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Original Article

Expression pattern of FGFR2, Grb2 and Plcγ1 acts as a novel prognostic marker of recurrence recurrence-free survival in lung adenocarcinoma

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Abstract: Lung adenocarcinoma is characterized by complex biology involving alterations at the genomic and protein expression levels. FGFR2 mutation and/or amplification are key drivers of disease progression and drug resistance in lung adenocarcinoma patients. These genetic alterations drive oncogenic downstream signalling due to the deregulated activity of the receptor. We have previously reported that wild type FGFR2 provides a binding site for which two proteins, Grb2 and Plcγ1, compete in a concentration-dependent manner. Metastasis and invasion ensue when Plcγ1 prevails on the receptor giving rise to oncogenic outcome in the absence of gene mutation/deletion. The effect of this signalling mechanism on FGFR2-driven lung adenocarcinoma has not previously been considered. In this study we show that fluctuation in the combinatorial expression levels of FGFR2, Grb2 and Plcγ1 modulates cell invasive properties, tumor formation and is linked to recurrence-free survival in 150 lung adenocarcinoma patients. High levels of expression of FGFR2 and Plcγ1 in a low background of Grb2 significantly correlates with poor prognosis. On the other hand, low levels of expression of FGFR2 and Plcγ1 in a high background of Grb2 correlates with favourable prognosis. This study defines the expression pattern of FGFR2, Plcγ1 and Grb2 as a novel prognostic marker in human lung adenocarcinoma. Thus, consideration of the Grb2 and Plcγ1-mediated mechanism of FGFR2 regulation will enhance the therapeutic targeting of aberrant FGFR2 activity to provide the much-needed improvement to the treatment regimen of this high mortality disease.

Keywords: SH3 domain, receptor tyrosine kinase, proline-rich domain, oncogenesis, cancer of non-genetic origin

Introduction

Lung cancer is the leading cause of neoplasia-related mortality worldwide with 1.6 million deaths per year [1]. The majority of the cases are non-small cell lung cancer (NSCLC) which is split into 3 subtypes, adenocarcinoma (LUAD) (representing 40% of total lung cancer cases), squamous cell (epidermoid) carcinoma (30%) and large cell (undifferentiated) carcinoma (15%). These cases have a 5-year survival rate of only 15% due to late stage diagnosis, recurrence and metastasis [2-7]. Adopting conventional treatments for LUAD, such as surgical resection followed by adjuvant chemotherapy, are limited based on the difficulties in ensuring

optimal benefit from therapeutic decisions [8-15]. As such, identifying novel biomarkers of disease progression would allow for better prognosis leading to therapeutic gain, personalized medical treatment and management of LUAD patients [16-20].

Fibroblast growth factor receptor 2 (FGFR2) is a cell surface receptor with a complex network of downstream signalling pathways fundamental to normal cellular processes [21]. Aberrant signalling caused by the mutation and/or amplification of FGFR2 have been commonly observed in multiple cancer types like colon, gastric cancer and lung cancer [7, 22-26]. Constitutive activation of the kinase occurring in a defined population of LUAD patients, along with the tumor's sensitivity to pan-FGFR2 inhibition make it a good therapeutic target, however a highly effective method for targeting FGFR2 has not yet been achieved [7, 27]. Furthermore, increased FGFR2 expression as a drug resistance mechanism make it a suitable prognostic marker and it has been previously reported that FGFR2 expression is a valuable indicative of LUAD clinical outcome [22, 28] yet the level of expression of FGFR2 binding partners and their role as LUAD prognostic markers in a combinatorial fashion have never been investigated.

We have previously shown that under growth factor-deprived conditions FGFR2 function can be regulated by two proteins, growth factor receptor bound protein 2 (Grb2) and phospholipase C gamma 1 (Plcγ1). These proteins compete for the same proline-rich binding site on the C-terminus of the receptor via their Src homology 3 (SH3) domains in a concentration-dependent manner [29, 30]. In cells expressing elevated levels of Grb2, Plcγ1 cannot be recruited to the receptor and cells maintain normal FGFR2-mediated functionality. However, in cells with depleted Grb2 concentration, Plcγ1 is able to bind to the receptor resulting in phosphorylation-independent activation of the lipase driving oncogenic outcomes such as cellular migration and invasion [29, 30]. Two important questions emerge from these studies; is the combinatorial expression level of FGFR2, Grb2 and Plcγ1 of any clinical relevance in cancer patients? And will the inclusion of Grb2 and Plcγ1 in FGFR2 survival studies enhance the precision of the prognostic potential of FGFR2 expression? In this work, we investigate the mRNA and protein levels of FGFR2, Grb2 and Plcγ1 in cell lines, a xenograft mouse model and LUAD patients to test for their correlation with oncogenic behaviour and clinical outcome. We report that recurrence-free survival of patients (adjusted for characteristics, e.g. age, gender, tobacco history, adjuvant therapy) correlates with the expression levels of FGFR2, Grb2 and Plcγ1 forming the basis for a critical combinatorial prognostic marker. FGFR2, Plcγ1 and Grb2 respectively being at high, high and low concentrations results in significantly poorer prognosis. However, with concentrations respectively being low, low and high, the prognosis is improved. The results obtained from patient profiles are in agreement with the markers' molecular signature and functional effects in lung adenocarcinoma cell lines. Therefore, combinational data on relative expression levels of these proteins provides the opportunity for better prognosis in patients and an improved understanding of the contributing factors and potential causes behind FGFR2 drug/inhibitor resistance. This allows for an informed decision on the choice and success rate of therapy (in this case adjuvant therapy).

Materials and methods

Cells

Human embryonic kidney 293T (HEK293T) and human NSCLC cell lines, were maintained in Dulbecco's modified Eagle's high-glucose medium (DMEM) supplemented with 10% (v/v) FBS and 1% antibiotic/antimycotic (Lonza) in a humidified incubator at 37°C with 10% CO_2. HEK293T cells were stably transfected with a C-terminal GFP fusion (FGFR2-GFP) and knockdown cells were generated by infecting cells with either scrambled shRNA, Grb2 shRNA or Plcγ1 shRNA containing lentiviral particles. Knockdowns were confirmed by western blotting. For experimental protocols cells were serum starved then left untreated or stimulated with FGF9 for 30 min. Cultured cells were grown in 10 cm dishes, serum-starved overnight, and left unstimulated or were stimulated with 20 ng/ml FGF9 (R&D Systems) for 30 min. Cells were lysed with HEPES lysis buffer (50 mM HEPES, pH 7.5, 1% (v/v) IGEPAL-C630, 1 mg/ml bacitracin, 1 mM EDTA, 10 mM NaF, 1

mM sodium orthovanadate, 10% (v/v) glycerol, 50 mM NaCl, 1 mM PMSF, and Protease Inhibitor Cocktail Set III (EMD Millipore)). Protein concentration was quantified by Bradford assay, and 50 μg of total proteins was used for every assay.

Xenograft mouse model

Adult female nude mice were injected with 1 × 10^6 cells in PBS (5 mice per group). Cells used were stably transfected with pGL3-basic empty luciferase vector (Promega). Luciferase expression was confirmed by luciferase-reporter assay (Promega) and IVIS imaging system of the cell lines following addition of Luciferin (System Biosciences). Following tumor growth, Luciferin was injected in the mice according to the manufacturer's protocol followed by periodic quantitative imaging of tumor growth using IVIS. Next, mice were sacrificed, nodules were counted and tumors were weighed. Weights were averaged in each group and plotted as a bar graph where the standard deviation of the mean is denoted by error bars.

IHC staining

IHC staining of the TMA (MTU 951, LUC1021 Pantomics), was carried out using Access Retrieval Buffer Menarini Diagnostics (Berkshire UK) Plcγ1 (Cell Signalling Technology, D9H10 XP® Rabbit mAb #5690) diluted 1:25, FGFR2 (Santa Cruz Biotechnology, C-17; sc-122) diluted 1:250 and Grb2 (Santa Cruz Biotechnology, C-23) diluted 1:100. All antibodies were diluted in Invitrogen antibody diluent, Life Technologies (Paisley UK). Detection was with the Menarini X-Cell Plus detection system.

Wound-healing and invasion assays

Confluent cells seeded in a 12-well plate were serum starved overnight. The surface of the cells was then scratched with a pipette tip and the cells were washed with PBS. One group of cells was incubated in 1% serum for 6 h and another was stimulated with FGF9. The average distance traveled by the cells along the wound was quantified in three independent experiments. The percentage of wound closure was calculated, averaged and normalized. Matrigel Invasion Chambers in two 24-well plates with 8.0-μm pores were purchased from BD Biosciences. The experiment was carried out as previously described [29].

Statistical analysis

A total of 150 adenocarcinoma lung cancer patients (The Profiling of Resistance patterns and Oncogenic Signaling Pathways in Evaluation of Cancers of the Thorax (PROSPECT) trial) who did not receive neo-adjuvant therapy were included in the analysis. Patient characteristics and clinical outcomes are summarized using descriptive statistics, including mean (SD) and median (minimum, maximum) for continuous variables and frequency (%) for categorical variables. Wilcoxon rank-sum test or Kruskal-Wallis test was used to compare differences in continuous variables between groups. Chi-square test or Fisher's exact test was used to compare differences in categorical variables between groups. Overall survival (OS) was defined as the time interval from surgery to the time of death or last contact and recurrence-free survival (RFS) was defined as the time interval from surgery to the time of recurrence or last contact. Both OS and RFS were censored at 60 months if a patient was alive or died beyond 60 months for OS or if a patient was known to be recurrence-free at 5 years or had recurrence after 5 years for RFS. Kaplan-Meier Method and Cox-proportional hazards regression analyses were conducted on OS and RFS. For the K-M curves we used the median as a cut-off point and the binary cut-off points of biomarkers were also identified using a recursive partitioning algorithm (rpart function in R) in which a cut-off point is determined for each predictor variable such that resulting subgroups are the most different in their RFS. A P value of less than 0.05 indicated a statistical significance. SAS 9.3 (SAS Institute Inc. Cary. NC) was used for data analysis.

Results

Protein expression levels of FGFR2, Grb2 and Plcγ1 modulate tumor growth in a xenograft mouse model

Prior to investigating the role of FGFR2, Grb2 and Plcγ1 as disease prognostic markers, we tested their ability to modulate tumor growth and progression in a xenograft mouse model. We utilized the highly characterized human embryonic kidney cell line, HEK293T [29]. This cell line provides an excellent and simple model

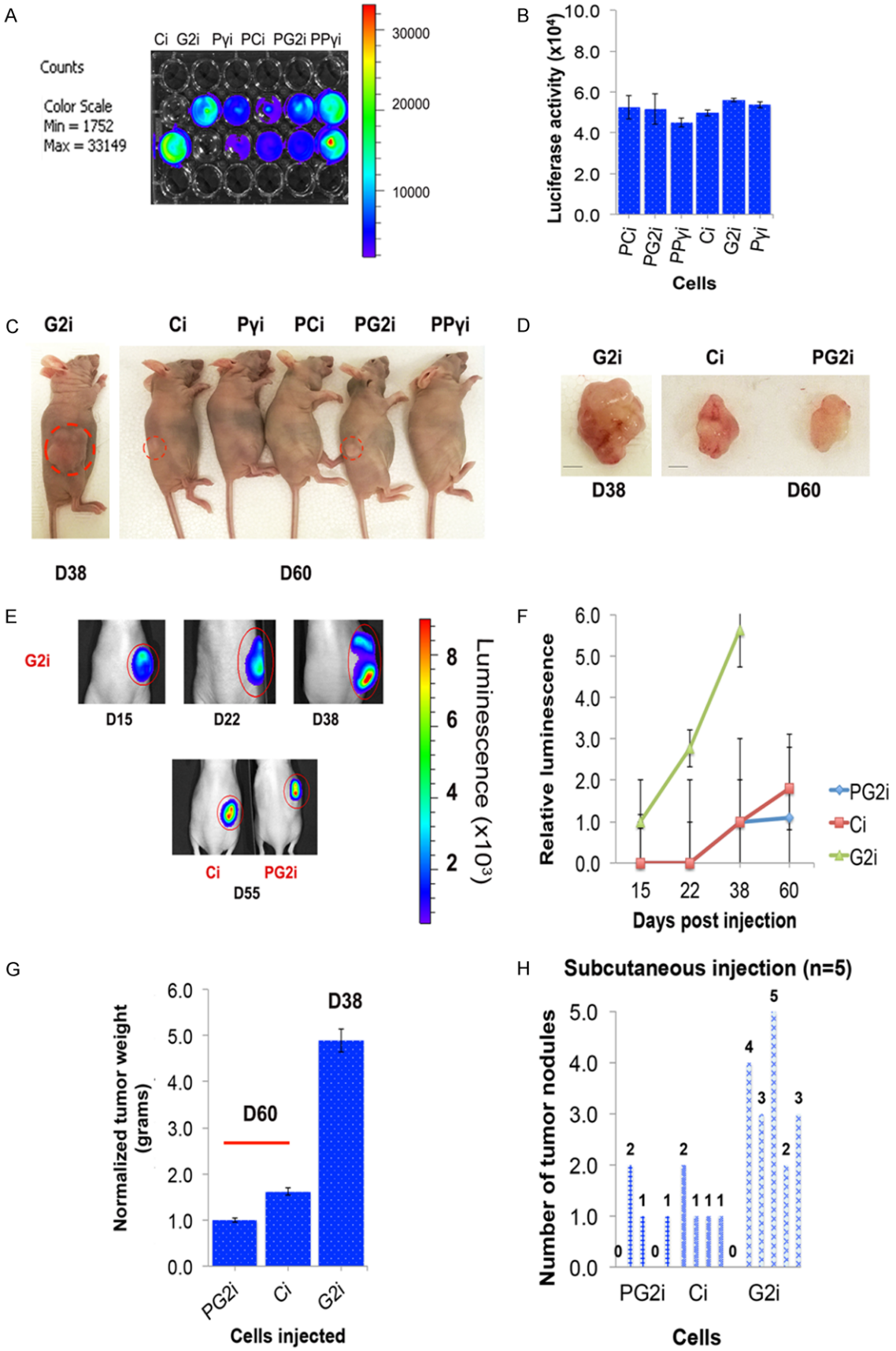
A
Ci G2i Pγi PCi PG2i PPγi
Counts
Color Scale
Min = 1752
Max = 33149
30000
20000
10000
B
Luciferase activity (x10⁴)
10.0
8.0
6.0
4.0
2.0
0.0
PCi
PG2i
PPγi
Ci
G2i
Pγi
Cells
C
G2i
Ci
Pγi
PCi
PG2i
PPγi
D38
D60
D
G2i
Ci
PG2i
D38
D60
E
G2i
D15
D22
D38
Ci
PG2i
D55
Luminescence (x10³)
8
6
4
2
F
Relative luminescence
6.0
5.0
4.0
3.0
2.0
1.0
0.0
15
22
38
60
Days post injection
PG2i
Ci
G2i
G
Normalized tumor weight (grams)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
D38
D60
PG2i
Ci
G2i
Cells injected
H
Subcutaneous injection (n=5)
Number of tumor nodules
5.0
4.0
3.0
2.0
1.0
0.0
0
2
1
0
1
2
1
1
0
4
3
5
2
3
PG2i
Ci
G2i
Cells

Figure 1. FGFR2, Grb2 and Plcγ1 expression trend regulates tumor formation in xenograft mouse model of HEK 293T model cell lines. A, B. Efficiency of transfection of luciferase vector in the indicated cell lines was determined by treating the cells in a 12-well plate with luciferin and measuring the activity via IVIS imaging system. Results were validated by luciferase reporter assay to measure luciferase activity. C. Mice injected with the indicated cell lines were imaged and tumor growth identified (see red circles). G2i mice had an increased tumour burden and had to be euthanized at day 38 (D38). The other mice were sacrificed at day 60 (D60). D. Tumours were then resected and imaged to show the burden where the scale-bar represents 1 cm. E. IVIS system based luminescence was measured at the indicated time points to monitor tumour development. F. Relative luminescence was plotted against days post-injection. The error bars represent the standard deviation of the mean. G, H. Tumor weight and number of nodules/tumor were determined and averaged per group of mice.

system to test whether these three proteins can drive general, not cancer specific, tumor formation. This model system is also highly relevant since the parental cells express very low levels of FGFR2 (can thus be used as a partial negative control) and the cells are highly transfectable (for protein overexpression or shRNA-mediated knockdown of proteins of interest).

HEK293T cells with and without FGFR2 overexpression were used for shRNA knock down experiments. These cells were stably transfected with Grb2 shRNA (to give Grb2 knock down cells G2i and (parental) PG2i respectively) or Plcγ1 shRNA (to give Plcγ1 knock down cells Pγi and PPγi respectively). These two cell lines were also stably transfected with scrambled shRNA (Ci and PCi cells respectively) to provide control cell lines. The knock down efficiencies for both Grb2 and Plcγ1 are shown elsewhere [29]. The 6 cell lines were also stably transfected with an empty luciferase vector and treated with luciferin (luciferase enzyme substrate) to detect the efficiency of luciferase vector transfection and select for comparable expression levels (**Figure 1A** and **1B**).

1×10^6 cells/cell line were injected into female nude mice (5 mice/cell line) to monitor tumour development. Tumors were only observed in G2i, Ci and PG2i injected mice (**Figure 1C** and **1D**), G2i tumors in the absence of Grb2 progressed to excessive burden and the mice had to be euthanized at day 38. By monitoring luciferase-based luminescence at multiple time points, we observed that G2i tumors were unique in their ability to develop fast and form secondary tumors (**Figure 1E** and **1F**). Tumor weight and number of nodules/tumor were also dramatically higher in G2i cells compared to Ci cells with PG2i cells forming the smallest tumors with a limited number of nodules (**Figure 1G** and **1H**). These data clearly indicate that tumor formation and malignancy are dependent on FGFR2 expression and are enhanced with Plcγ1 expression and Grb2 depletion.

Expression levels of FGFR2, Grb2 and Plcγ1 are indicators of invasive potential in LUAD cell lines

In order to investigate whether the established model system is relevant to cancer progression, we stained a multi-tumor tissue microarray (95 cases of 40 cancer types from 27 organs) and a LUAD tissue array (102 cases) with FGFR2, Grb2 and Plcγ1 antibodies. We observed that FGFR2 and Plcγ1 staining intensity is higher in LUAD compared to normal lung tissue (**Figure 2A**) while it is lower for Grb2. This was also validated by the results obtained from The Human Protein Atlas Database (human Protein Atlas databses http://www.proteinatlas.org/) where Grb2 expression is minimal unlike FGFR2/Plcγ1 which are medium to high in lung cancer tissues.

Based on the above, and taking into consideration that a LUAD mouse model fails to successfully mimic metastatic human adenocarcinoma [31], we decided to investigate the correlation of our protein markers with cancer progression at a cellular level. We screened for cell lines that express variable levels of FGFR2, Grb2 and Plcγ1 and hence provide adequate controls (**Figure 2B** and **2C**). We found that three LUAD cell lines (HCC1793, HCC44 and HCC515; also referred to as 1793, 44 and 515 respectively) show differential expression levels of FGFR2, Grb2 and Plcγ1. HCC44 and HCC515 express lower levels of FGFR2 compared to HCC1793. Grb2 expression was considerably higher in HCC44 and HCC515 compared to HCC1793. Plcγ1 appeared higher in HCC44 and, HCC1793 compared to HCC515, but after adjusting for loading (**Figure 2C**) the difference was not significant.

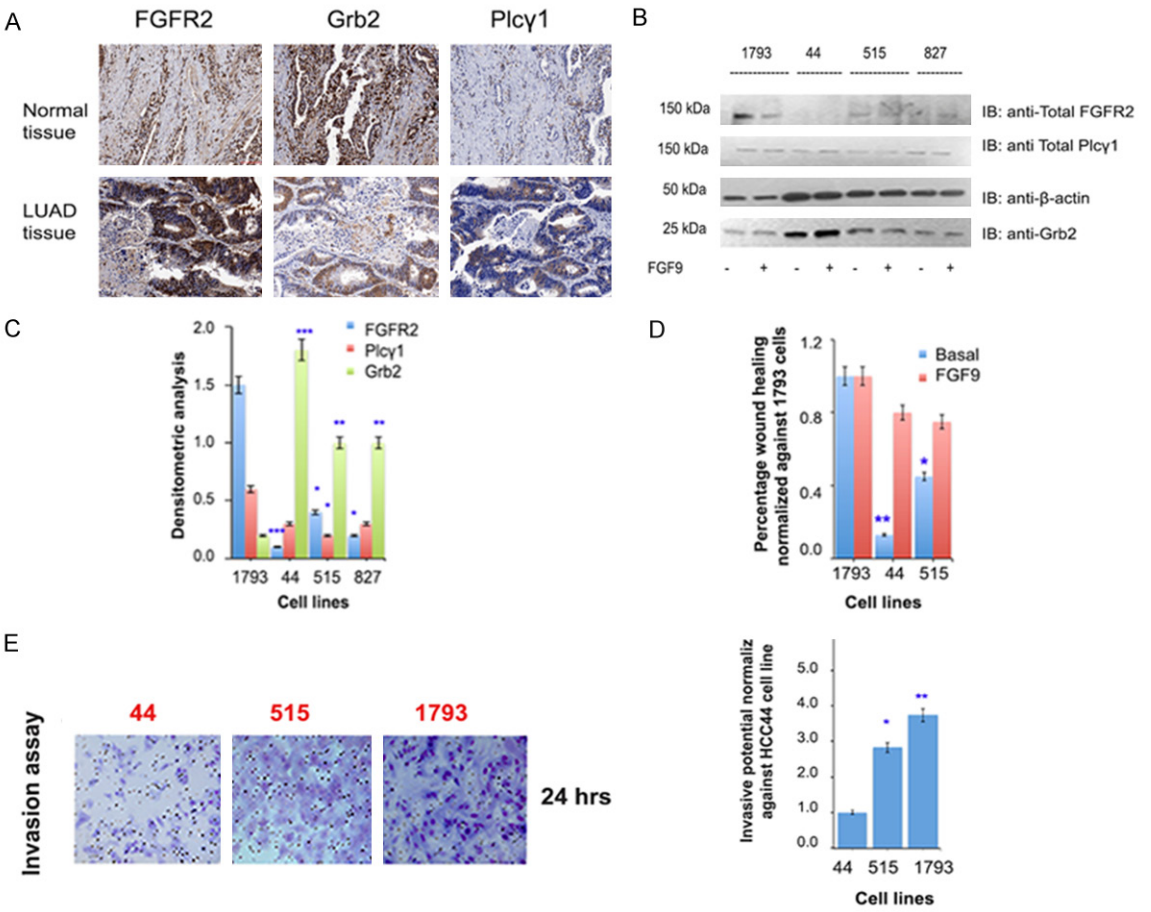

Figure 2. FGFR2, Grb2 and Plcγ1 expression regulate the invasive potential of LUAD cell lines. A. Patient TMAs (multi-organ and lung cancer TMAs) were stained with antibodies against the 3 markers and pictures were taken at 20× magnification to show the intensity of the staining. B. Lung adenocarcinoma cell lines were serum starved, left unstimulated or stimulated with FGF9 for 30 minutes then used in western blot analysis. The blots were probed with the indicated antibodies. C. Results obtained in densitometric analysis from 3 independent experiments (n = 3) were averaged and then used to generate a bar graph. Error bars denote the standard deviation of the calculated mean. Statistical significance was determined by Student's t-Test- with *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. D. The wound healing experiment was carried out as explained in the Materials and Methods section. Results from 3 independent experiments (n = 3) were averaged. Error bars denote the standard deviation of the calculated mean. Statistical significance was determined by Student's t-Test- with *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. E, F. The invasion assay was carried out as explained in the Materials and Methods section. The number of cells that invaded to the lower chamber containing 1% serum was counted, averaged and normalized from 3 independent experiments to measure the invasive potential of the cell lines used. Error bars denote the standard deviation of the calculated mean. Statistical significance was determined by Student's t-Test- with *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

Cells were left unstimulated or stimulated with fibroblast growth factor 9 (FGF9) to detect the effects of FGFR2, Grb2 and Plcγ1 protein concentration and FGFR2 activation (as an additional control) on the cells' migratory and invasive potentials (**Figure 2D-F**). A wound healing assay was used to measure the migratory potential of HCC1793 (high FGFR2, high Plcγ1 and low Grb2), HCC515 (lower FGFR2, lower Plcγ1 and higher Grb2) and HCC44 cell line that expresses negligible level of FGFR2 and high Grb2 level (**Figure 2B-D**). Predictably based on protein expression levels the percentage wound healing was significantly different in the basal non-stimulated state with HCC1793 having the highest migratory potential followed by HCC515 with HCC44 having the lowest percentage of wound healing (**Figure 2D**). Because our previous observations on the effects of FGFR2/Grb2 and Plcγ1 on oncogenesis had been made

Table 1. A-C Association of biomarkers with patient characteristics with (A) showing the Spearman Correlation

A

	Spearman Correlation Coefficients, N = 150 Prob > |r| under H0: Rho=0		
	Plcγ1	Grb2	FGFR2
Age3	0.01493	-0.05529	0.03636
	0.8561	0.5016	0.6587
Tumor_Size_Principal_cm	-0.08397	0.13915	0.01494
	0.3070	0.0895	0.8561

B

Marker	Covariate	Levels	n	Mean	Std	Stderr	Min	Max	Median	q1	q3	*p* Value	*P* value <0.05
FGFR2	ADJ	N	94	3.95	0.48	0.05	3.05	5.05	3.88	3.62	4.24	0.504	
		Y	56	3.97	0.52	0.07	2.96	5.55	4.02	3.75	4.30	.	
	Agegrp_1	≥ Median	75	4.01	0.51	0.06	3.05	5.55	3.93	3.70	4.28	0.476	
		< Median	75	3.91	0.48	0.06	2.96	4.90	3.95	3.57	4.26	.	
	Gender	F	73	3.93	0.47	0.05	3.05	5.05	3.88	3.62	4.27	0.469	
		M	77	3.98	0.52	0.06	2.96	5.55	4.00	3.68	4.26	.	
	Tobacco History	N	19	3.84	0.51	0.12	3.05	4.90	3.85	3.35	4.28	0.326	
		Y	131	3.97	0.49	0.04	2.96	5.55	3.95	3.68	4.26	.	
	race2_1	Non Caucasian	19	4.10	0.55	0.13	3.09	5.02	4.24	3.72	4.33	0.129	
		Caucasian	131	3.94	0.48	0.04	2.96	5.55	3.90	3.62	4.23	.	
Grb2	ADJ	N	94	7.96	0.66	0.07	3.28	9.16	8.05	7.74	8.23	0.522	
		Y	56	8.01	0.59	0.08	5.69	9.18	8.11	7.67	8.37	.	
	Agegrp_1	≥ Median	75	8.00	0.45	0.05	6.47	9.16	8.07	7.72	8.22	0.597	
		< Median	75	7.95	0.79	0.09	3.28	9.18	8.05	7.74	8.42	.	
	Gender	F	73	7.93	0.58	0.07	5.69	8.84	8.05	7.73	8.29	0.455	
		M	77	8.02	0.69	0.08	3.28	9.18	8.07	7.75	8.25	.	
	Tobacco History	N	19	7.74	0.65	0.15	6.47	9.18	7.81	7.29	8.05	0.022	*
		Y	131	8.01	0.63	0.06	3.28	9.16	8.09	7.75	8.29	.	*
	race2_1	Non Caucasian	19	7.76	1.22	0.28	3.28	9.18	8.05	7.55	8.34	0.588	
		Caucasian	131	8.01	0.50	0.04	5.69	9.16	8.07	7.75	8.27	.	
Plcγ1	ADJ	N	94	7.56	0.62	0.06	6.22	9.29	7.54	7.21	7.93	0.130	
		Y	56	7.64	0.86	0.11	3.81	9.02	7.80	7.37	8.12	.	
	Agegrp_1	≥ Median	75	7.61	0.64	0.07	6.22	9.08	7.57	7.15	8.01	0.848	
		< Median	75	7.57	0.79	0.09	3.81	9.29	7.63	7.28	8.07	.	
	Gender	F	73	7.58	0.73	0.09	3.81	9.02	7.58	7.31	7.98	0.937	
		M	77	7.60	0.71	0.08	6.20	9.29	7.65	7.21	8.08	.	
	Tobacco History	N	19	7.67	0.73	0.17	6.22	8.99	7.82	7.11	8.09	0.508	
		Y	131	7.58	0.72	0.06	3.81	9.29	7.57	7.24	8.01	.	
	race2_1	Non Caucasian	19	7.75	0.74	0.17	6.43	9.29	7.90	7.11	8.07	0.343	
		Caucasian	131	7.57	0.71	0.06	3.81	9.08	7.57	7.24	8.01	.	

C

Marker	Covariate	Levels	n	Mean	Std	Stderr	Min	Max	Median	q1	q3	*p* Value	*p* Value<0.05
FGFR2	Final Stage IASLC	I	82	3.94	0.45	0.05	3.02	5.05	3.89	3.67	4.24	0.850	
		II	38	3.96	0.57	0.09	2.96	4.96	3.91	3.39	4.37	.	
		III	30	4.00	0.50	0.09	3.14	5.55	3.98	3.70	4.24	.	
	Pathological N	N0	101	3.97	0.49	0.05	2.96	5.05	3.95	3.67	4.28	0.862	
		N1	28	3.96	0.59	0.11	3.14	5.55	3.89	3.51	4.25	.	
		N2	21	3.89	0.38	0.08	3.14	4.52	3.94	3.59	4.16	.	
	T IASLC 1	T1	46	3.91	0.47	0.07	3.07	5.05	3.86	3.70	4.15	0.554	
		T2	74	3.95	0.46	0.05	3.02	4.94	3.97	3.62	4.28	.	
		T3	25	4.00	0.64	0.13	2.96	5.55	4.05	3.45	4.32	.	
		T4	5	4.16	0.35	0.16	3.70	4.56	4.07	4.02	4.45	.	
	Type of smoker	Current	64	3.87	0.43	0.05	2.96	4.76	3.91	3.62	4.17	0.146	
		Former	67	4.07	0.53	0.06	3.14	5.55	3.97	3.74	4.45	.	

		Never	19	3.84	0.51	0.12	3.05	4.90	3.85	3.35	4.28	.	
Grb2	Final Stage IASLC	I	82	7.95	0.50	0.05	6.47	9.03	8.00	7.69	8.19	0.226	
		II	38	7.97	0.99	0.16	3.28	9.18	8.11	7.72	8.46	.	
		III	30	8.07	0.36	0.07	7.15	8.84	8.13	7.79	8.27	.	
	Pathological N	N0	101	7.99	0.49	0.05	6.47	9.18	8.04	7.72	8.25	0.562	
		N1	28	7.86	1.10	0.21	3.28	9.16	8.14	7.76	8.29	.	
		N2	21	8.07	0.41	0.09	7.15	8.84	8.16	7.79	8.28	.	
	T IASLC 1	T1	46	7.81	0.81	0.12	3.28	8.74	8.04	7.57	8.19	0.123	
		T2	74	8.01	0.58	0.07	5.69	9.16	8.04	7.73	8.46	.	
		T3	25	8.14	0.41	0.08	7.62	9.18	8.12	7.78	8.28	.	
		T4	5	8.21	0.24	0.11	7.86	8.53	8.18	8.17	8.30	.	
	Type of smoker	Current	64	8.03	0.80	0.10	3.28	8.85	8.12	7.80	8.50	0.016	*
		Former	67	7.99	0.42	0.05	6.55	9.16	8.04	7.70	8.23	.	*
		Never	19	7.74	0.65	0.15	6.47	9.18	7.81	7.29	8.05	.	*
Plcγ1	Final Stage IASLC	I	82	7.59	0.61	0.07	6.22	9.29	7.54	7.26	7.94	0.123	
		II	38	7.44	0.90	0.15	3.81	9.08	7.57	6.92	7.93	.	
		III	30	7.79	0.69	0.13	6.20	9.02	7.91	7.44	8.13	.	
	Pathological N	N0	101	7.54	0.65	0.06	6.20	9.29	7.52	7.10	7.93	0.046	*
		N1	28	7.56	0.93	0.18	3.81	8.86	7.67	7.26	8.06	.	*
		N2	21	7.88	0.68	0.15	6.21	9.02	8.01	7.50	8.13	.	*
	T IASLC 1	T1	46	7.58	0.66	0.10	6.22	9.29	7.52	7.07	8.01	0.517	
		T2	74	7.64	0.75	0.09	3.81	9.02	7.73	7.37	8.01	.	
		T3	25	7.52	0.75	0.15	6.21	9.08	7.49	7.07	8.03	.	
		T4	5	7.35	0.69	0.31	6.20	8.08	7.49	7.44	7.52	.	
	Type of smoker	Current	64	7.52	0.80	0.10	3.81	9.08	7.53	7.28	7.93	0.534	
		Former	67	7.64	0.63	0.08	6.40	9.29	7.70	7.15	8.09	.	
		Never	19	7.67	0.73	0.17	6.22	8.99	7.82	7.11	8.09	.	

*Cutoff point determined by Martingale residual.

under basal cell conditions, this study is focused on the non-stimulated state. However, it is worth noting that upon stimulation, the difference in migratory potential between the different cell lines was minimal (**FIgure 2D**). The FGFR2 concentration in HCC1793 decreases upon stimulation (**Figure 2B**) which is likely to be the effect of rapid receptor processing/degradation of the receptor in this cell line.

A Matrigel invasion assay was performed where cells were incubated in serum-deprived culture media in the upper chamber with 1% serum containing media in the lower chamber. Again HCC1793 had the highest invasive potential followed by HCC515 with HCC44 being the least invasive (**Figure 2E** and **2F**). Therefore the invasive potential of the cell lines mirrored their migratory ability.

FGFR2, Grb2 and Plcγ1 are prognostic markers in LUAD patients

To examine the relevance of our identified markers to LUAD progression and clinical outcome, we utilized the "The Profiling of Resistance patterns and Oncogenic Signaling Pathways in Evaluation of Cancers of the Thorax (PROSPECT)" trial data set from the Department of Thoracic/Head and Neck Medical Oncology at MD Anderson Cancer Center. We analyzed the processed gene expression levels in 150 surgically resected lung adenocarcinomas, stages I-III, to test whether FGFR2, Grb2 and Plcγ1 concentrations varied significantly between patients. The primary objectives of the study were to correlate the biomarkers with patients' characteristics (age, gender, tobacco history, type of smoker, race, tumor size, final stage in International Association for the Study of Lung Cancer (IASLC) database, adjuvant therapy, tumor (T) and node (N) pathological staging) and clinical outcomes (with respect to (overall survival (OS) and recurrence-free survival (RFS)). The Patient Characteristics for Entire Cohort (N = 150) and the general Kaplan-Meier Survival Curves (overall (OS) and recurrence free survival (RFS)) are shown in Supplementary Figure 1A (for OS) and 1B (for RFS) and Supplementary Table 1 where the median follow-up for those who survived at 5 years is 60 months, After investigating the association of the biomarkers with patient

Table 2. Association of biomarkers with RFS

A

		For Entire Cohort			Excluding each outlier		
Variable		HR (95% CI)		*P*-value	HR (95% CI)		*P*-value
Plcγ1	Per 1 unit increase	1.166	(0.774, 1.758)	0.4627	1.095	(0.695, 1.725)	0.6964
Grb2	Per 1 unit increase	0.937	(0.562, 1.564)	0.8044	0.936	(0.560, 1.564)	0.8003
FGFR2	Per 1 unit increase	0.892	(0.501, 1.585)	0.6958	0.798	(0.440, 1.447)	0.4576
Plcγ1	> Median vs. ≤ 7.6035	1.449	(0.799, 2.628)	0.2223	1.416	(0.781, 2.566)	0.2519
Grb2	> Median vs. ≤ 8.0675	0.597	(0.345, 1.031)	0.0641	0.596	(0.345, 1.031)	0.0640
FGFR2	> Median vs. ≤ 3.94	1.024	(0.585, 1.791)	0.9336	0.992	(0.564, 1.742)	0.9763
Plcγ1	> 7.58 vs. ≤ 7.58	1.556	(0.855, 2.834)	0.1479	1.520	(0.835, 2.766)	0.1708
Grb2	> 8.072 vs. ≤ 8.072	0.557	(0.321, 0.968)	0.0379	0.557	(0.321, 0.968)	0.0378
FGFR2	> 3.9955 vs. ≤ 3.9955	1.193	(0.684, 2.081)	0.5332	1.163	(0.664, 2.036)	0.5973

B

		For Entire Cohort			Excluding each outlier		
Variable		HR (95% CI)		*P*-value	HR (95% CI)		*P*-value
Plcγ1	Per 1 unit increase	1.151	(0.767, 1.728)	0.4980	1.078	(0.687, 1.692)	0.7443
Grb2	Per 1 unit increase	0.935	(0.562, 1.556)	0.7957	0.933	(0.559, 1.556)	0.7903
FGFR2	Per 1 unit increase	0.924	(0.522, 1.634)	0.7848	0.823	(0.456, 1.485)	0.5171
Plcγ1	> Median vs. ≤ 7.6035	1.395	(0.773, 2.516)	0.2692	1.361	(0.755, 2.456)	0.3053
Grb2	> Median vs. ≤ 8.0675	0.612	(0.356, 1.052)	0.0759	0.612	(0.356, 1.052)	0.0758
FGFR2	> Median vs. ≤ 3.94	1.026	(0.590, 1.786)	0.9270	0.989	(0.565, 1.729)	0.9680
Plcγ1	> 7.58 vs. ≤ 7.58	1.494	(0.824, 2.706)	0.1859	1.457	(0.804, 2.640)	0.2143
Grb2	> 8.072 vs. ≤ 8.072	0.571	(0.331, 0.987)	0.0450	0.571	(0.331, 0.987)	0.0449
FGFR2	> 3.9955 vs. ≤ 3.9955	1.198	(0.690, 2.077)	0.5210	1.161	(0.666, 2.023)	0.5980

C

		For Entire Cohort			Excluding outliers		
Variable		HR (95% CI)		*P*-value	HR (95% CI)		*P*-value
Plcγ1	> 7.58 vs. ≤ 7.58	1.497	(0.814, 2.754)	0.1940	1.427	(0.773, 2.634)	0.2551
Grb2	> 8.072 vs. ≤ 8.072	0.574	(0.330, 1.000)	0.0499	0.580	(0.333, 1.012)	0.0552
FGFR2	> 3.9955 vs. ≤ 3.9955	1.222	(0.698, 2.139)	0.4833	1.209	(0.690, 2.121)	0.5072
		For Entire Cohort			Excluding outliers		
		HR (95% CI)		*P*-value	HR (95% CI)		*P*-value
Level of Composite variable[2]				0.0153[1]			0.0224[1]
Plcγ1 Low, FGFR2 Low, Grb2 High		Reference			Reference		
Plcγ1 High, FGFR2 High, Grb2 Low		9.297	(1.992, 43.404)	0.0046	8.781	(1.866, 41.312)	0.0060
Other[3]		5.271	(1.250, 22.223)	0.0236	5.563	(1.317, 23.506)	0.0196

D

		For Entire Cohort			Excluding outliers		
Variable		HR (95% CI)		*P*-value	HR (95% CI)		*P*-value
Plcγ1	> 7.58 vs. ≤ 7.58	1.426	(0.781, 2.604)	0.2485	1.360	(0.742, 2.495)	0.3201
Grb2	> 8.072 vs. ≤ 8.072	0.586	(0.338, 1.016)	0.0571	0.590	(0.340, 1.026)	0.0617
FGFR2	> 3.9955 vs. ≤ 3.9955	1.226	(0.706, 2.129)	0.4704	1.202	(0.690, 2.093)	0.5162
		For Entire Cohort			Excluding outliers		
		HR (95% CI)		*P*-value	HR (95% CI)		*P*-value
Level Composite variable				0.0194[1]			0.0293[1]
Plcγ1 Low, FGFR2 Low, Grb2 High		Reference			Reference		
Plcγ1 High, FGFR2 High, Grb2 Low		8.528	(1.856, 39.193)	0.0059	7.986	(1.721, 37.069)	0.0080
Other		5.044	(1.200, 21.209)	0.0272	5.290	(1.257, 22.260)	0.0231

A. Association of each Biomarker with RFS in Multivariable Cox adjusting for age group, gender, tumor size group, final Stage IASLC, and adjuvant therapy. B. Association of each Biomarker with RFS in Multivariable Cox Model adjusting for tumor size group. final stage IASLC. and adjuvant therapy. C. Association of three Biomarkers with RFS in Multivariable Cox Model Adjusting for age group, gender, tumor size group, final stage IASLC, and adjuvant therapy. Upper panel: 3 biomarkers in the model. Lower panel: one composite variable to represent combinations of 3 biomarkers. [1]*P*-value for overall effect. [2]Composite variable has 8 possible responses (each of three markers have 2 levels (high/low); low/low/low, low/low/high, low/high/low, low/high/high, high/low/low, high/low/high, high/high/low, high/high/high). [3]other combinations of biomarkers. D. Association of three Biomarkers with RFS in Multivariable Cox Model adjusting for tumor size group, final Stage IASLC, and adjuvant therapy. Upper panel: 3 biomarkers in the model. Lower panel: one composite variable to represent combinations of 3 biomarkers. [1]*P*-value for overall effect.

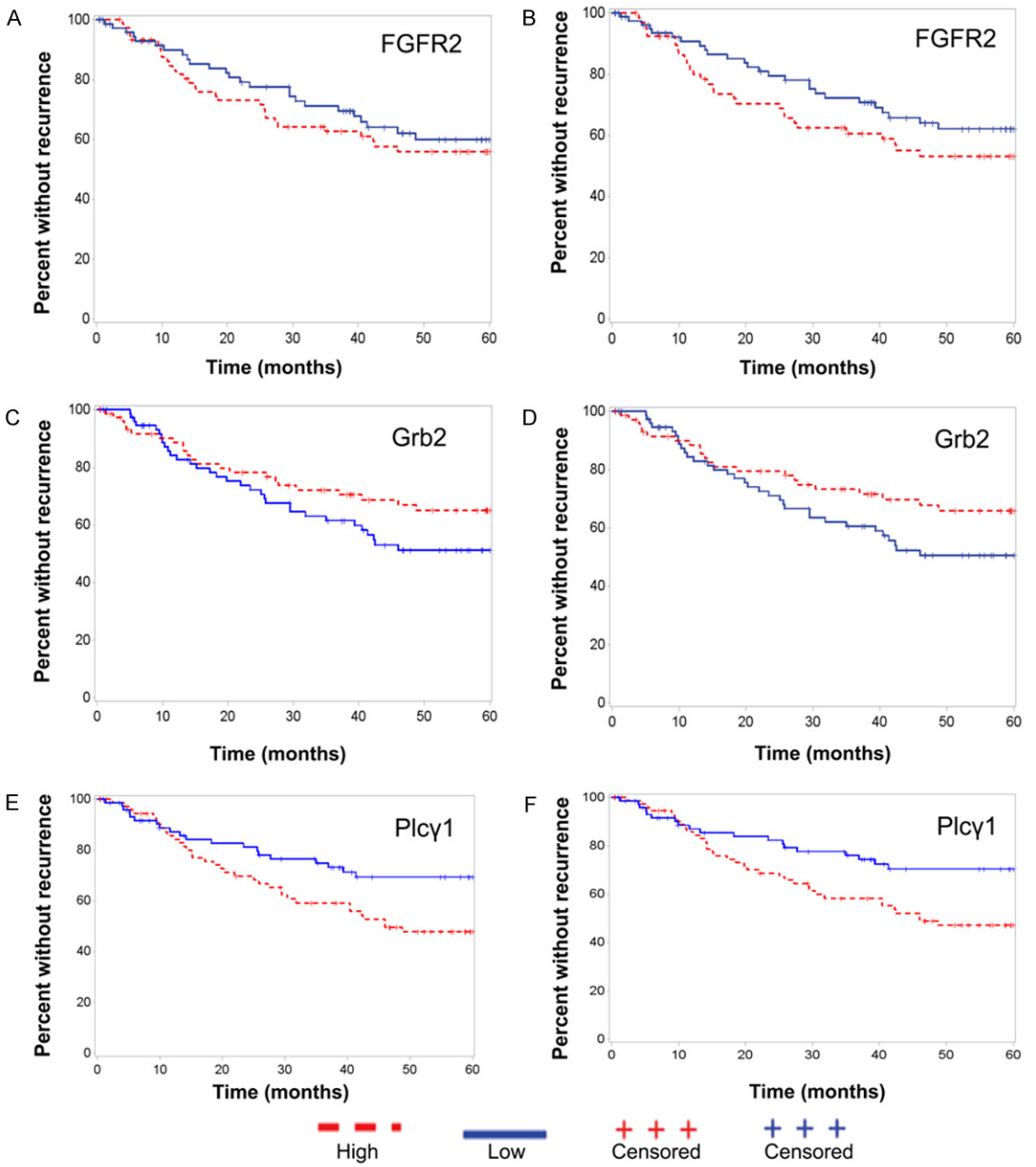

Figure 3. Independent gene expression of FGFR2, Grb2 and Plcγ1 correlate with patient survival. (A, B) Median was used as a cut-off point in the first Kaplan-Meier Recurrence-Free Survival curve (A. Log rank test *P*-value = 0.4897)

and cut-off by recursive partitioning for the second graph (B. Log rank test *P*-value = 0.2063). (C, D) Median was used as a cut-off point in the first Kaplan-Meier Recurrence-Free Survival curve (C. Log rank test *P*-value = 0.1636) and cut-off by recursive partitioning for the second graph (D. Log rank test *P*-value = 0.1114). (E, F) Median was used as a cut-off point in the first Kaplan-Meier Recurrence-Free Survival curve (E. Log rank test *P*-value = 0.0294) and cut-off by recursive partitioning for the second graph (F. Log rank test *P*-value = 0.0165).

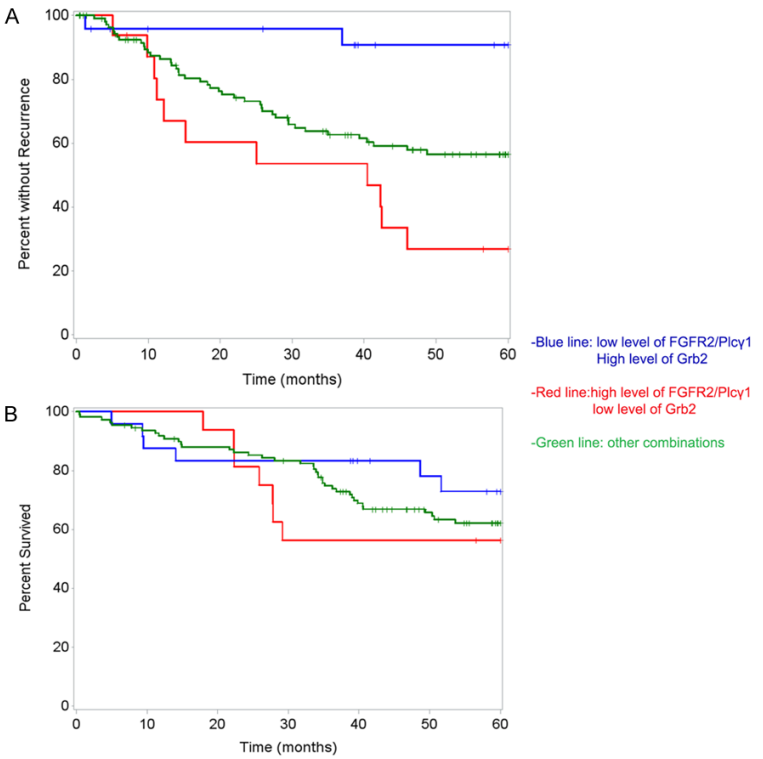

Figure 4. Combinatorial expression level of FGFR2, Grb2 and Plcγ1 correlate with patient survival. (A, B) For each marker, a cut-off of low/high was determined by recursive partitioning using RFS for (A) and OS for (B) as described in the Materials and Methods section.

characteristics in **Table 1** (Spearman correlation is shown in **Table 1A**) and distribution of biomarkers across the entire cohort before and after excluding outlying measurements (Supplementary Table 2), we made two observations: 1) the Grb2 level is considerably higher in patients with smoking history with P = 0.022 **Table 1B**). Significant differences in Grb2 is detected among three types of smokers (current, former, never) with P = 0.016 (**Table 1C**). Current and former smokers have higher Grb2 levels than never smokers, 2) Higher pathological N is associated with higher Plcγ1 (P = 0.046) (**Table 1C**).

Next we investigated the association of the biomarkers with RFS for univariate analysis of RFS (Supplementary Table 3A) and for multivariable model with clino-pathological/reduced clino-pathological factors (Supplementary Table 3B and 3C). In these cases the 5-year recurrence status was 55 (36.67%) with recurrence vs. 95 (63.33%) without recurrence. Six of the patients without recurrence at 5 years had recurrence in the subsequent 5 years. After looking at each marker and covariates in the multivariable model, we noticed that the Grb2 group (> 8.072 vs. ≤ 8.072) showed significant association with RFS in all patients, adjusting for age group, gender, tumor size group, overall tumor stage and adjuvant therapy (HR (95% CI), 0.557 (0.321. 0.968); P = 0.0379) (**Table 2A**). The association remained significant after excluding one patient with outlying measurement of Grb2 (HR (95% CI), 0.557 (0.321. 0.968); P = 0.0378). The high Grb2 group showed lower hazard of recurrence compared to the low Grb2 group (**Table 2A**). The Grb2 group (> 8.072 vs. ≤ 8.072) showed significant association with RFS in all patients, adjusting for tumor size group, overall tumor stage and adjuvant therapy (**Table 2B**). The association remained significant after excluding one patient with outlying measurement of Grb2. The high Grb2 group showed lower hazard of recurrence compared to the low Grb2 group (**Table 2B**). After including all three markers and covariates in the multivariable model, we noticed that, after adjusting for age group, gender, tumor size group, final stage IASLC, and adjuvant therapy, the Plcγ1 and FGFR2 groups were not significant but the Grb2 group was significant (P = 0.0499) (**Table 2C** upper panel). The Plcγ1 group, the Grb2 group and the FGFR2 group were not significant when tumor size group, final stage

IASLC, and adjuvant therapy were adjusted (**Table 2C** upper panel). A composite variable representing combinations of three biomarkers with 3 levels (Plcγ1, FGFR2, Grb2 being low, low, high (27 patients) versus high, high, low (14 patients) versus the rest of combinations), and after adjusting for age group, gender, tumor size group, final stage IASLC, and adjuvant therapy, showed significant differences between relative concentrations of Plcγ1 high, FGFR2 high, Grb2 low and Plcγ1 low, FGFR2 low, Grb2 high. The same result was obtained when comparing other combinations with Plcγ1 low, FGFR2 low, Grb2 high (**Table 2C** lower panel). The same significant associations were observed following the adjustment for multiple characteristics (tumour size, final stage IASCLC and adjuvant therapy) in different combinations like the one shown in **Table 2D** (upper and lower panel) before and after excluding patients with outlying measurements. As an additional control, we looked at the association of the biomarkers with OS (Supplementary Table 4) where the median follow-up of surviving patients is 60 months. 5 year survival status was 52 (34.67%) deceased versus 98 (65.33%) alive. Nine patients in the "alive group" were deceased after 5 years. In this case the biomarkers did not show significant association with survival even after adjusting for patient characteristics. Kaplan-Meier-RFC curves for each marker (with median as cut-off point or cut-off performed by recursive partitioning) are shown in **Figure 3**. In order to clearly depict the correlation of the combinatorial expression level of the markers with RFS in LUAD patients, we plotted 2 Kaplan-Meier curves for all markers in **Figure 4A** (percent without recurrence) and **4B** (percent survived). In both cases it is clear that patients with FGFR2/Plcγ1/Grb2 relative concentrations as: high/high/low have the poorest prognosis whilst those having FGFR2/Plcγ1/Grb2 as: low/low/high drastically enhances the prognosis.

Discussion

Lung cancer, and specifically LUAD remain the main cause of cancer-related death in the world. This stems from the late stage diagnosis and the lack of personalized therapies limiting the treatment options to the existing palliative ones. FGFR2 signalling-related LUAD progression is specific to a subset of patients and even though the current design of FGFR2 targeting drugs seems promising, the challenge remains in identifying the patients that will benefit from such a treatment [32, 33]. Hence, appropriate selection and classification of patients is critical in minimizing drug toxicity and/or unnecessary administration of an irrelevant drug. Although aberrant activation of FGFR2 has been revealed as a novel driver in LUAD [7, 34, 35], there has been no study that factors in other proteins associated with FGFR2-mediated signalling. Such a study is critical since we have shown that mutations and/or amplification of FGFR2 are not the sole drivers of oncogenic FGFR2 signalling [29, 30].

Our study, which is based on a xenograft mouse model, cell lines and accumulated clinical data, identifies FGFR2, Grb2 and Plcγ1 as novel prognostic markers that will allow for a better understanding of patients' profiles to determine the group that will benefit from an FGFR2-based treatment regimen while sparing the others from the negative side effects. Based on the relative protein concentrations dictating whether Plcγ1 can bind to FGFR2 and become activated, it defines patients with relative concentrations of FGFR2/Plcγ1/Grb2: high/high/low (HHL cases) as having the poorest prognosis while patients with FGFR2/Plcγ1/Grb2: low/low/high (LLH cases) as having a more favourable one. As a result drugs that aim to inhibit FGFR2 kinase activity or modulate its expression might not be successful in treating HHL cases unless Plcγ1 and Grb2 are also therapeutically targeted. In HHL cases, inhibiting Plcγ1 binding to FGFR2 might be sufficient to maintain homeostasis and inhibit disease progression. This study introduces a combinatorial protein prognostic marker for LUAD raising the exciting prospect of a novel approach to determining patient outcomes.

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Disclosure of conflict of interest

None.

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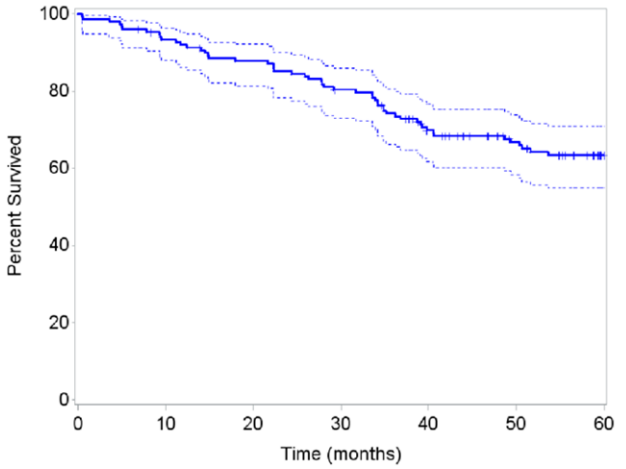
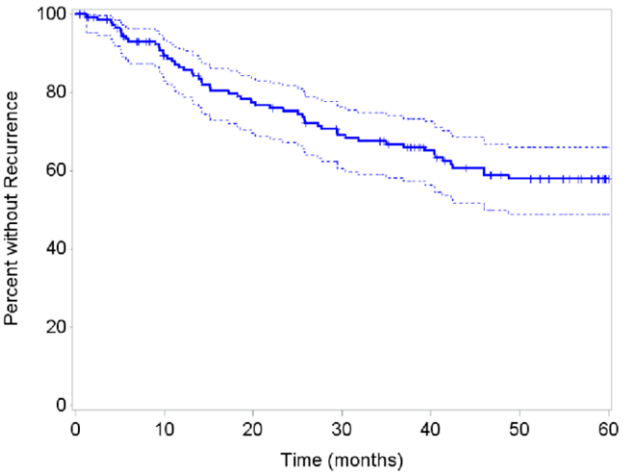
A



	Time (Months)					
Time (Months)	0	12	24	36	48	59.66
Number at Risk	150	136	125	107	84	63

B



	Time (Months)					
Time (Months)	0	12	24	36	48	59.66
Number at Risk	150	117	100	83	62	46

Supplementary Figure 1. Kaplan-Meier Survival Curves. A. OS, median follow-up for those who survived at 5 years is 60 months. B. Recurrence free survival (RFS).

Supplementary Table 1. Patient characteristics for entire cohort (N = 150)

Variable	n	Mean SD, Median (Minimum, Maximum)
Age (in year)	150	64.92 ± 10.33, 64.91 (40.99, 85.86)
Tumor Size Principal (cm)	150	3.8 ± 2.07, 3.35 (1, 14)
	Category	Frequency (%)
5-year Survival	Death	52 (34.7%)
	Survived	98 (65.3%)
5-year Recurrence	Recurrence	55 (36.7%)
	Without Recurrence	95 (63.3%)
Gender	Male	77 (51.3%)

	Female	73 (48.7%)
Age Group	< 65 years	75 (50.0%)
	≥ 65 years	75 (50.0%)
Race	African American	8 (5.3%)
	Asian	6 (4.0%)
	Caucasian	131 (87.3%)
	Hispanic	5 (3.3%)
Tobacco History	No	19 (12.7%)
	Yes	131 (87.3%)
Type of smoker	Current	64 (42.7%)
	Former	67 (44.7%)
	Never	19 (12.7%)
Final Stage IASLC	I	82 (54.7%)
	II	38 (25.3%)
	III	30 (20%)
T IASLC 1	T1	46 (30.7%)
	T2	74 (49.3%)
	T3	25 (16.7%)
	T4	5 (3.3%)
Pathological N	N0	101 (67.3%)
	N1	28 (18.7%)
	N2	21 (14%)
KRAS MUT[1]	Mutant	36 (25%)
	WT	108 (75%)
EGFR MUT[2]	Mutant	21 (15.2%)
	WT	117 (84.8%)
ADJ	No	94 (62.7%)
	Yes	56 (37.3%)

[1]Frequency missing = 6. [2]Frequency missing = 12.

Supplementary Table 2. Distributions of biomarkers for Entire Cohort (N = 150)

A		
Variable	n	Mean SD, Median (Minimum, Maximum)
Plcγ1	150	7.59 ± 0.72, 7.60 (3.81, 9.29)
Grb2	150	7.98 ± 0.64, 8.07 (3.28, 9.18)
FGFR2	150	3.96 ± 0.49, 3.94 (2.96, 5.55)
B		
Variable	n	Mean SD, Median (Minimum, Maximum)
Plcγ1	149	7.62 ± 0.65, 7.63 (6.20, 9.29)
Grb2	149	8.01 ± 0.51, 8.07 (5.69, 9.18)
FGFR2	149	3.95 ± 0.48, 3.94 (2.96, 5.05)

A. All Measurements. B. Excluding outlying Measurements.

Supplementary Table 3. Association of biomarkers with Recurrence-free Survival (RFS)

A				
Variable			HR (95% CI)	*P*-value
Age (in year)	Per Year increase	1.0138	(0.9870, 1.0414)	0.3162
Age Group	> Median vs. ≤ 64.91	1.2719	(0.7478, 2.1633)	0.3748
Gender	Male vs. Female	1.1425	(0.6732, 1.9390)	0.6215
Race	Caucasian vs. Others	0.7875	(0.3854, 1.6093)	0.5124
Tobacco History	Yes vs. No	0.8517	(0.4024, 1.8025)	0.6747
Tumor Size Group1	> 2.7* cm vs. ≤ 2.7	2.1499	(1.1709, 3.9473)	0.0136
Tumor Size Group2	> Median vs. ≤ 3.35	2.1463	(1.2623, 3.6494)	0.0048
KRAS Mutation[1]	Yes vs. No	0.9367	(0.4999, 1.7550)	0.8383
EGFR Mutation[2]	Yes vs. No	1.1428	(0.5525, 2.3639)	0.7189
Adjuvant	Yes vs. No	0.7218	(0.4235, 1.2302)	0.2307
Plcγ1	Per 1 unit increase	1.3426	(0.9082, 1.9848)	0.1396
Grb2	Per 1 unit increase	1.0261	(0.6228, 1.6905)	0.9194
FGFR2	Per 1 unit increase	1.0784	(0.6234, 1.8655)	0.7872
Plcγ1	> Median vs. ≤ 7.6035	1.8253	(1.0534, 3.1627)	0.0319
Grb2	> Median vs. ≤ 8.0675	0.6847	(0.4005, 1.1704)	0.1661
FGFR2	> Median vs. ≤ 3.94	1.2055	(0.7089, 2.0501)	0.4903
Plcγ1	> 7.58[3] vs. ≤7 .58	1.9499	(1.1180, 3.4007)	0.0186
Grb2	> 8.072[3] vs. ≤ 8.072	0.6472	(0.3771, 1.1105)	0.1142
FGFR2	> 3.9955[3] vs. ≤ 3.9955	1.4048	(0.8271, 2.3858)	0.2085
Final Stage IASLC				0.0011[4]
	II vs. I	1.5514	(0.7980, 3.0162)	0.1954
	III vs. I	3.2123	(1.7299, 5.9651)	0.0002
Pathological N				0.0290[4]
	N1 vs. N0	1.7337	(0.8899, 3.3776)	0.1059
	N2 vs. N0	2.3282	(1.1938, 4.5403)	0.0131
T IASLC				0.0087[4]
	T2 vs. T1	1.1738	(0.6155, 2.2385)	0.6266
	T3 vs. T1	2.7887	(1.3220, 5.8828)	0.0071
	T4 vs. T1	3.9913	(1.1534, 13.8115)	0.0289
Type of smoker				0.9056[4]
	Current vs. Never	0.8329	(0.3725, 1.8624)	0.6561
	Former vs. Never	0.8704	(0.3910, 1.9377)	0.7340
B				
Variable			HR (95% CI)	*P*-value
Age Group	> Median vs. ≤ 64.91	1.407	(0.802, 2.469)	0.2340
Gender	Male vs. Female	0.900	(0.516, 1.569)	0.7093
Tumor Size Group2	> Median vs. ≤ 3.35	1.890	(1.052, 3.396)	0.0333
Final Stage IASLC				0.0014
	II vs. I	1.376	(0.696, 2.721)	0.3585
	III vs. I	3.952	(1.876, 8.323)	0.0003
Adjuvant	Yes vs. No	0.762	(0.383, 1.517)	0.4391
C				
Variable			HR (95% CI)	*P*-value
Tumor Size Group2	> Median vs. ≤ 3.35	1.890	(1.052, 3.396)	0.0333
Final Stage IASLC				0.0014
	II vs. I	1.376	(0.696, 2.721)	0.3585
	III vs. I	3.952	(1.876, 8.323)	0.0003
Adjuvant	Yes vs. No	0.762	(0.383, 1.517)	0.4391

5-year recurrence status: 55 (36.67%) with recurrence vs. 95 (63.33%) without recurrence (6 patients without recurrence at 5 years had recurrence after 5 years). A. Univariate analysis of RFS. *Cutoff point determined by Martingale residual. [1]Frequency missing = 6. [2]Frequency missing = 12. [3]Cutoff point determined by recursive partitioning (rpart function in R, First knot) using RFS. [4]*P*-value for overall effects. B. Multivariable Model with Clinicopathological Factors. C. Multivariable Model with reduced Clinicopathological Factors.

Supplementary Table 4. Association of biomarkers with OS where median follow-up of surviving patients is 60 months

A				
Variable			HR (95% CI)	*P*-value
Age (in year)	Per 1 Year increase	1.0297	(1.0010, 1.0593)	0.0425
Age Group	> Median vs. ≤ Median	1.3821	(0.7994, 2.3894)	0.2466
Gender	Male vs. Female	1.6285	(0.9356, 2.8347)	0.0846
Race	Caucasian vs. Others	1.5984	(0.6354, 4.0207)	0.3190
Tobacco History	Yes vs. No	1.2213	(0.5213, 2.8611)	0.6453
Tumor Size Group 1	> 2.7 cm vs. ≤ 2.7	3.6306	(1.7084, 7.7155)	0.0008
Tumor Size Group 2	> 3.5cm vs. ≤ 3.5	2.4753	(1.4214, 4.3106)	0.0014
KRAS Mutation[1]	Yes vs. No	0.7889	(0.4039, 1.5411)	0.4877
EGFR Mutation[2]	Yes vs. No	0.4487	(0.1608, 1.2519)	0.1258
Adjuvant Therapy	Yes vs. No	1.1493	(0.6604, 2.0001)	0.6225
Plcγ1	Per 1 unit increase	1.2611	(0.8469, 1.8779)	0.2534
Grb2	Per 1 unit increase	0.9821	(0.6321, 1.5258)	0.9358
FGFR2	Per 1 unit increase	0.7193	(0.4142, 1.2492)	0.2420
Plcγ1	> Median vs. ≤ 7.6035	1.4075	(0.8114, 2.4415)	0.2239
Grb2	> Median vs. ≤ 8.0675	1.0157	(0.5896, 1.7496)	0.9554
FGFR2	> Median vs. ≤ 3.94	0.8293	(0.4806, 1.4311)	0.5014
Plcγ1	> 7.58[3] vs. ≤ 7.58	1.3590	(0.7835, 2.3573)	0.2750
Grb2	> 8.072[3] vs. ≤ 8.072	0.9575	(0.5556, 1.6502)	0.8758
FGFR2	> 3.9955[3] vs. ≤ 3.9955	0.8668	(0.4999, 1.5030)	0.6108
Final Stage IASLC				<0.0001[4]
	II vs. I	2.3131	(1.1686, 4.5783)	0.0161
	III vs. I	4.7286	(2.4244, 9.2227)	0.0000
Pathological N				0.0037[4]
	N1 vs. N0	2.1027	(1.0978, 4.0276)	0.0250
	N2 vs. N0	2.9451	(1.4834, 5.8472)	0.0020
T IASLC				0.0002[4]
	T2 vs. T1	1.3791	(0.6686, 2.8446)	0.3842
	T3 vs. T1	3.5118	(1.6105, 7.6576)	0.0016
	T4 vs. T1	7.8358	(2.4778, 24.7795	0.0005
Type of smoker				0.8976[4]
	Current vs. Never	1.2331	(0.5019, 3.0295)	0.6478
	Former vs. Never	1.2097	(0.4922, 2.9730)	0.6782
B				
Variable		HR (95% CI)		*P*-value
Age Group	> Median vs. ≤ Median	1.550	(0.877, 2.738)	0.1313
Gender	Male vs. Female	1.207	(0.677, 2.150)	0.5232
Tumor Size Group 2	> Median vs. ≤ Median	2.119	(1.159, 3.876)	0.0148
Final Stage IASLC				<0.0001[1]
	II vs. I	1.929	(0.954, 3.903)	0.0676
	III vs. I	6.480	(3.013, 13.937)	<.0001
Adjuvant	Yes vs. No	0.537	(0.277, 1.040)	0.0653
C				
Variable			HR (95% CI)	*P*-value
Tumor Size Group 2	> Median vs. ≤ Median	2.012	(1.119, 3.617)	0.0194
Final Stage IASLC				<0.0001[1]
	II vs. I	6.637	(3.111, 14.160)	<.0001
	III vs. I	1.928	(0.951, 3.907)	0.0685

Adjuvant		Yes vs. No	0.511	(0.264, 0.986)		0.0454	
D							
		For Entire Cohort			Excluding each outlier		
Variable		HR (95% CI)		*P*-value	HR (95% CI)		*P*-value
Plcγ1	Per 1 unit increase	1.110	(0.725, 1.699)	0.6309	1.060	(0.670, 1.679)	0.8031
Grb2	Per 1 unit increase	0.849	(0.545, 1.323)	0.4700	1.124	(0.629, 2.008)	0.6931
FGFR2	Per 1 unit increase	0.620	(0.352, 1.094)	0.0989	0.549	(0.307, 0.983)	0.0437
Plcγ1	> Median vs. ≤ 7.6035	1.133	(0.630, 2.040)	0.6763	1.117	(0.621, 2.011)	0.7119
Grb2	> Median vs. ≤ 8.0675	0.876	(0.501, 1.530)	0.6409	0.908	(0.517, 1.595)	0.7379
FGFR2	> Median vs. ≤ 3.94	0.698	(0.393, 1.241)	0.2206	0.670	(0.375, 1.198)	0.1770
Plcγ1	> 7.58 vs. ≤ 7.58	1.073	(0.599, 1.923)	0.8117	1.057	(0.590, 1.894)	0.8529
Grb2	> 8.072 vs. ≤ 8.072	0.817	(0.467, 1.428)	0.4772	0.845	(0.481, 1.485)	0.5579
FGFR2	> 3.9955 vs. ≤ 3.9955	0.718	(0.401, 1.285)	0.2645	0.691	(0.383, 1.245)	0.2181
E							
		For Entire Cohort			Excluding each outlier		
Variable		HR (95% CI)		*P*-value	HR (95% CI)		*P*-value
Plcγ1	Per 1 unit increase	1.124	(0.742, 1.704)	0.5802	1.063	(0.674, 1.676)	0.7930
Grb2	Per 1 unit increase	0.887	(0.572, 1.373)	0.5898	1.162	(0.653, 2.068)	0.6095
FGFR2	Per 1 unit increase	0.675	(0.388, 1.172)	0.1627	0.594	(0.337, 1.048)	0.0720
Plcγ1	> Median vs. ≤ 7.6035	1.159	(0.648, 2.072)	0.6192	1.135	(0.634, 2.031)	0.6692
Grb2	> Median vs. ≤ 8.0675	0.935	(0.541, 1.616)	0.8096	0.972	(0.559, 1.689)	0.9199
FGFR2	> Median vs. ≤3.94	0.729	(0.413, 1.284)	0.2737	0.695	(0.392, 1.233)	0.2138
Plcγ1	> 7.58[3] vs. ≤ 7.58	1.078	(0.606, 1.918)	0.7988	1.055	(0.592, 1.879)	0.8566
Grb2	> 8.072[3] vs. ≤ 8.072	0.872	(0.504, 1.507)	0.6231	0.904	(0.520, 1.572)	0.7213
FGFR2	> 3.9955[3] vs. ≤ 3.9955	0.753	(0.425, 1.336)	0.3324	0.720	(0.403, 1.287)	0.2678
F							
		For Entire Cohort			Excluding outliers		
Variable		HR (95% CI)		*P*-value	HR (95% CI)		*P*-value
Plcγ1	> 7.58 vs. ≤ 7.58	1.077	(0.599, 1.935)	0.8052	1.008	(0.554, 1.833)	0.9797
Grb2	> 8.072 vs. ≤ 8.072	0.837	(0.476, 1.472)	0.5372	0.883	(0.496, 1.569)	0.6704
FGFR2	> 3.9955 vs. ≤ 3.9955	0.726	(0.405, 1.300)	0.2808	0.719	(0.397, 1.302)	0.2758
G							
		For Entire Cohort			Excluding outliers		
		HR (95% CI)		*P*-value	HR (95% CI)		*P*-value
Level of Composite variale				0.7817[1]			0.7946[1]
Plcγ1 Low, FGFR2 Low, Grb2 High		Reference			Reference		
Plcγ1 High, FGFR2 High, Grb2 Low		1.456	(0.459, 4.619)	0.5236	1.306	(0.396, 4.304)	0.6607
Other		1.350	(0.549, 3.317)	0.5129	1.366	(0.554, 3.368)	0.4979
H							
		For Entire Cohort			Excluding outliers		
Variable		HR (95% CI)		*P*-value	HR (95% CI)		*P*-value
Plcγ1	>7.58 vs. ≤7.58	1.087	(0.609, 1.942)	0.7776	1.013	(0.560, 1.833)	0.9650
Grb2	>8.072 vs. ≤8.072	0.897	(0.516, 1.560)	0.7002	0.941	(0.535, 1.656)	0.8342
FGFR2	>3.9955 vs. ≤3.9955	0.758	(0.426, 1.348)	0.3451	0.747	(0.415, 1.343)	0.3295
I							
		For Entire Cohort			Excluding outliers		
		HR (95% CI)		*P*-value	HR (95% CI)		*P*-value
Level of Composite variale				0.7560[1]			0.7994[1]

Plcγ1 Low, FGFR2 Low, Grb2 High	Reference			Reference		
Plcγ1 High, FGFR2 High, Grb2 Low	1.513	(0.492, 4.657)	0.4702	1.329	(0.415, 4.252)	0.6319
Other	1.339	(0.546, 3.282)	0.5234	1.358	(0.553, 3.338)	0.5047

5-year survival status: 52 (34.67%) deceased vs. 98 (65.33%) alive (9 patients in alive group were deceased after 5 years). A. Univariate analysis of OS.1Frequency missing = 6. 2 Frequency missing = 12. [3]Cutoff point determined by recursive partitioning (rpart function in R, First knot) using RFS. [4]*P*-value for overall effects. B. Multivariable Model with clinicopathological Factors [1]*P*-value for overall effects. C. Multivariable Model with significant clinicopathological Factors. [1]*P*-value for overall effects. D. Association of each biomarker with OS in Multivariable Cox Model adjusting for age group, gender, tumor size group, final stage IASLC, and adjuvant therapy. E. Association of each biomarker with OS in Multivariable Cox Model adjusting for tumor size group, final stage IASLC, and adjuvant therapy. F. Association of 3 biomarkers with OS in Multivariable Cox Model adjusting for age group, gender, tumor size group, final Stage IASLC, and adjuvant therapy (all 3 markers were included in the same multivariable model. Correlations between pair of 3 markers were not significant). Upper panel: Three biomarkers in the model. Lower panel: Composite variable to represent combinations of three biomarkers. G. Association of 3 biomarkers with OS in Multivariable Cox Model adjusting for tumor size group, final stage IASLC, and adjuvant (all three markers were included in the same multivariable model). Upper panel: 3 biomarkers in the model. Lower panel: Composite variable to represent combinations of three biomarkers. [1]*P*-value for overall effect. H, I. Association of 3 biomarkers with OS in Multivariable Cox Model adjusting for tumor size group, final stage IASLC, and adjuvant (all three markers were included in the same multivariable model). H. 3 biomarkers in the model. I. Composite variable to represent combinations of three biomarkers. [1]*P*-value for overall effect.