a consistent trend when analyzed as a continuous variable (HR:1.04; 95%CI:0.997-1.084; p<0.0726; Appendix Table A3A and A3B). A similar analysis of the OS data using a Cox proportional hazards model with interaction terms (Appendix Tables A4A & A4B) showed that GLS1 expression significantly interacts with age and has a significant association with OS when analyzed as a dichotomous variable (HR:348.3; 95%CI:3.3-36433.4; p=0.0136) but not as a continuous variable (HR:1.04; 95%CI:0.994-1.08; p=0.0976).

DISCUSSION/CONCLUSIONS

SCLC remains a fatal disease due to limited understanding of disease biology and a lack of effective treatment options. This work attempted to bridge the knowledge gap in this disease by systematically evaluating the impact of GLS1 expression in this disease. Using tissue samples from 48 patients, we demonstrated significantly higher expression of GLS1 in adjacent normal lung in comparison to tumor tissue. There was no significant different by age, gender or race in the level of GLS1 expression in this patient population. Furthermore, contrary to our initial hypothesis, high GLS1 expression was not significantly associated with worse PFS or OS. Indeed, we observed a trend of higher level of GLS1 in association with better FPS and OS.

The finding of a reduced expression of GLS1 in SCLC relative to adjacent normal tissue was contrary to our initial hypothesis formulated based on cell line work where we had predicted that SCLC, especially the highly malignant poor prognosis subtypes, will have high GLS1 expression rendering them less responsive to standard chemotherapy. We also observed that the intensity of staining (feasible with tiny FNA samples) achieved comparable results to immunoscore. These two findings are very important to guide future studies where only small tissue samples will be available for analysis.

Interestingly, we observed the opposite trend where lower GLS1 was associated with reduced PFS and OS. While our result is not conclusive, due to limited sample size, the generally higher expression of GLS1 in adjacent normal lung relative to SCLC supports the finding that reduced GLS1 expression is associated with a worse outcome perhaps due to a more aggressive tumor biology following malignant transformation. This finding is also consistent with the published report by other groups showing high expression of

GLS1 in normal lung and reduced expression in non small cell lung cancer tissue samples and cell lines.(18)

We observed no significant differences in the expression pattern of GLS1 across different patient demographic subgroups including age, gender and race. The superior PFS and OS for female patients are consistent with the established prognostic advantage of female gender in SCLC.(19, 20) Expectedly, patients treated with chemotherapy or radiation had improved OS and PFS compared with untreated patients. Based on the pre-specified primary analysis using the definition of high or low expression level of GLS1 based on median immunoscore or intensity of 0/1+ vs. 2+/3+, we did not observe any significant survival differences in patients with low versus high expression contrary to our hypothesis. This finding was not altered when other known prognostic factors in SCLC were included in the model. However, additional analyses using other definitions of low vs. high intensity of expression as well as analysis of the immunoscore as a continuous variable rather than a dichotomous variable defined by the median immunoscore revealed that GLS1 expression significantly impacts PFS and OS especially when we added the interaction terms for age and GLS1 expression. This observation supports the hypothesis that survival differences exist between patients with different GLS1 expression levels in their tumor. Moreover, the interaction with age implies that the impact of GLS1 on patient outcome will vary by age. While the biological basis for this interaction remains to be elucidated, age-related changes in cellular repair capacity could offer plausible explanation. The use of immunoscore as the measure of GLS1 expression performed better than the use of intensity in demonstrating the survival impact of GLS1. This would therefore be preferable in future studies designed to replicate or validate the findings in

the current study. However, in clinical settings where the calculation of immunoscore may not be practical, an intensity of staining of 2 or higher would be a reasonable cut point for identifying patients with high GLS expression.

Our limited ability in this study to conclusively demonstrate a survival difference between patients with high and low GLS1 expression might reflect a true lack of association between GLS1 expression and patient survival. Alternatively, because we only enrolled 48 patients instead of the 58 patients required by the a priori estimation, it is possible that the study lacked sufficient power to establish an association if one truly exists. Finally, since the role of GLS1 is predicated on its impact of chemotherapy efficacy, the study population was suboptimal to test the hypothesis since not all the patients received chemotherapy. Exploratory analysis within the subset of patients treated with chemotherapy suggests a trend towards increased risk of death in patients with high GLS expression. This finding is consistent with our original hypothesis but requires careful interrogation in a properly designed study in the future.

One of the major strengths of this study is the use of surgical samples that allowed careful characterization of the expression pattern of GLS1 in SCLC and adjacent normal tissue. Furthermore, careful validation of original diagnosis and joint assessment of the staining pattern by two investigators in a blinded fashion also ensured an objective evaluation of the staining characteristics.

Nonetheless, some real and potential weaknesses of the study are recognized and their impacts on the study outcome would be worthy of proper discussion. These include possible selection bias as a single institution study because the selected patients probably

reflected the practice style and referral pattern to our institutions. The requirement for surgical specimen might also have selected for good prognosis patients who were able to undergo surgery because of their overall superior health condition. The pre-specified inclusion and exclusion criteria enabled us to limit the impact of this type of error. Case misclassification was also a possible source of error due to the retrospective study design either because of an erroneous diagnosis in the archival record or because of changes in the histopathologic classification over time given the long interval of time covered by the study. In order to guide against this potential source of error, all cases were re-examined by a board-certified pathologist who confirmed the diagnosis of SCLC for all selected cases. Known prognostic factors in SCLC include age, gender, performance status, brain metastasis and LDH levels, which were not fully measured and controlled for due to the retrospective study design. Furthermore, we had no reliable record of treatment obtained outside our institution and co-morbid illnesses in the selected cases.

One of the major impediments to progress in SCLC is lack of tumor sample for analytic research. We encountered this challenge in our study where we could only identify 48 cases with usable tumor specimens over a period of time spanning almost 3 decades. This is an ongoing problem with no easy solution because the vast majority of patients with SCLC are diagnosed at more advanced stages where surgical resection is not an option. Moreover, most cases are diagnosed on the basis of relatively tiny tissue sample obtained via fine needle aspiration biopsy. This type of sample typically only provides enough tissue sample needed for establishing the diagnosis and with minimal amount of tissue left behind for research purposes. In order to appropriately characterize GLS1 protein expression, we required all enrolled patients to have adequate archival surgical

specimens. While this requirement could have resulted in a biased sample selection if patient group going for surgery represent a different category than the general SCLC patient population. However, a close analysis of the patient demographics and other characteristics revealed that the patients selected for this study were generally comparable to the lung cancer population. For instance, the age of the patients were normally distributed with a median age of 60 years, which is comparable to the general lung cancer patient population where the median age at diagnosis is 63 years.(21, 22) Approximately 19% of the study population was of African American race, which is reflective of the racial diversity of the referral population base for our institution. While the slight female preponderance in our study population is unusual for the general SCLC population, this could be a random event especially because a large proportion of patients were excluded on various grounds of ineligibility.

In conclusion, this is the first study characterizing the pattern of GLS1 expression in SCLC. While the overall small sample size did not allow for definitive conclusion to be drawn, the findings are generally consistent with other report indicating that GLS1 expression is low in non-small cell lung cancer, the more prevalent type of lung cancer. While this is contrary to our original hypothesis, if this preliminary observation can be further validated in future studies, the potential association between high (preserved) GLS1 expression and better PFS and OS may offer a tool for assigning prognosis and tailoring treatment option in SCLC.

Our future plans include conducting additional preclinical work to carefully elucidate the biology of GLS1 in SCLC; especially with the observation that GLS1 expression is lower in SCLC relative to normal lung tissue.

A replication of the current study in an independent retrospective cohort of Emory patients diagnosed using fine needle biopsy samples as well as a prospective study in newly diagnosed will be pursued in the future. Since we have already established that GLS1 is homogenously expressed and that staining intensity is a reliable measure of protein expression, it should be feasible to employ fine needle aspiration sample for the follow-up studies. The planned retrospective study will also assess the impact of GLS1 expression on survival in patients treated primarily with chemotherapy in line with contemporary practice pattern. Finally, tumor samples collected as part of an ongoing prospective study of chemotherapy in SCLC patients will be employed to further characterize the clinical relevance of GLS1 in SCLC patients.

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TABLES / FIGURES

Table 1 – Patient Distribution by Demographics and Treatment (n=48)

Variable	*Group*	*Mean (± SD) or N (%)*
Age		60.33 (± 11.99)
Gender	Female	28 (58.33)
	Male	20 (41.67)
Race/Ethnicity	African American	8 (18.6)
	Asian	1 (2.33)
	Caucasian	34 (79.07)
	Missing	5
Chemotherapy/Radiation	Yes	25 (52.08)
	No	23 (47.92)
	Chemo: Cisplatin/carboplatin, etoposide	9 (36)
	Chemo (other types)	5 (20)
	Radiation ± Chemo	11 (44)

Table 2 - Expression of GLS1, GLS2 and GS by patient demographics and treatment

Variable	*Subgroups*	*GLS1**	*P-value*	*GLS2#*	*P-value*	*GS**	**P-value**
Age	<Mean	140 (15 - 285)	0.367	99 (± 64)	0.041	210 (20 - 300)	0.985
	≥Mean	97.5 (30 - 255)		144 (± 71)		225 (20 - 285)	
Gender	Female	95 (15 - 270)	0.144	104 (± 62)	0.104	180 (20 - 300)	0.298
	Male	170 (30 - 285)		140 (± 76)		270 (40 - 300)	
Race	African American	140 (30 - 170)	0.351	115 (± 69)	0.902	150 (20 - 285)	0.089
	Caucasian	100 (15 - 285)		119 (± 68)		263 (40 - 300)	
Chemotherapy	Yes	122.5 (30 - 285)	0.827	134 (± 65)	0.186	263 (20 - 300)	0.102
Radiation							
	No	120 (15 - 285)		105 (± 74)		165 (20 - 300)	

There was no significant difference in the expression GLS1, GLS2 or GS by age, gender, race or treatment with chemotherapy or radiation.

*: median with range; #: Mean with standard deviation

Table 3 - Correlation between Glutaminase (GLS1) and other associated proteins (GLS2 and GS)

Variable1	***Variable2***	***N***	***Spearman CC***	**Spearman P-value**
GLS1	GLS2	39	0.034	0.835
GLS1	GS	30	0.125	0.510
GLS2	GS	28	0.551	0.002

The probability that the observed correlation or an even stronger correlation between GLS1 and GLS2 could have been due to chance given that the null hypothesis is true is greater than 0.1. Therefore we do not have sufficient evidence to reject the null hypothesis that the correlation between GLS1 and GLS2 is 0.

Similarly, the probability that the observed correlation between GLS1 and GS could have been due to chance given that the null hypothesis is true is greater than 0.1. Therefore we do not have sufficient evidence to reject the null hypothesis that the correlation between GLS1 and GS is 0.

However, since the probability that the observed correlation between GLS2 and GS could have been due to chance given that the null hypothesis is true is 0.002, which is less than 0.1, we have sufficient evidence to reject the null hypothesis that the correlation between GLS2 and GS is 0.

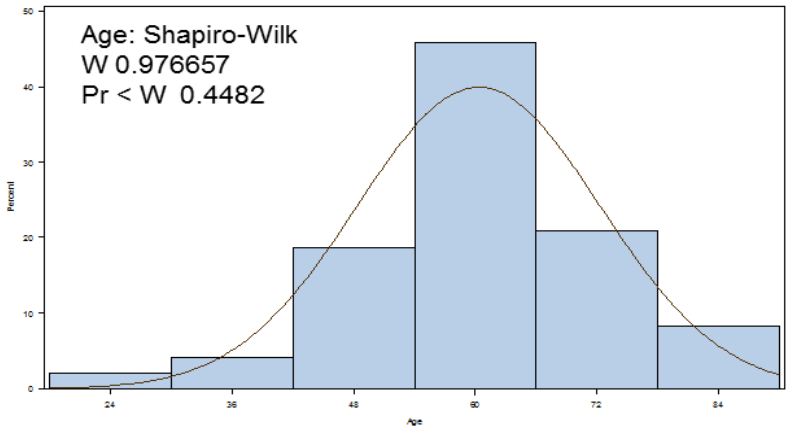
Table 4 - Multivariable analysis of GLS1 expression and OS

Covariates	*Group*	*Hazard Ratio*	*95%CI*	*HR P-value*
GLS1 (high >= 120)	**High**	**1.07**	**0.5-2.29**	**0.867**
	Low	**(Ref)**	-	-
Age		1.01	0.95-1.07	0.82
Gender	Female	**0.30**	0.09-1.02	0.06
	Male	**(Ref)**	-	-
Race binary	African American	0.99	0.25-3.98	0.99
	Caucasian	(Ref)	-	-
Chemotherapy/Radiation	Yes	0.63	0.23-1.75	0.37
	No	(Ref)	-	-

Table 5 - Multivariable analysis of GLS1 expression and OS without interaction terms in the model

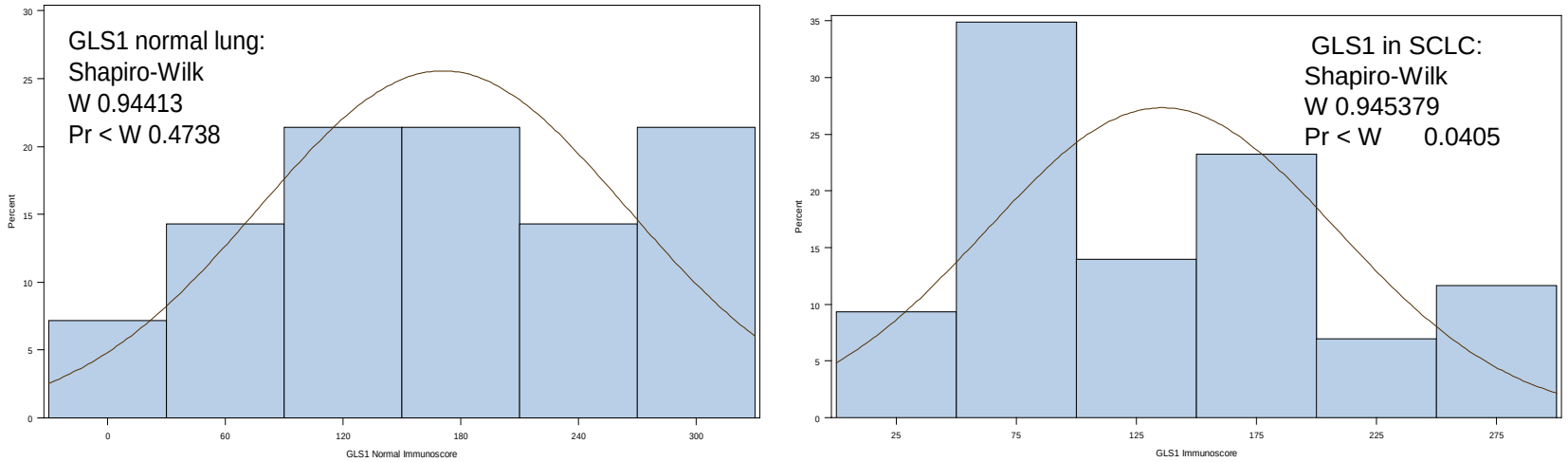
Covariates	Group	Hazard Ratio	95%CI	HR P-value
GLS1 score (high >= 120)	**High**	**1.00**	**0.19-5.31**	**0.997**
	Low	**(Ref)**	-	-
Age		1.00	0.94-1.06	0.995
Gender	Female	**0.24**	0.06-0.98	**0.047**
	Male	**(Ref)**	-	-
Race	African American	1.12	0.25-4.93	0.883
	Caucasian	(Ref)	-	-
Chemotherapy/Radiation	Yes	0.38	0.12-1.18	0.094
	No	(Ref)	-	-

Figure 5 - Normality assumptions for age and GLS1 distribution



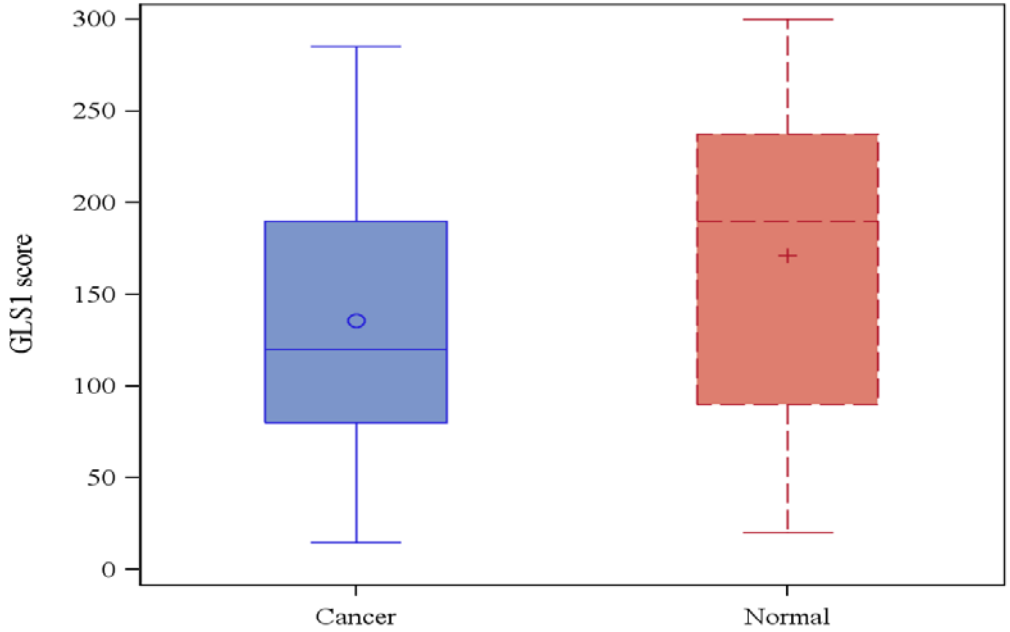
Normality assumption testing for age distribution of the study population

Normal Lung



Normality assumption testing for GLS1 expression in normal lung (Left) and SCLC (Right) showing a normal distribution of the immunoscore values for normal lung but not for SCLC

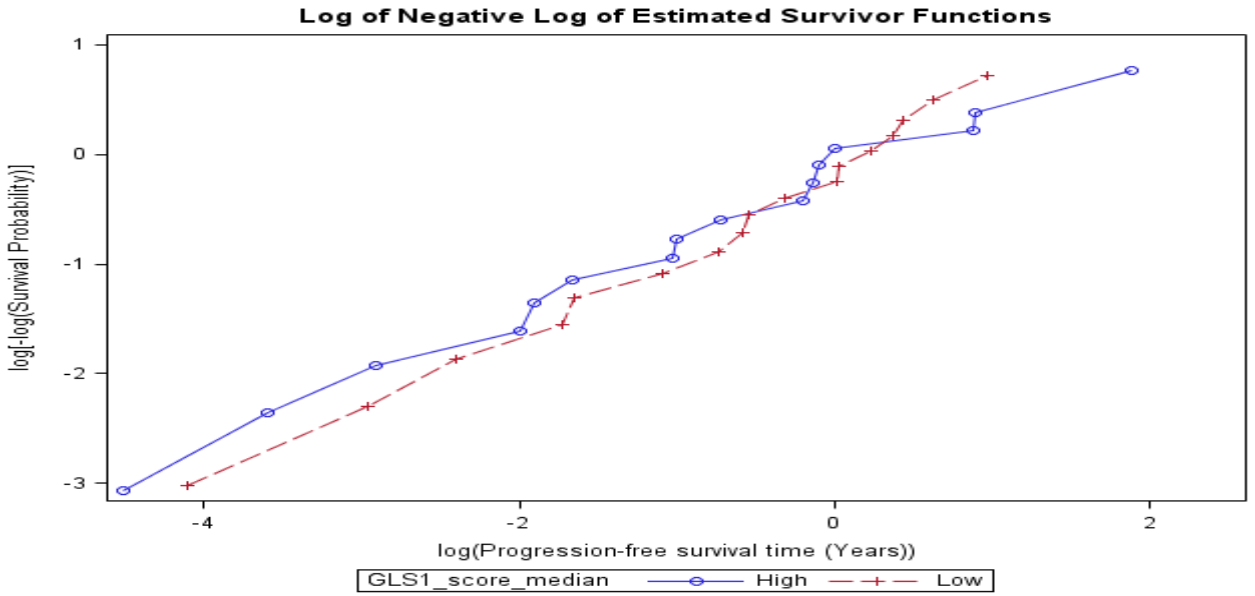
Figure 6 - Comparison of GLS1 expression in normal lung and SCLC showing a higher median GLS1 expression in normal lung versus SCLC



Variable	Normal	Cancer	P-value
GLS1	190 (20 – 300)	120 (15 – 285)	0.054

Data presented as median (range)

Figure 7: Log-log plot



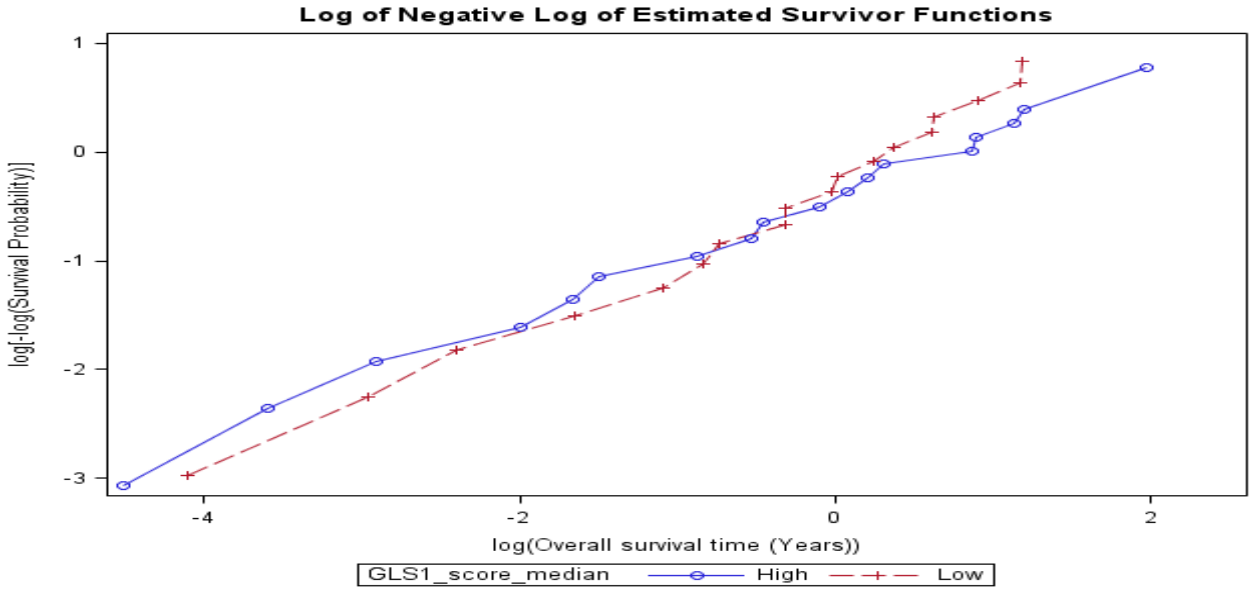
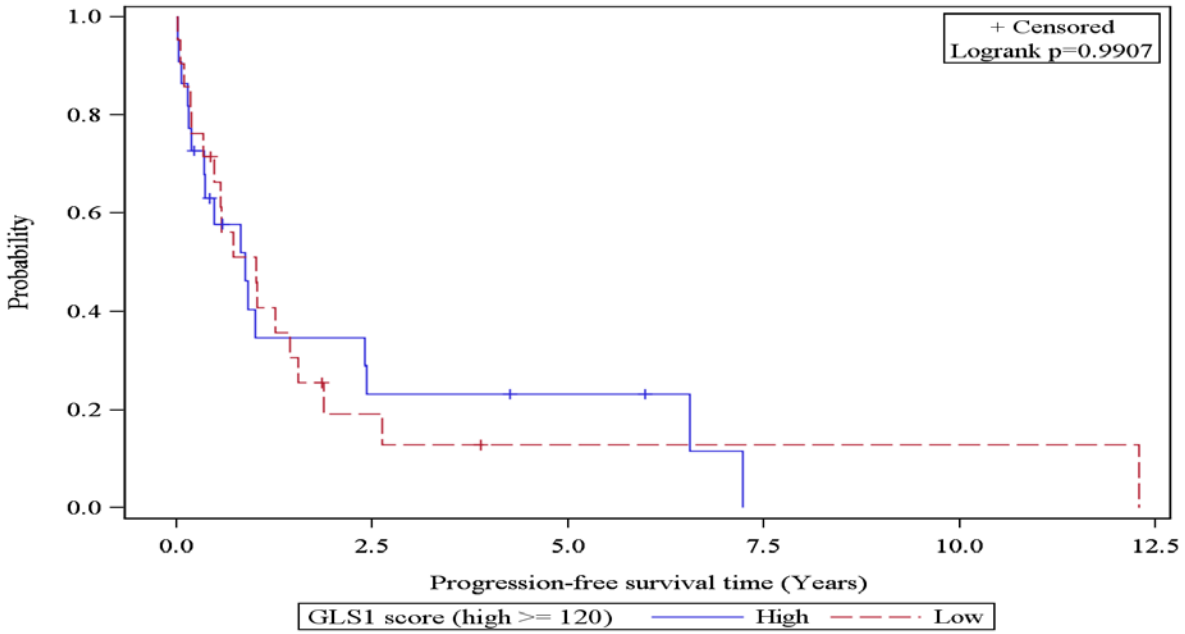


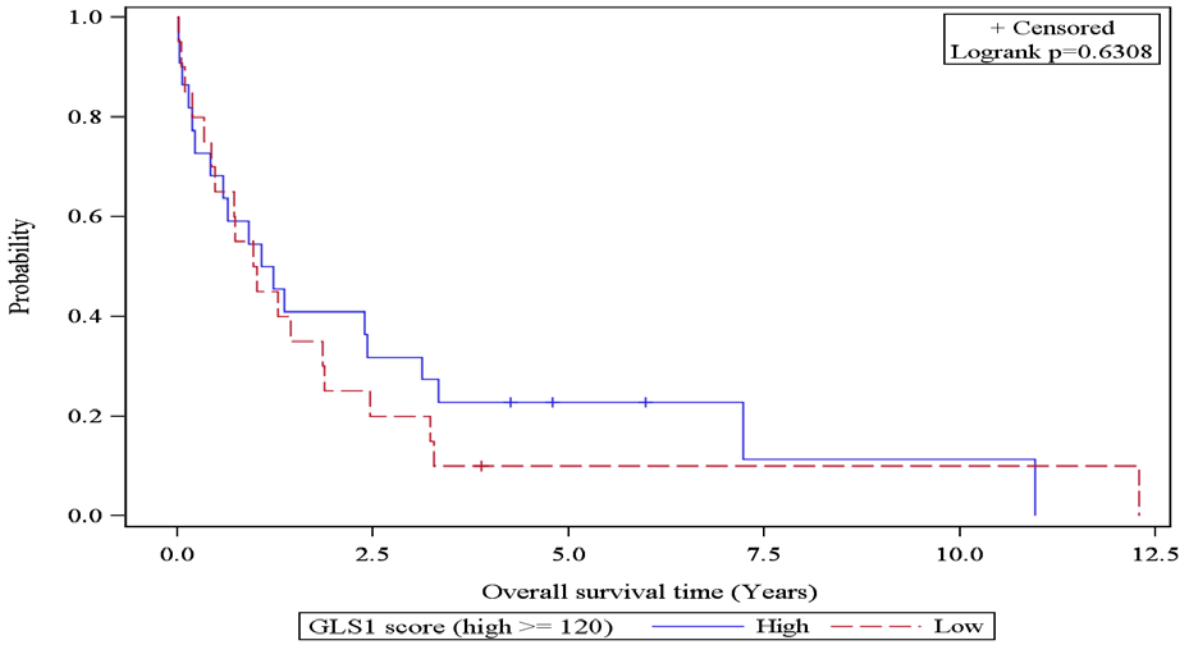
Figure 8 - Univariate analysis of GLS1 expression on PFS



Covariate	*Group*	*N**	*Hazard Ratio*	*95%CI Low*	*95%CI Up*	*HR P-value*
GLS1 score (high >= 120)	High	22	1.00	0.51	1.98	0.991
	Low	21	(Ref)	-	-	-

* 5 patients censored

Figure 9 - Univariate analysis of GLS1 expression (Dichotomized based on median immunoscore) on OS



Covariate	*Group*	*N**	*Hazard Ratio*	*95%CI Low*	*95%CI Up*	*HR P-value*
GLS1 (high >= 120)	High	22	0.85	0.45	1.63	0.631
	Low	20	(Ref)	-	-	-

* 6 censored patients

APPENDIX OF TABLES AND FIGURES:

Table A1A - Multivariable analysis of progression free survival by GLS1 expression (measured as intensity of 2+/3+ vs. 0/1+) adjusted for known prognostic factors (age and gender), race, other treatment, and interaction terms between expression and age, gender, race, and any initial treatment in SCLC

Covariates	*Group*	*Hazard Ratio*	*95%CI*	*P-value*
GLS1	2+/3+ vs. 0/1+	6.22	0.098-393.4	0.3879
Age		1.01	0.957-1.064	0.7480
Gender	Female vs. Male	0.67	0.216-2.073	0.4863
Race	African American vs. Caucasian	0.54	0.103-2.872	0.4741
Chemotherapy/Radiation	Yes vs. No	0.59	0.154-2.261	0.4418
GLS1*Age	2+/3+ vs. 0/1+	0.97	0.221-4.221	0.3436
GLS1*Gender	2+/3+ vs. 0/1+	6.58	0.142-305.6	0.9410
GLS1*Race	2+/3+ vs. 0/1+	20.12	0.187-2159.5	0.2839
GLS1*Treatment	2+/3+ vs. 0/1+	3.75	0.042-331.2	0.5527

Table A1B - Multivariable analysis of progression free survival by GLS1 expression (measured as intensity of 0/1+/2+ vs. 3+) adjusted for known prognostic factors (age and gender), race, other treatment, and interaction terms between expression and age, gender, race, and any initial treatment in SCLC

Covariates	Group	Hazard Ratio	95%CI	P-value
GLS1	0/1+/2+ vs. 3+	2.57E9	0.0001-1.90E22	0.1604
Age		1.31	0.877-1.933	0.1765
Gender	Female vs. Male	1105.9	0.310-2522262	**0.0844**
Race	African American vs. Caucasian	1.18	0.420-3.565	0.7538
Chemotherapy/Radiation	Yes vs. No	0.14	0.010-2.253	0.1603
GLS1*Age	0/1+/2+ vs. 3	151.8	0.187-85231.5	0.1727
GLS1*Gender	0/1+/2+ vs. 3	1246476	0.0001-7.33E15	**0.0636**
GLS1*Race	0/1+/2+ vs. 3	2.57E9	0.0001-1.90E22	NA
GLS1*Treatment	0/1+/2+ vs. 3	6.48E9	0.0001-1.44E23	0.5169

Table A1C - Multivariable analysis of progression free survival by GLS1 expression (measured as intensity of 3+ or 2+ vs. 1+) adjusted for known prognostic factors (age and gender), race, other treatment, and interaction terms between expression and age, gender, race, and any initial treatment in SCLC

Covariates	***Group***	***Hazard Ratio***	***95%CI***	***P-value***
GLS1	3+ vs. 1+	3.86E-10	2.37E-23-6291.4	0.1626
	2+ vs. 1+	17.17	0.194-1521.1	0.2140
Age		1.01	0.958-1.067	0.6990
Gender	Female vs. Male	0.61	0.195-1.931	0.4039
Race	African American vs. Caucasian	0.53	0.10-2.793	0.4520
Chemotherapy/Radiation	Yes vs. No	0.55	0.141-2.177	0.3976
GLS1*Age	3+ vs. 1+	0.01	8.66E-6-4.555	0.1769
	2+ vs. 1+	2.79E8	0.008-9.47E18	0.3211
GLS1*Gender	3+ vs. 1+	9.538E-7	1.01E-16-9018.4	**0.0603**
	2+ vs. 1+	42423.0	3.75-4.798E8	0.4912
GLS1*Race	3+ vs. 1+	3.86E-10	2.37E-23-6291.4	NA
	2+ vs. 1+	17.17	0.194-1521.1	0.1477
GLS1*Treatment	3+ vs. 1+	9.28E-11	1.65E-24-5222.7	0.3539
	2+ vs. 1+	5.80	0.052-651.7	0.2407

Table A2A - Multivariable analysis of overall survival by GLS1 expression (measured as intensity of 2+/3+ vs. 0/1+) adjusted for demographics, treatment, and interaction terms

Covariates	***Group***	***Hazard Ratio***	***95%CI***	***P-value***
GLS1	2+/3+ vs. 0/1+	28.59	0.420-1944.4	0.1194
Age		1.02	0.969-1.072	0.4554
Gender	Female vs. Male	0.63	0.202-1.985	0.4325
Race	African American vs. Caucasian	0.36	0.065-2.008	0.2443
Chemotherapy/Radiation	Yes vs. No	0.45	0.113-1.771	0.2520
GLS1*Age	2+/3+ vs. 0/1+ at mean Age	0.98	0.222-4.341	0.0927
GLS1*Gender	2+/3+ vs. 0/1+ at mean Female	20.06	0.416-967.7	0.6621
GLS1*Race	2+/3+ vs. 0/1+ at mean African American	261.4	1.983-34457.5	0.0536
GLS1*Treatment	2+/3+ vs. 0/1+ at mean Treatment	9.89	0.114-857.4	0.2341

Table A2B - Multivariable analysis of overall survival by GLS1 expression (measured as intensity of 0/1+/2+ vs. 3+) adjusted for demographics, treatment, and interaction terms

Covariates	*Group*	*Hazard Ratio*	*95%CI*	*P-value*
GLS1	0/1+/2+ vs. 3+	1.3E10	0.001-2.60E23	0.1361
Age		1.34	0.903-2.004	0.1453
Gender	Female vs. Male	706.3	0.227-2198105	0.1099
Race	African American vs. Caucasian	1.31	0.441-3.905	0.6261
Chemotherapy/Radiation	Yes vs. No	0.07	0.005-1.115	0.0599
GLS1*Age	0/1+/2+ vs. 3+ at mean Age	224.3	0.271-185898	0.1459
GLS1*Gender	0/1+/2+ vs. 3+ at mean Female	9079138	0.001-9.59E16	0.0803
GLS1*Race	0/1+/2+ vs. 3+ at	1.30E10	0.001-2.60E23	NA
GLS1*Treatment	0/1+/2+ vs. 3+ at mean Treatment	3.88E10	0.001-2.11E24	0.4429

Table A2C - Multivariable analysis of overall survival by GLS1 expression (measured as intensity of 3+ or 2+ vs. 1+) adjusted for demographics, treatment, , and interaction terms

Covariates	*Group*	*Hazard Ratio*	*95%CI*	*P-value*
GLS1	3+ vs. 1+	6.39E-12	1.84E-25-221.6	0.1051
	2+ vs. 1+	122.2	1.030-14497.1	0.0486
Age		1.02	0.973-1.079	0.3643
Gender	Female vs. Male	0.58	0.182-1.859	0.3612
Race	African American vs. Caucasian	0.35	0.062-1.952	0.2301
Chemotherapy/Radiation	Yes vs. No	0.39	0.094-1.615	0.1938
GLS*Age	3+ vs. 1+	0.002	2.05E-6-2.129	0.1169
	2+ vs. 1+	4.00E10	0.538-2.974E21	0.0703
GLS1*Gender	3+ vs. 1+	2.46E-8	1.6E-18-379.8	0.0534
	2+ vs. 1+	471244	22.630-9.81E9	0.2667
GLS1*Race	3+ vs. 1+	6.39E-12	1.84E-25-221.6	NA
	2+ vs. 1+	122.2	1.030-14497.1	0.0208
GLS1*Treatment	3+ vs. 1+	7.6E-13	7.49E-27-77.088	0.1721
	2+ vs. 1+	22.95	0.183-2881.6	0.0903

Table A3A - Multivariable analysis of progression free survival by GLS1 expression immunoscore (treated as a dichotomous variable) adjusted for demographics, treatment, percentage cell staining and interaction terms

Covariates	***Group***	***Hazard Ratio***	***95%CI***	***P-value***
GLS1	High vs. Low	265.6	2.981-23675.9	0.0148
Age		1.02	0.976-1.057	0.4350
Gender	Female vs. Male	1.07	0.357-3.209	0.9027
Race	African American vs. Caucasian	0.77	0.140-4.185	0.7588
Chemotherapy/Radiation	Yes vs. No	0.38	0.130-1.112	0.0773
% Cell Staining		1.01	0.987-1.026	0.5417
GLS1*Age	High vs. Low	1.64	0.353-7.635	0.0132
GLS1*Gender	High vs. Low	69.36	1.312-3668.6	0.1372
GLS1*Race	High vs. Low	520.7	3.049-88944.9	0.5406
GLS1*Treatment	High vs. Low	427.2	3.755-48602.6	0.5591

Table A3B - Multivariable analysis of progression free survival by GLS1 expression immunoscore (treated as a continuous variable) adjusted for demographics, treatment, percentage cell staining and interaction terms

Covariates	*Group*	*Hazard Ratio*	*95%CI*	*P-value*
GLS1		1.04	0.997-1.084	0.0726
Age		1.07	0.985-1.168	0.1085
Gender	Female vs. Male	0.68	0.108-4.342	0.6877
Race	African American vs. Caucasian	0.48	0.024-9.435	0.6283
Chemotherapy/Radiation	Yes vs. No	0.22	0.036-1.378	0.1061
% Cell Staining		1.01	0.986-1.035	0.4069
GLS1*Age	GLS1	1.00	0.990-1.011	0.0408
GLS1*Gender	GLS1	1.04	0.999-1.075	0.6982
GLS1*Race	GLS1	1.05	0.998-1.103	0.4192
GLS1*Treatment	GLS1	1.04	0.998-1.093	0.4375

Table A4A - Multivariable analysis of OS by GLS1 expression immunoscore (treated as a dichotomous variable) adjusted for demographics, treatment, percentage cell staining and interaction terms

Covariates	***Group***	***Hazard Ratio***	***95%CI***	***P-value***
GLS1	High vs. Low	348.3	3.330-36433.4	0.0136
Age		1.02	0.981-1.066	0.2863
Gender	Female vs. Male	1.09	0.334-3.548	0.8886
Race	African American vs. Caucasian	0.69	0.108-4.428	0.6982
Chemotherapy/Radiation	Yes vs. No	0.27	0.082-0.882	0.0302
% Cell Staining		1.01	0.986-1.035	0.4027
GLS1*Age	High vs. Low	1.51	0.299-7.651	0.0109
GLS1*Gender	High vs. Low	63.95	1.142-3581.8	0.0816
GLS1*Race	High vs. Low	1581.3	6.617-377911	0.1842
GLS1*Treatment	High vs. Low	345.9	2.5453-47010.3	0.9935

Table A4B - Multivariable analysis of OS by GLS1 expression immunoscore (treated as a continuous variable) adjusted for demographics, treatment, percentage cell staining and interaction terms

Covariates	***Group***	***Hazard Ratio***	***95%CI***	***P-value***
GLS1		1.04	0.994-1.0815	0.0976
Age		1.08	0.988-1.1824	0.0906
Gender	Female vs. Male	0.77	0.111-5.355	0.7924
Race	African American vs. Caucasian	0.43	0.020-9.227	0.5877
Chemotherapy/Radiation	Yes vs. No	0.14	0.018-1.049	0.0557
% Cell Staining		1.02	0.994-1.050	0.1204
GLS1*Age	GLS1	0.997	0.986-1.008	0.0438
GLS1*Gender	GLS1	1.03	0.993-1.068	0.3980
GLS1*Race	GLS1	1.05	0.999-1.105	0.2414
GLS1*Treatment	GLS1	1.04	0.994-1.090	0.4905

Figure A1A – Kaplan-Meier Curves for GLS1 (0/1+ vs. 2+/3+) on PFS

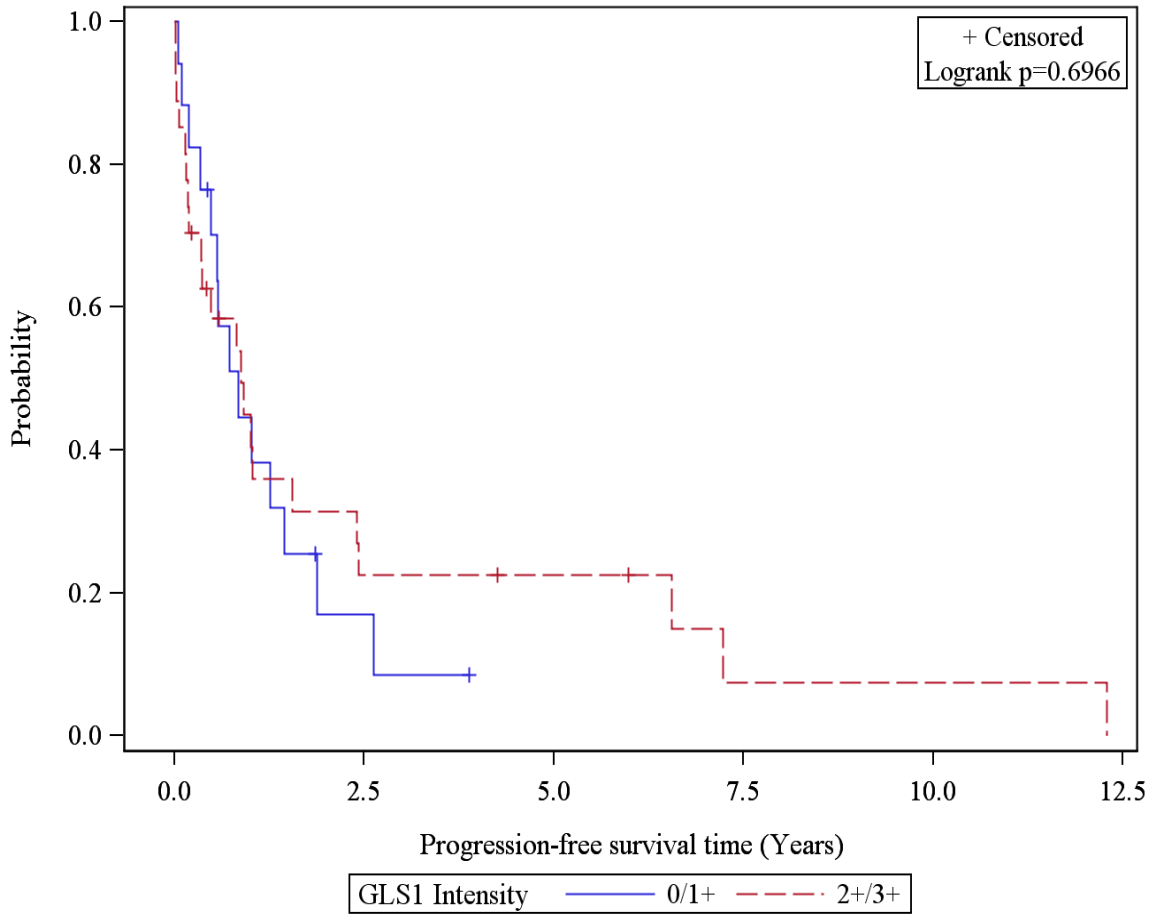


Figure A1B – Kaplan-Meier Curves for GLS1 (0/1+/2+ vs. 3+) on PFS

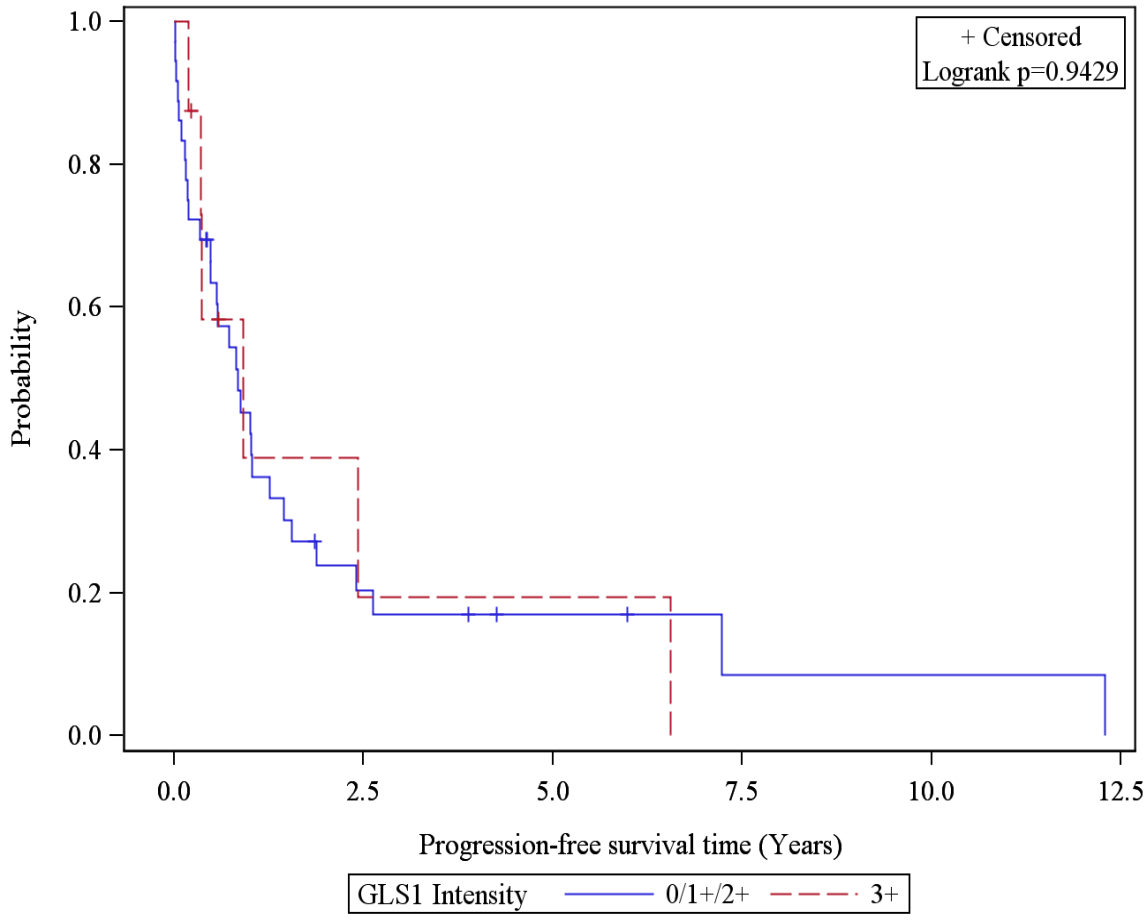


Figure A1C – Kaplan-Meier Curves for GLS1 (1+ vs. 2+ vs. 3+) on PFS

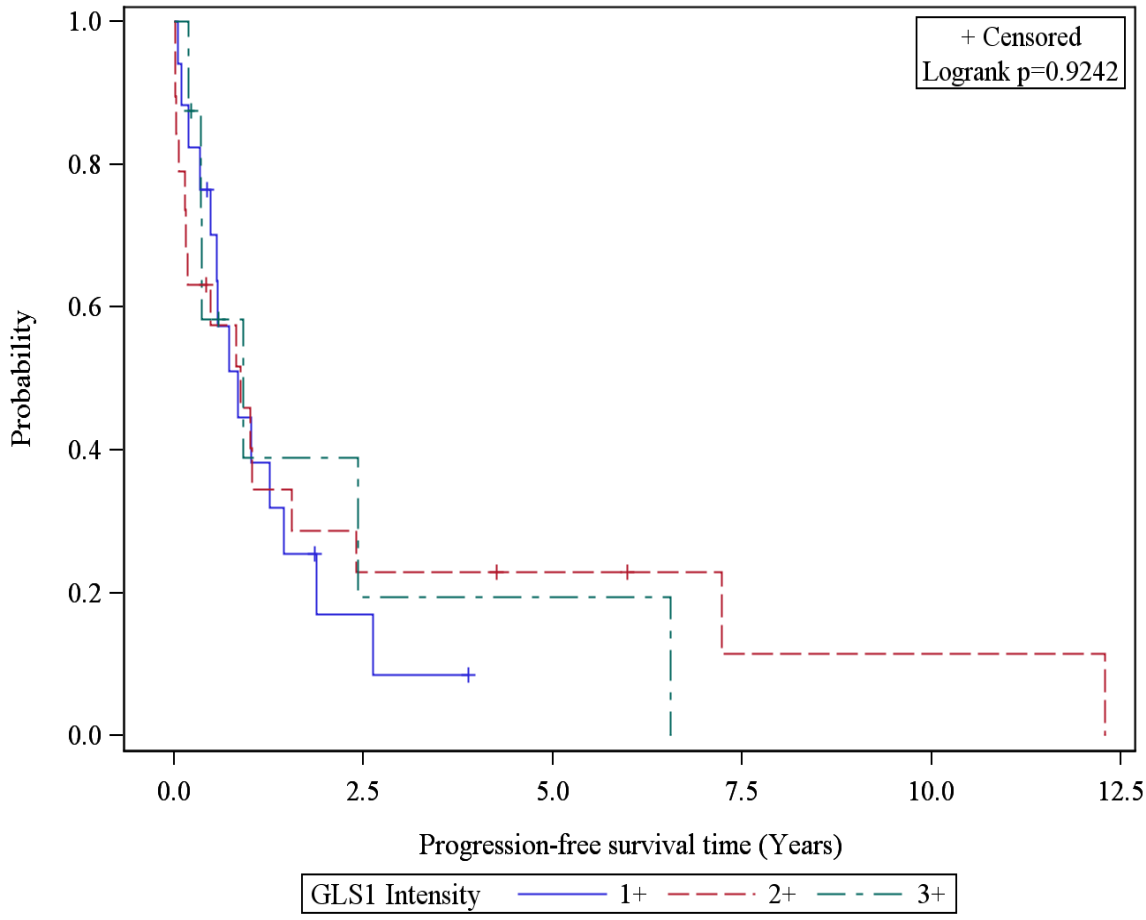


Figure A2A – Kaplan-Meier Curves for GLS1 (0/1+ vs. 2+/3+) on OS

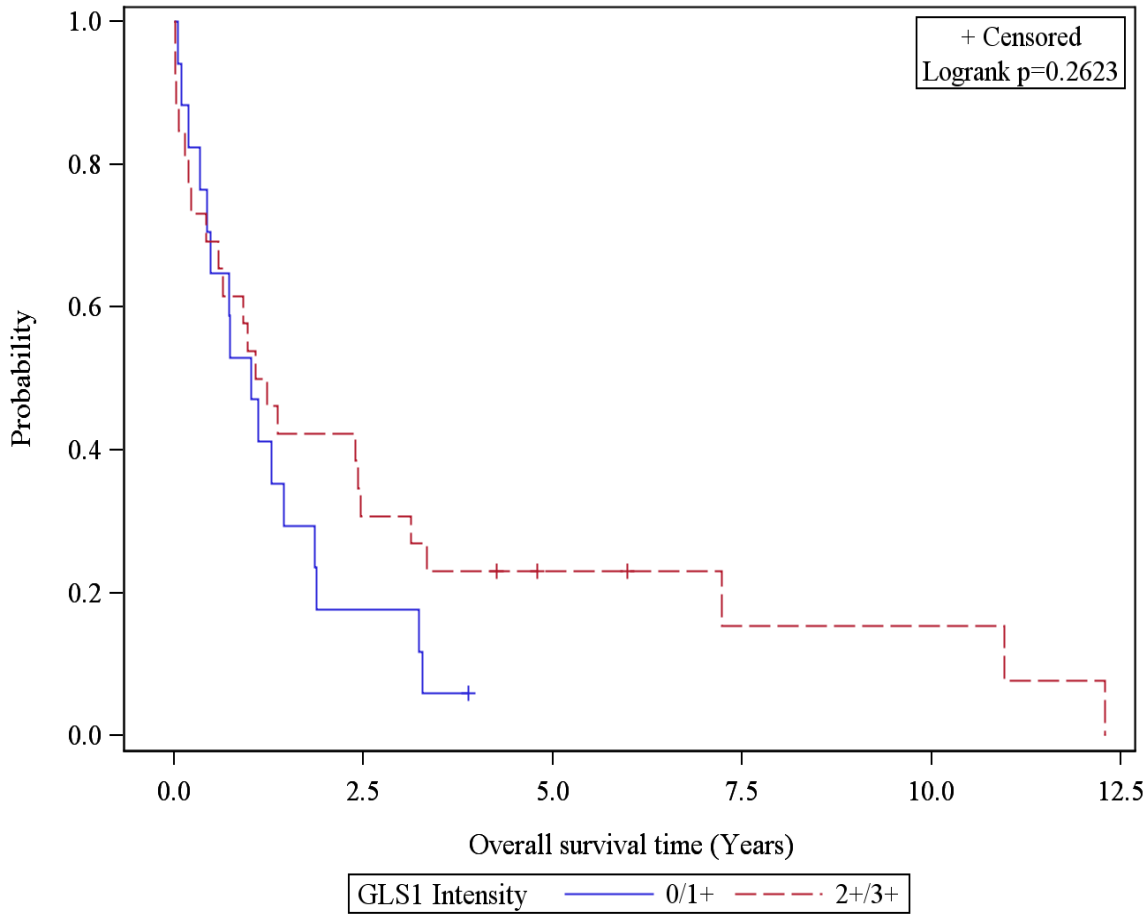


Figure A2B – Kaplan-Meier Curves for GLS1 (0/1+/2+ vs. 3+) on OS

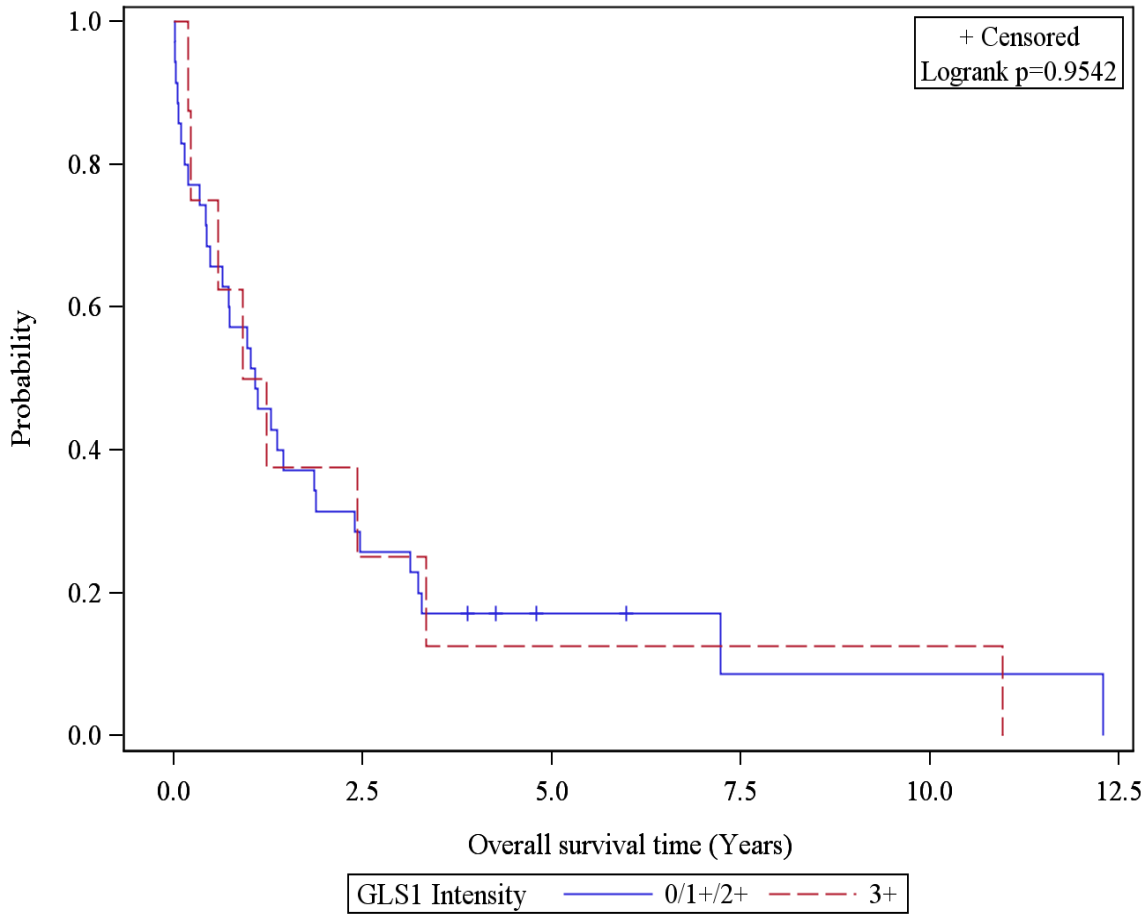


Figure A2C – Kaplan-Meier Curves for GLS1 (1+ vs. 2+ vs. 3+) on OS