Herreros-Pomares, A.; Doria, P.; Gallach, S.; Meri-Abad, M.; Guijarro, R.; Calabuig-Fariñas, S.; Camps, C.... (2022). A Sonic Hedgehog Pathway Score to Predict the Outcome of Resected Non-Small Cell Lung Cancer Patients. Annals of Surgical Oncology. 30(2):1225-1235. https://doi.org/10.1245/s10434-022-12565-2

Establishment of a Sonic Hedgehog Pathway Score to Predict the Outcome of Resected Non-Small Cell Lung Cancer Patients

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Simple summary

In recent years, considerable progress has been achieved in clinical trials for Hedgehog (Hh) pathway inhibitors, resulting in regulatory approvals of several molecules targeting Hh components for cancer treatment. Unfortunately, the link between Hh signaling pathway and lung cancer, which is the leading cause of cancer death in the world, is less clear, with contradictory results reported that have hampered the usage of Hh inhibitors. In this study, the gene expression of the main components of Hh signaling was evaluated in non-small cell lung cancer (NSCLC) patients. Our results indicate that Hh

pathway plays an important role in NSCLC prognosis and suggest that their components could constitute a potential target with major implications in patients' survival.

Abstract

Mutations and deregulations in the components of the Hedgehog (Hh) pathway have been associated to cancer onset and tumor growth in some malignancies, but their role in non-small cell lung cancer (NSCLC) remains unclear. This study aims to investigate the expression pattern of the main components of Hh pathway in tumor and adjacent normal tissue biopsies of resectable NSCLC patients. The relative expression of *GLI1*, *PTCH1*, *SHH* and *SMO* was analyzed by quantitative PCR and associated with clinicopathological information. Significant variations in the expression levels of the genes analyzed were found for tumor and normal tissues and for patients with different ECOG and histology. In addition, patients with higher expression levels of *PTCH1* presented better outcomes. A gene expression score, called Hedgehog score, was then calculated using the absolute regression coefficients of a multivariate model including the components of Hh signaling analyzed. Kaplan–Meier analysis showed that patients with high Hedgehog score have shorter Relapse-Free Survival (RFS) [39.13 vs. 81.23 months (mo), p = 0.025] and overall survival (OS) [44.50 vs. 95.40 mo, p = 0.039]. Similarly, patients in the adenocarcinoma (ADC) subcohort had shorther RFS [29.83 vs. 71.63 mo, p = 0.036] and OS [29.83 vs. 90.43 mo, p = 0.012]. Multivariate analysis indicated that the Hedgehog score is an independent biomarker of prognosis for OS in both the entire cohort [hazard ratio (HR): 1.564; 95% confidence interval (CI), 1.052–2.326; p = 0.027] and the ADC subcohort [HR: 2.399; 95% CI, 1.164–4.946; p = 0.018]. This score was validated in an independent cohort of NSCLC patients from The Cancer Genome Atlas (TCGA), which confirmed its prognostic value. Our findings provide relevant prognostic information for NSCLC patients and support future trials targeting Hh pathway.

Keywords: Lung cancer; Hedgehog pathway; Cancer Stem Cells; CSC targeting; Tumor treatment; SMO antagonist

INTRODUCTION

Lung cancer is the second most commonly diagnosed form of cancer, with more than 2.2 million new cases (11.4%) in the world in 2020, and the leading cause of cancer-related death, with 1.80 million deaths (18.0%) (1). Histologically, lung cancer patients are classified into non-small cell lung cancer (NSCLC), which represents the 85% of diagnosed patients and includes adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large-cell carcinoma (LCC) and small cell lung cancer (SCLC), which accounts for a 15% of all cases. There have been notable improvements in cancer diagnostics and therapeutics over the past decades (2,3), but many patients still develop treatment resistance, progress, and die (4,5). Surgery is still the standard of care for early-stage NSCLC patients with a good ECOG, but the recurrence rate ranges from 35 to 50% and, after an apparently successful surgical intervention, the development of secondary tumors frequently leads to the relapse of resected patients (6). This heightened rate of lung cancer related mortality highlights the importance of gaining a better understanding of this disease through extensive new researches.

The hedgehog (Hh) signaling pathway is an important component on the regulation of stem cells properties during the embryonic development and in adult tissues (7). In lung tissue, Hh signaling pathway seems to be inactive in all cells of the human adult lung epithelium except for the progenitor cells (8). The persistence of Hh signaling in the epithelial progenitor cells seems to facilitate these cells maintenance and play a decisive role in tissue response to injuries in the airway epithelia (9,10). However, mutations and deregulations of genes related to Hh pathway have been reported in several solid tumors, including lung cancer, which contribute to the onset of cancer and accelerate its growth (11). The first connection between aberrant Hh signaling and cancer was the discovery of a mutation in the transmembrane receptor *PTCH1* that causes a rare condition, named Gorlin syndrome (12). Gorlin syndrome patients suffer from various basal cell carcinomas (BCC) throughout their lifetimes and are predisposed towards other types of cancer. Additionally, increased Hh signaling has been reported in a third of all human medulloblastoma cases, frequently due to *PTCH1* and *SUFU* mutations (13,14). In all these cases, deregulated Hh signaling have been proven to increase cell proliferation and tumor formation, resulting in regulatory approvals of several SMO antagonists for tumor treatment. Unfortunately, the link between Hh pathway and lung cancer is less clear. Activation of Hh pathway has been

clearly reported on small cell lung cancer (SCLC) cell lines and tumors (15,16), but not in non-small cell lung cancer (NSCLC), although the blockade of Hh signaling increases sensitivity to EGFR-TKIs in NSCLC cell lines (17,18).

The objective of this study was to provide new insight into the role of Hh signaling pathway in NSCLC. Tumor and adjacent normal tissue biopsies were obtained from non-pretreated early-stage NSCLC patients at the time of surgery. We identified significant differences in the expression of core Hh components between samples (tumor and adjacent healthy) and patients and investigated their prognostic implications. A gene signature based on the four Hh components analyzed was established, constituting an independent prognostic biomarker for OS in NSCLC. The results obtained were further validated using an independent cohort of NSCLC patients from The Cancer Genome Atlas (TCGA).

MATERIALS AND METHODS

Patients and sample collection

This study included 245 patients from the General University Hospital of Valencia who underwent surgery between 2004 and 2017 and who fit the eligibility criteria: resected, non-pretreated stage I–IIIA patients (according to the American Joint Committee on Cancer staging manual) with a histological diagnosis of NSCLC. The study was conducted in accordance with the Declaration of Helsinki, and the institutional ethical review board approved the protocol. The most relevant demographic and clinicopathological characteristics of the cohort are shown in **Table 1**. Tumor and adjacent normal tissue specimens were obtained at the time of surgery and frozen at −80 °C in RNAlater® (Applied Biosystems, USA) to avoid degradation of RNA. Patients with post-surgical complications were excluded and only those patients who had at least 1 month of follow-up were included.

Gene expression analysis

RNA from frozen tissue samples was extracted using standard TRIZOL (Invitrogen, USA) method. Reverse transcription reactions were performed from 1.0 μg of total RNA using random hexanucleotides and a High-Capacity complementary DNA (cDNA) Reverse Transcription Kit (Applied Biosystems, USA) following the manufacturer's instructions. The thermal cycling conditions were as follows: 10 min at 25 °C, 120 min at 37 °C, and 5 s at 85 °C. The relative gene expression of *GLI1*, *PTCH1*, *SHH* and *SMO* was analyzed by RTqPCR using 1 μL of cDNA, TaqMan Gene Expression Master Mix (Applied Biosystems, USA) and the corresponding TaqMan Gene Expression Assay (Hs01110766_m1, Hs00181117_m1, Hs00179843_m1 and Hs01090242_m1, respectively) in a 5 μL final reaction volume. The RTqPCR was performed on a Roche LightCycler®480 II system (Roche Ltd., Basel, Switzerland) with the following thermal cycling parameters: 2 min at 50 °C and 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. For efficiency calculations, we used random-primed qPCR Human Reference cDNA (Clontech, USA). *ACTB*, *GUSB*, and *CDKN1B* were selected as endogenous controls using GeNorm software. Relative gene expression levels were expressed as the ratio of target gene expression to the geometric mean of the endogenous gene expressions according to Pfaffl formula (19). It was considered a gene to be overexpressed when the median of the relative gene expression of the pathological

area referred to the adjacent healthy tissue was higher than 2 and underexpressed when it was less than 0.5. Gene expression levels were dichotomized as “high” and “low” according to the median of each case.

Bioinformatic analysis

Expression levels of *GLI1*, *PTCH1*, *SHH* and *SMO* were evaluated in two lung cancer data sets from The Cancer Genome Atlas (TCGA) consortium (20,21). Clinical and RNA-sequencing (Illumina HiSeq platform) information was directly downloaded from the ICGC Data Portal (22), https://dcc.icgc.org/releases/current/projects/LUAD-US, and https://dcc.icgc.org/releases/current/projects/LUSC-US.

Statistical analyses

Continuous variables were compared by non-parametric Mann–Whitney U and Kruskal–Wallis tests. Survival analyses were performed using univariate Cox regression analysis and Kaplan–Meier (log-rank) test method with clinicopathological variables and dichotomized gene expression levels. Relapse-Free Survival (RFS) spans from surgery to relapse or exitus dates and and overall survival (OS) from surgery to exitus dates, following the Response Evaluation Criteria in Solid Tumors (RECIST). For patients who neither relapsed nor died, the last recorded follow-up was considered. To assess the independent value of the tested biomarkers, a Cox proportional hazard model for multivariate analyses was used. All significant variables from the univariate were entered into the multivariate analyses in a forward stepwise Cox regression analysis. Furthermore, we also calculated gene expression score based on multi-gene signature using a method previously reported (23,24). Univariate Cox regression analysis was used to select genes associated with mortality (Z-score >1.5), which were afterwards included in a multivariate risk model. All genes were included for these purposes, and expression values for all analyses were continuous variables. A probability of 95% ($p < 0.05$) was considered statistically significant for all analyses. Statistical analyses and boxplots were performed using the IBM® SPSS Statistics version 23.0 and R version 3.6.2.

RESULTS

Hedgehog pathway molecules are differentially expressed along resected NSCLC samples

The demographic and clinicopathological data of the 245 resected NSCLC patients included in this part of the study is available at **Table 1**. The median patient age was 65 years [range: 54-83], 82.4% were males, 46.5% had ADC, and 54.3% of them were diagnosed at stage I of the disease. During the follow-up (median 34.2 months), 101 patients relapsed (41.4%) and 117 died (48.0%).

Table 1. Clinicopathological characteristics of the patients included in the study.

Characteristics	**N (245)**	**%**
Age at surgery (median, range)	65 [26-85]	
Gender		
Male	202	82.4
Female	43	17.6
Stage		
I	133	54.3
II	70	28.6
IIIA	42	17.1
Histology		
SCC	111	45.3
ADC	114	46.5
Others	20	8.2
ECOG Performance Status		
0	154	62.9
1/2	91	37.1
Differentiation grade		
Poor	57	23.3
Moderate	96	39.2
Well	46	18.8
NS	46	18.8
Smoking habits		
Current	116	47.3
Former	101	41.2
Never	28	11.4

ADC, adenocarcinoma; SCC, Squamous Cell Carcinoma

We measured the expression of components of HH signaling pathway (**Figure 1A** and **1B**) in primary lung tumor and paired non-cancerous tissues (adjacent healthy lung tissue) using RTqPCR. We found that *SMO* (2.66X) and *GLI1* (1.52X) were overexpressed in the tumor compared with normal-paired tissue, whereas *PTCH1* (0.81X) and *SHH* (0.34X) were underexpressed (**Figure 2A**). Non-parametric tests were conducted to determine associations between the relative gene expressions and clinicopathological variables (**Supplementary Table S1**). The Mann-Whitney U test revealed that the expression of *PTCH1* and *SHH* was significantly higher in patients with ECOG 1/2 than in patients with ECOG 0 (**Figure 2B** and **2C**). In addition, the expression of *PTCH1* was significantly higher in patients with SCC histology than in patients with ADC (**Figure 2D**). Similarly, the expression of *GLI1* and *SMO* was significantly higher in patients with SCC histology than in patients with ADC or other histologies (**Figure 2E** and **2F**).

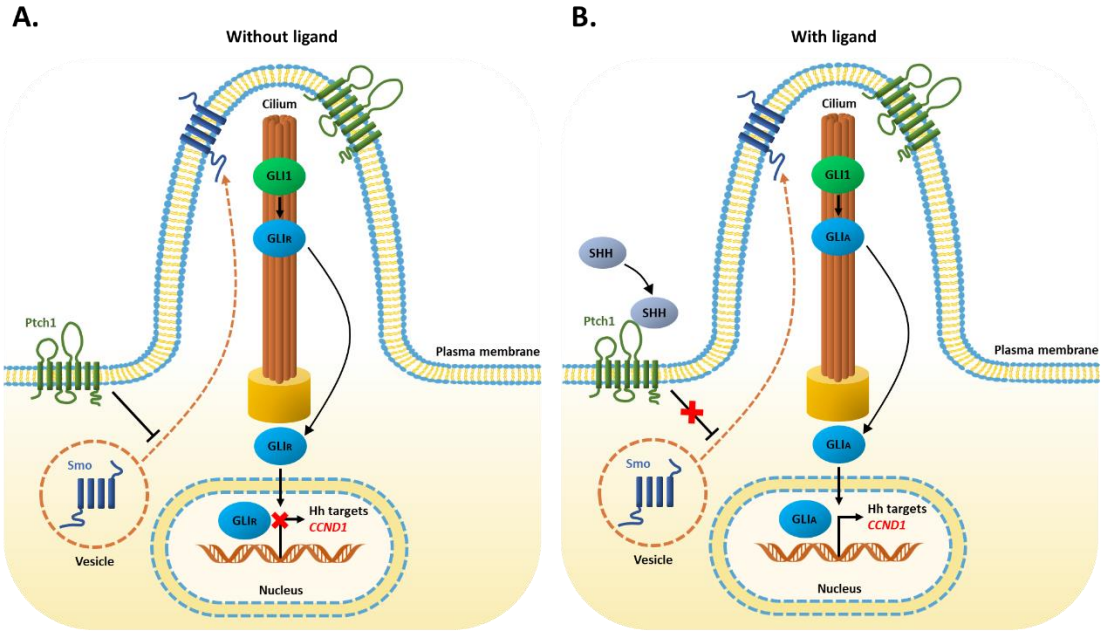

Figure 1. Hedgehog signaling pathway. In the absence of SHH, PTCH1 represses the activity of SMO, preventing its localization to the cell surface from intracellular endosomes. Under these circumstances, the transcription factor GLI1 is phosphorylated and prevented from transactivating Hedgehog targets (**A**). Upon binding of the ligand, PTCH1 is internalized, allowing the intracellular accumulation and activation of SMO, which in turn activates GLI1 to exert its effect in the nucleus (**B**).

Afterwards, survival data was used to associate components of HH pathway with NSCLC patients' prognosis. Cox regression and Kaplan–Meier analyses revealed that

patients with high *PTCH1* had longer RFS (44.50 vs. 88.23 months, p = 0.003, **Figure 2G**). A statistical trend toward better OS was also detected (49.63 vs. 95.40 months, p = 0.071). Additionally, survival analyses were applied according to patient histology, associating high *PTCH1* with better RFS and OS in ADC patients (42.90 vs. 81.23 months, p = 0.016, for RFS and 42.90 vs. 84.77 months, p = 0.022, for OS, respectively, **Figure 2H** and **2I**). No other significant associations were found between gene expression and survival (**Supplementary Table S2**).

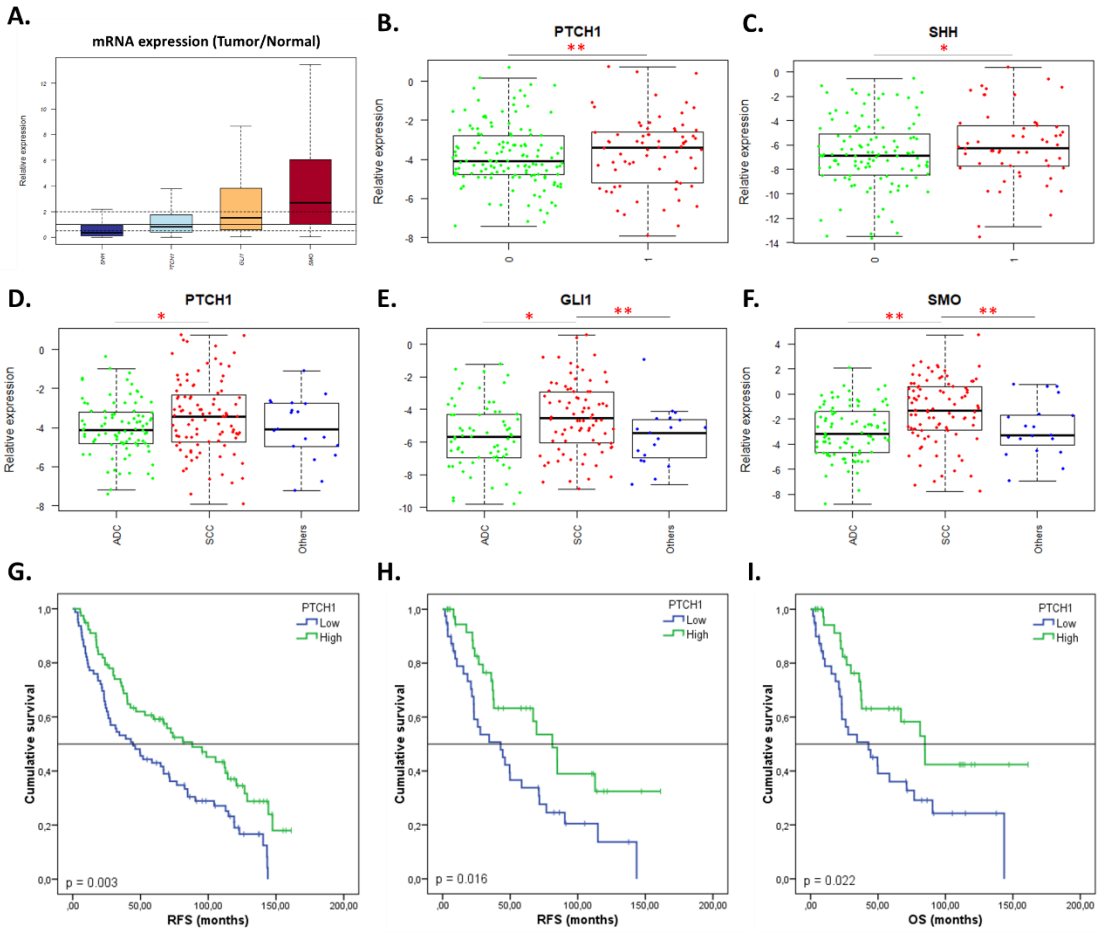

Figure 2. Expression of the components of HH signaling pathway in lung cancer. Ratio between the transcription levels of *SHH*, *PTCH1*, *GLI1* and *SMO* in lung cancer and adjacent normal tissues (**A**). Representation of *PTCH1* (**B**) and *SHH* (**C**) expressions according to ECOG Performance Status and *PTCH1* (**D**), *GLI1* (**E**) and *SMO* (**F**) expressions according to the tumor histology. Kaplan–Meier plots for RFS in the entire cohort (**G**) and for RFS and OS in the ADC subcohort (**H-I**) according to *PTCH1* expression.

Hedgehog Score is a prognostic biomarker for RFS and OS in NSCLC

Thereafter, we intended to create a gene expression score that can provide more accurate predictions for patients' prognostic (23,24). We constructed a model based on the relative contribution of HH pathway components in the multivariate analysis (considering absolute regression coefficients, see **Supplementary Table S3**), and the resulting score was named Hedgehog Score, with the following equation: (*PTCH1*x-0.170) + (*SHH*x0.013) + (*GLI1*x0.074) + (*SMO*x0.007). No associations between Hedgehog Score and clinicopathological variables were found (**Supplementary Table S4**). Kaplan–Meier analysis showed that patients with high Hedgehog Score (> median) had shorter RFS (39.13 vs. 81.23 months, p = 0.025; **Figure 3A**) and OS (44.50 vs. 95.40 months, p = 0.039; **Figure 3B**). We also performed stratified analyses by histology and found a similar association between high Hedgehog score and prognosis for ADC patients (RFS: 29.83 vs. 71.63 months, p = 0.036; **Figure 3C** and OS: 29.83 vs. 90.43 months, p = 0.012; **Figure 3D**). To evaluate the potential use of the Hedgehog Score as an independent prognostic biomarker, a multivariate analysis was performed including all the significant variables from the univariate analyses (age, tumor node metastasis (TNM) staging, ECOG, KRAS mutation, *PTCH1*, and the Hedgehog Score). Results obtained from this multivariate analysis indicated that ECOG and the Hedgehog Score in the entire cohort and age, KRAS mutation and the Hedgehog Score in the ADC cohort were independently associated with survival (see **Table 2**).

Table 2. Results from the multivariate Cox regression model for OS.

	Global cohort			**ADC subcohort**		
Variables	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Performance Status *1/2 vs. 0*	1.670	1.092-2.553	0.018*	-	-	-
Age *>65 vs. <65*	-	-	-	2.269	1.124-4.581	0.022*
KRAS mutation *Mutated vs. Wild Type*	-	-	-	2.206	1.007-4.834	0.048*
Hedgehog Score *High vs. low*	1.564	1.052-2.326	0.027*	2.399	1.164-4.946	0.018*

ADC, adenocarcinoma; HR, hazard ratio; CI, confidence interval

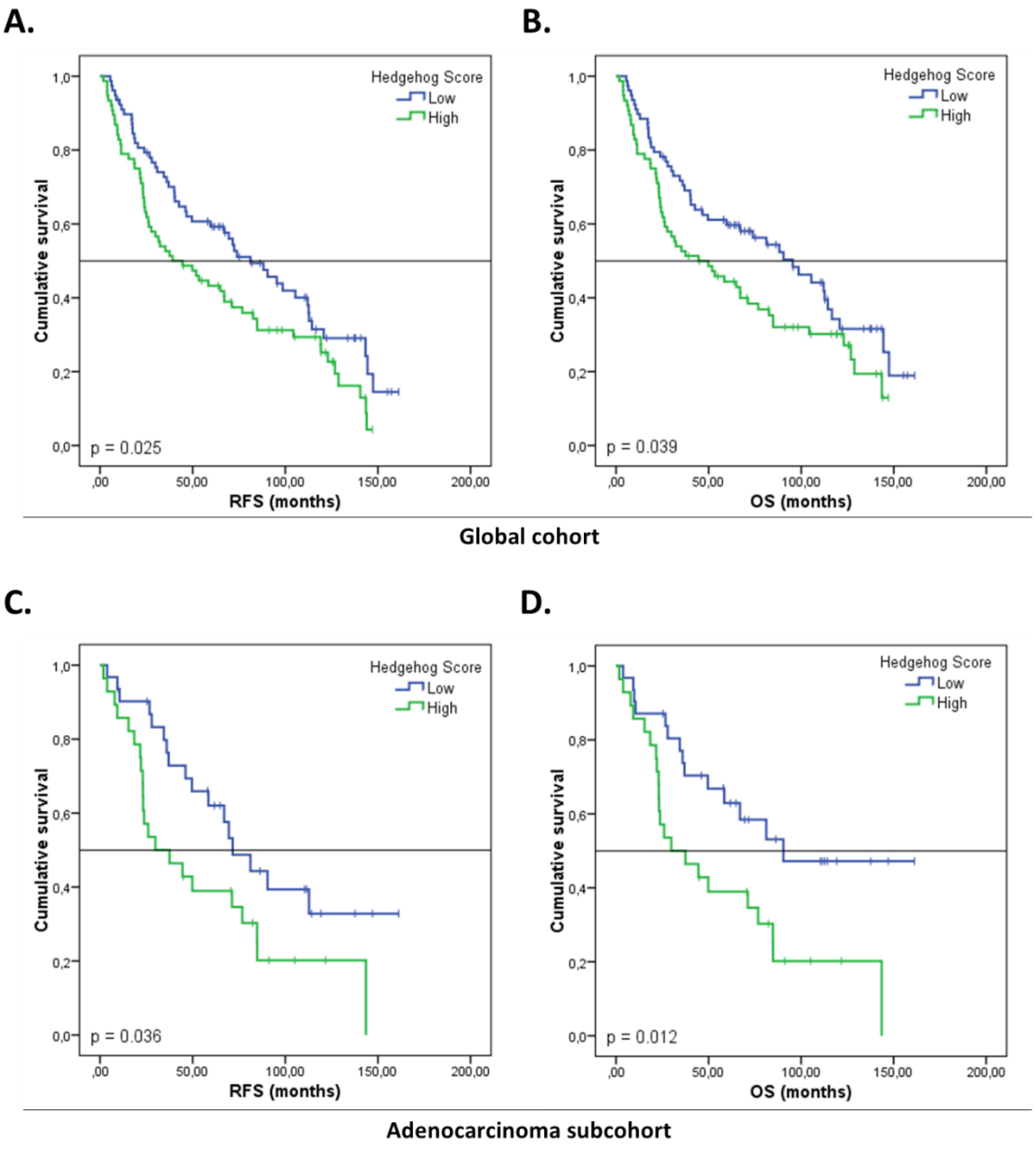

Figure 3. Prognostic value of the Hedgehog Score. Kaplan–Meier plots for RFS and OS according to the CSC score in the entire cohort (**A-B**) and the adenocarcinoma subcohort (**C-D**).

An independent cohort of NSCLC patients from TCGA was then used for the validation of the Hedgehog Score. Clinicopathological characteristics of these patients are summarized in **Supplementary Table S5**. Cox regression and Kaplan-Meier analyses of individual genes indicated that NSCLC patients with high expression of *PTCH1* have better RFS (**Supplementary Table S6**). In addition, ADC patients with high expression of *PTCH1* exhibited longer OS as well. Similarly, the association

between high Hedgehog Score and worse RFS and OS was confirmed in both the NSCLC cohort and the ADC subcohort (**Figure 4**).

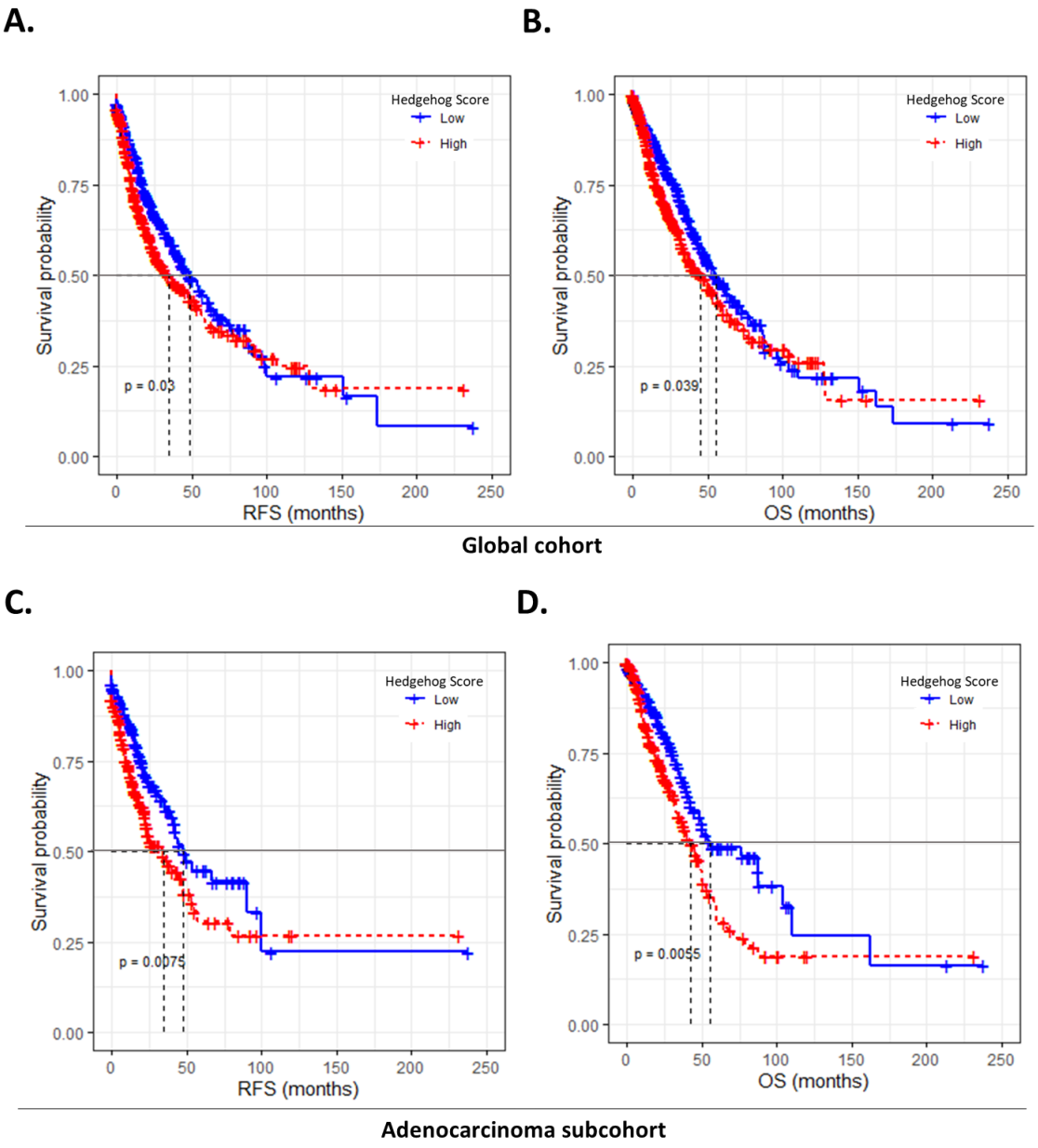

Figure 4. Prognostic value of the Hedgehog Score. Kaplan–Meier plots for RFS and OS according to the CSC score in the entire cohort (**A-B**) and the adenocarcinoma subcohort (**C-D**) from TCGA.

DISCUSSION

The management of NSCLC has evolved substantially over the last 15 years. Specific anti-target therapies have emerged, including inhibitors of EGFR (gefitinib, erlotinib, afatinib, dacomitinib and osimertinib) (25–27), ALK and ROS1 (crizotinib, lorlatinib, ceritinib, brigatinib and entrectinib) (28,29), and BRAF and MEK (dabrafenib and trametinib) (30), which have increased patients' survival and decreased the toxicity produced by conventional chemotherapy. Additionally, cancer immunotherapy has set a new standard in the treatment of NSCLC with the approvals of monoclonal antibodies that block the immune checkpoint molecule programmed cell death 1 (PD1) (pembrolizumab and nivolumab) and its ligand (PD-L1) (atezolizumab) (31). In spite of all these advances, lung cancer remains as the leading cause of cancer-related death in the world due to treatment resistance (1).

There is strong evidence pointing out that treatment resistance is highly associated to populations of tumor cells with stem-like properties, named cancer stem-like cells (CSCs), which are able to survive using different mechanisms, including self-renewal, asymmetric division capacity, aberrant regulation of cell cycling, and enhanced tumorigenic activity (32). These characteristics are a direct result of the expression of signaling pathways which are essential for stem cell populations (Herreros-Pomares 2022). Among these pathways, Hh signaling constitutes an important component on the regulation of stem cells properties. Indeed, considerable progress has been achieved in clinical trials targeting Hh pathway, especially for the treatment of basal cell carcinoma (BCC) and acute myeloid leukemia (AML), for which SMO antagonists (vismodegib, sonidegib and glasdegib) have received regulatory approvals (33–35). Unfortunately, the role of Hh pathway in lung cancer remains elusive (36).

Thus, we evaluated the expression of the main components of Hh signaling in tumor and adjacent normal biopsies from NSCLC patients. *SMO* and *GLI1* were found overexpressed in tumor tissue, whereas the expression of *PTCH1* and *SHH* was higher in the adjacent normal tissue. Overexpression of *SMO* and *GLI1* has been previously reported in tumor tissues from breast and pancreatic cancer, being associated with tumor size, lymph node metastasis and postoperative recurrence (37–39). In contrast, loss of the tumor suppressor *PTCH1* has been reported in some tumors, including BBC (40), medulloblastoma (41) colorectal (42), and breast (43) cancers. In NSCLC, disparate results have been published. An immunohistochemical analysis of 81 NSCLC samples

reported negative to weak expression of Shh, Gli-1, SMO and Ptch-1 compared with normal lung epithelial cells (44). An opposite observation was reported in another study including 80 NSCLC cases which concluded that all the HH-signaling molecules examined were overexpressed in tumor samples compared with the adjacent non-neoplastic lung parenchyma (45). The reason behind these contrasting results remains unknown, but clinical and pathological features, such as the smoking habit, have been linked to the activation of the pathway (46). Therefore, we evaluated the associations between the relative gene expressions and the clinicopathological variables of patients. We found that those with worse ECOG (1/2) had higher expression of *PTCH1* and *SHH* and that the expression of *PTCH1*, *GLI1* and *SMO* was higher in SCC than in ADC and other histologies. Again, results from previous studies range from those that find no correlations (47) to those that associated high levels of Hh components with SCC histology (PTCH1 and SMO), tumor grade (PTCH1), node metastasis (SMO) and visceral pleural invasion (Shh) (45,48).

In parallel, several studies have tried to evaluate if Hh components are associated with lung cancer patients' survival (44,47–50). In a study including 248 early-stage NSCLC, no significant association were found between RFS or OS and any of the Hh components analyzed by immunohistochemistry (IHC) (47). Similar results were found by Savani and colleagues, who analyzed the expression of Gli1, Shh, Smo and Ptch1 in a tissue microarray including 42 NSCLC patients (44). In contrast with these results, two independent studies reported that the expression of Shh was significantly associated with shorter OS (48,49), whereas the study conducted by Kim and colleagues concluded that the high expression of SHH and GLI-1 was related to better progression-free survival (PFS) and OS. In our study, only the expression of *PTCH1* was found associated to better prognosis. In consonance with this finding, the loss of *PTCH1* was previously linked to poor survival in SCC (51). Unfortunately, these studies focus on single genes with limited prognostic value. Finding gene expression signatures that identify altered pathways in carcinogenesis could lead to the discovery of molecular subclasses and predict patients' outcomes better (52,53). We created a score combining the expression of Hh components, which was an independent prognostic biomarker for resectable NSCLC patients. To validate it, the expression of these genes was evaluated in an independent cohort of lung ADC and SCC patients from TCGA, finding that patients with elevated Hedgehog score had shorter RFS and OS. These

results are of great importance because current clinicopathological staging methods have limited success in predicting patient survival and today we still cannot predict which patients will be cured, and which ones will relapse after surgery. Gene expression scores based on RTqPCR have demonstrated being useful for classifying tumors and predicting prognosis, being even approved as prognostic tools in clinical practice (54). This technology is a well-implemented methodology in our group for biomarkers' research, previously reporting CSC, angiogenesis and immune checkpoint scores for NSCLC (24,55,56). The Hedgehog Score proposed can help in future clinical practice, since high scores may reflect an activation of the Hh signaling pathway that may indicate which patients should be closely followed after a successful surgery because they have a higher risk to relapse and die and that could potentially benefit from Hh pathway inhibitors. The development of targeted therapies against this signaling pathway might be essential to prevent relapse of patients and improve their future outcome.

CONCLUSIONS

Treatment resistance makes lung cancer a global health challenge that needs to be addressed. Our results indicate that the activation of Hh signaling, a potential mechanism of treatment resistance, is associated to worse outcome in NSCLC, representing an independent prognostic biomarker for patients' survival. Thus, the clinical implementation of the Hh score could help in distinguishing which patients have more risk to relapse and die. Future clinical trials should be carried out trying to determine the safety and efficacy of the new therapeutic strategies against Hh components, since they could have major implications in NSCLC patients' survival.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021 May;71(3):209–49.

2. Hirsch FR, Suda K, Wiens J, Bunn PAJ. New and emerging targeted treatments in advanced non-small-cell lung cancer. Lancet. 2016 Sep;388(10048):1012–24.

3. Rizvi NA, Peters S. Immunotherapy for Unresectable Stage III Non-Small-Cell Lung Cancer. Vol. 377, The New England journal of medicine. United States, United States; 2017. p. 1986–8.

4. Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. Cell. 2017 Feb;168(4):707–23.

5. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. Nature. 2018 Jan;553(7689):446–54.

6. Raman V, Yang C-FJ, Deng JZ, D'Amico TA. Surgical treatment for early stage non-small cell lung cancer. J Thorac Dis. 2018 Apr;10(Suppl 7):S898–904.

7. Clara JA, Monge C, Yang Y, Takebe N. Targeting signalling pathways and the immune microenvironment of cancer stem cells - a clinical update. Nat Rev Clin Oncol. 2020 Apr;17(4):204–32.

8. Velcheti V, Govindan R. Hedgehog signaling pathway and lung cancer. J Thorac Oncol [Internet]. 2007 Jan [cited 2016 Feb 4];2(1):7–10. Available from: http://www.sciencedirect.com/science/article/pii/S1556086415300101

9. Peng T, Frank DB, Kadzik RS, Morley MP, Komal S, Wang T, et al. Hedgehog actively maintains adult lung quiescence and regulates repair and regeneration. Nature. 2015;526(7574):578–82.

10. Metcalfe C, Siebel CW. The Hedgehog Hold on Homeostasis. Cell Stem Cell [Internet]. 2015 Nov 5;17(5):505–6. Available from: http://www.sciencedirect.com/science/article/pii/S1934590915004683

11. Gonnissen A, Isebaert S, Haustermans K. Targeting the Hedgehog signaling pathway in cancer: beyond Smoothened. Oncotarget [Internet]. 2015;6(16):13899–913. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4546439&tool=pmcentrez&rendertype=abstract

12. Hahn H, Wicking C, Zaphiropoulos PG, Gailani MR, Shanley S, Chidambaram A, et al. Mutations of the Human Homolog of Drosophila patched in the Nevoid Basal Cell Carcinoma Syndrome. Cell [Internet]. 1996 Jun [cited 2016 Jan 12];85(6):841–51. Available from: http://www.sciencedirect.com/science/article/pii/S0092867400812684

13. Thalakoti S, Geller T. Basal cell nevus syndrome or Gorlin syndrome. Handb Clin Neurol. 2015;132:119–28.

14. Shanley S, McCormack C. Diagnosis and Management of Hereditary Basal Cell Skin Cancer. Recent results cancer Res Fortschritte der Krebsforsch Prog dans

les Rech sur le cancer. 2016;205:191–212.

15. Park K-S, Martelotto LG, Peifer M, Sos ML, Karnezis AN, Mahjoub MR, et al. A crucial requirement for Hedgehog signaling in small cell lung cancer. Nat Med. 2011;17(11):1504–8.

16. Kaur G, Reinhart RA, Monks A, Evans D, Morris J, Polley E, et al. Bromodomain and hedgehog pathway targets in small cell lung cancer. Cancer Lett [Internet]. 2016 Feb;371(2):225–39. Available from: http://dx.doi.org/10.1016/j.canlet.2015.12.001

17. Giroux Leprieur E, Antoine M, Vieira T, Rozensztajn N, Ruppert A-M, Rabbe N, et al. [Role of the Sonic Hedgehog pathway in thoracic cancers]. Rev Mal Respir. 2015 Oct;32(8):800–8.

18. Bai X-Y, Zhang X-C, Yang S-Q, An S-J, Chen Z-H, Su J, et al. Blockade of Hedgehog Signaling Synergistically Increases Sensitivity to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors in Non-Small-Cell Lung Cancer Cell Lines. PLoS One. 2016;11(3):e0149370.

19. Pfaffl MW, Duquenne M, François JM, Parrou J-L, Francois J, Gancedo C, et al. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res [Internet]. 2001 May 1 [cited 2017 Jun 7];29(9):45e – 45. Available from: https://academic.oup.com/nar/article-lookup/doi/10.1093/nar/29.9.e45

20. Hammerman PS, Lawrence MS, Voet D, Jing R, Cibulskis K, Sivachenko A, Stojanov P, McKenna A, Lander ES, Gabriel S, Getz G, Sougnez C, Imielinski M, Helman E, Hernandez B, Pho NH, Meyerson M, Chu A, Chun HJ, Mungall AJ, Pleasance E, Robertson A, Sipahimala TE, Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. Nature [Internet]. 2012 Sep 27 [cited 2014 Jul 11];489(7417):519–25. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3466113&tool=pmcentrez&rendertype=abstract

21. Cancer Genome Atlas Research Network, Collisson EA, Campbell JD, Brooks AN, Berger AH, Lee W, et al. Comprehensive molecular profiling of lung adenocarcinoma. Nature [Internet]. 2014 Jul 9 [cited 2014 Jul 9];511(7511):543–50. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4231481&tool=pmcentrez&rendertype=abstract

22. Zhang J, Baran J, Cros A, Guberman JM, Haider S, Hsu J, et al. International Cancer Genome Consortium Data Portal--a one-stop shop for cancer genomics data. Database (Oxford). 2011;2011:bar026.

23. Lossos IS, Czerwinski DK, Alizadeh AA, Wechser MA, Tibshirani R, Botstein D, et al. Prediction of Survival in Diffuse Large-B-Cell Lymphoma Based on the Expression of Six Genes. n engl j med. 2004;35018350(29):1828–37.

24. Herreros-Pomares A, De-Maya-Girones JD, Calabuig-Fariñas S, Lucas R, Martínez A, Pardo-Sánchez JM, et al. Lung tumorspheres reveal cancer stem cell-like properties and a score with prognostic impact in resected non-small-cell lung

461 cancer. Cell Death Dis [Internet]. 2019 Sep 10 [cited 2019 Sep 28];10(9):660.
462 Available from: http://www.ncbi.nlm.nih.gov/pubmed/31506430

463 25. Thress KS, Paweletz CP, Felip E, Cho BC, Stetson D, Dougherty B, et al.
464 Acquired EGFR C797S mutation mediates resistance to AZD9291 in non-small
465 cell lung cancer harboring EGFR T790M. Nat Med. 2015 Jun;21(6):560–2.

466 26. Planchard D, Loriot Y, Andre F, Gobert A, Auger N, Lacroix L, et al. EGFR-
467 independent mechanisms of acquired resistance to AZD9291 in EGFR T790M-
468 positive NSCLC patients. Ann Oncol Off J Eur Soc Med Oncol. 2015
469 Oct;26(10):2073–8.

470 27. Ichihara E, Westover D, Meador CB, Yan Y, Bauer JA, Lu P, et al. SFK/FAK
471 Signaling Attenuates Osimertinib Efficacy in Both Drug-Sensitive and Drug-
472 Resistant Models of EGFR-Mutant Lung Cancer. Cancer Res. 2017
473 Jun;77(11):2990–3000.

474 28. Lim SM, Kim HR, Lee J-S, Lee KH, Lee Y-G, Min YJ, et al. Open-Label,
475 Multicenter, Phase II Study of Ceritinib in Patients With Non-Small-Cell Lung
476 Cancer Harboring ROS1 Rearrangement. J Clin Oncol. 2017 Aug;35(23):2613–8.

477 29. Drilon A, Siena S, Ou S-HI, Patel M, Ahn MJ, Lee J, et al. Safety and Antitumor
478 Activity of the Multitargeted Pan-TRK, ROS1, and ALK Inhibitor Entrectinib:
479 Combined Results from Two Phase I Trials (ALKA-372-001 and STARTRK-1).
480 Cancer Discov. 2017 Apr;7(4):400–9.

481 30. Yu HA, Planchard D, Lovly CM. Sequencing Therapy for Genetically Defined
482 Subgroups of Non-Small Cell Lung Cancer. Am Soc Clin Oncol Educ book Am
483 Soc Clin Oncol Annu Meet. 2018 May;38:726–39.

484 31. Raju S, Joseph R, Sehgal S. Review of checkpoint immunotherapy for the
485 management of non-small cell lung cancer. ImmunoTargets Ther. 2018;7:63–75.

486 32. Hanahan D. Hallmarks of Cancer: New Dimensions. Cancer Discov [Internet].
487 2022 Jan 1;12(1):31 LP – 46. Available from:
488 http://cancerdiscovery.aacrjournals.org/content/12/1/31.abstract

489 33. Sekulic A, Migden MR, Oro AE, Dirix L, Lewis KD, Hainsworth JD, et al.
490 Efficacy and Safety of Vismodegib in Advanced Basal-Cell Carcinoma. N Engl J
491 Med. 2012;366(23):2171–9.

492 34. Basset-Séguin N, Hauschild A, Kunstfeld R, Grob J, Dréno B, Mortier L, et al.
493 Vismodegib in patients with advanced basal cell carcinoma: Primary analysis of
494 STEVIE, an international, open-label trial. Eur J Cancer. 2017 Nov;86:334–48.

495 35. Lear JT, Migden MR, Lewis KD, Chang ALS, Guminski A, Gutzmer R, et al.
496 Long-term efficacy and safety of sonidegib in patients with locally advanced and
497 metastatic basal cell carcinoma: 30-month analysis of the randomized phase 2
498 BOLT study. J Eur Acad Dermatol Venereol. 2018 Mar;32(3):372–81.

499 36. Pietanza MC, Litvak AM, Varghese AM, Krug LM, Fleisher M, Teitcher JB, et
500 al. A phase I trial of the Hedgehog inhibitor, sonidegib (LDE225), in
501 combination with etoposide and cisplatin for the initial treatment of extensive
502 stage small cell lung cancer. Lung Cancer. 2016 Sep;99:23–30.

503 37. Jeng K-S, Sheen I-S, Jeng W-J, Yu M-C, Hsiau H-I, Chang F-Y. High expression

of Sonic Hedgehog signaling pathway genes indicates a risk of recurrence of breast carcinoma. Onco Targets Ther. 2013;7:79–86.

38. Walter K, Omura N, Hong S-M, Griffith M, Vincent A, Borges M, et al. Overexpression of smoothened activates the sonic hedgehog signaling pathway in pancreatic cancer-associated fibroblasts. Clin cancer Res an Off J Am Assoc Cancer Res. 2010 Mar;16(6):1781–9.

39. Tao Y, Mao J, Zhang Q, Li L. Overexpression of Hedgehog signaling molecules and its involvement in triple-negative breast cancer. Oncol Lett. 2011 Sep;2(5):995–1001.

40. Campione E, Di Prete M, Lozzi F, Lanna C, Spallone G, Mazzeo M, et al. High-Risk Recurrence Basal Cell Carcinoma: Focus on Hedgehog Pathway Inhibitors and Review of the Literature. Chemotherapy [Internet]. 2020;65(1–2):2–10. Available from: https://www.karger.com/DOI/10.1159/000509156

41. Archer TC, Weeraratne SD, Pomeroy SL. Hedgehog-GLI pathway in medulloblastoma. J Clin Oncol Off J Am Soc Clin Oncol. 2012 Jun;30(17):2154–6.

42. Chung JH, Bunz F. A loss-of-function mutation in PTCH1 suggests a role for autocrine hedgehog signaling in colorectal tumorigenesis. Oncotarget. 2013 Dec;4(12):2208–11.

43. Wang C-Y, Chang Y-C, Kuo Y-L, Lee K-T, Chen P-S, Cheung CHA, et al. Mutation of the PTCH1 gene predicts recurrence of breast cancer. Sci Rep [Internet]. 2019;9(1):16359. Available from: https://doi.org/10.1038/s41598-019-52617-4

44. Savani M, Guo Y, Carbone DP, Csiki I. Sonic hedgehog pathway expression in non-small cell lung cancer. Ther Adv Med Oncol. 2012 Sep;4(5):225–33.

45. Gialmanidis IP, Bravou V, Amanetopoulou SG, Varakis J, Kourea H, Papadaki H. Overexpression of hedgehog pathway molecules and FOXM1 in non-small cell lung carcinomas. Lung Cancer. 2009 Oct;66(1):64–74.

46. Lemjabbar-Alaoui H, Dasari V, Sidhu SS, Mengistab A, Finkbeiner W, Gallup M, et al. Wnt and Hedgehog Are Critical Mediators of Cigarette Smoke-Induced Lung Cancer. Heisenberg C-P, editor. PLoS One [Internet]. 2006 Dec 20;1(1):e93. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1762353/

47. Raz G, Allen KE, Kingsley C, Cherni I, Arora S, Watanabe A, et al. Hedgehog signaling pathway molecules and ALDH1A1 expression in early-stage non-small cell lung cancer. Lung Cancer. 2012 May;76(2):191–6.

48. Huang L, Walter V, Hayes DN, Onaitis M. Hedgehog-GLI signaling inhibition suppresses tumor growth in squamous lung cancer. Clin cancer Res an Off J Am Assoc Cancer Res. 2014 Mar;20(6):1566–75.

49. Jiang WG, Ye L, Ruge F, Sun P-H, Sanders AJ, Ji K, et al. Expression of Sonic Hedgehog (SHH) in human lung cancer and the impact of YangZheng XiaoJi on SHH-mediated biological function of lung cancer cells and tumor growth. Anticancer Res. 2015 Mar;35(3):1321–31.

50. Kim JE, Kim H, Choe J-Y, Sun P, Jheon S, Chung J-H. High Expression of Sonic Hedgehog Signaling Proteins Is Related to the Favorable Outcome, EGFR Mutation, and Lepidic Predominant Subtype in Primary Lung Adenocarcinoma. Ann Surg Oncol [Internet]. 2013;20(3):570–6. Available from: https://doi.org/10.1245/s10434-013-3022-6

51. Zhao Y, Li Y, Lu H, Chen J, Zhang Z, Zhu Z-Z. Association of copy number loss of CDKN2B and PTCH1 with poor overall survival in patients with pulmonary squamous cell carcinoma. Clin Lung Cancer. 2011 Sep;12(5):328–34.

52. Huang E, Ishida S, Pittman J, Dressman H, Bild A, Kloos M, et al. Gene expression phenotypic models that predict the activity of oncogenic pathways. Nat Genet. 2003 Jun;34(2):226–30.

53. Raponi M, Zhang Y, Yu J, Chen G, Lee G, Taylor JMG, et al. Gene expression signatures for predicting prognosis of squamous cell and adenocarcinomas of the lung. Cancer Res. 2006 Aug;66(15):7466–72.

54. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A Multigene Assay to Predict Recurrence of Tamoxifen-Treated, Node-Negative Breast Cancer. N.Engl.J.Med. p.

55. Sanmartín E, Sirera R, Usó M, Blasco A, Gallach S, Figueroa S, et al. A Gene Signature Combining the Tissue Expression of Three Angiogenic Factors is a Prognostic Marker in Early-stage Non-small Cell Lung Cancer. Ann Surg Oncol [Internet]. 2014;21(2):612–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24145997

56. Usó M, Jantus-Lewintre E, Calabuig-Fariñas S, Blasco A, García del Olmo E, Guijarro R, et al. Analysis of the prognostic role of an immune checkpoint score in resected non-small cell lung cancer patients. Oncoimmunology. 2017;6(1):e1260214.

Supplementary Table S1. Results from the non-parametric tests to determine associations between the relative gene expression of *PTCH1*, *SHH*, *GLI1* and *SMO* and clinicopathological variables.

	PTCH1		***SHH***		***GLI1***		***SMO***	
	Mean ± SD	*p-value*	**Mean ± SD**	*p-value*	**Mean ± SD**	*p-value*	**Mean ± SD**	*p-value*
Gender								
Male	1.49 ± 2.07	0.777	2.35 ± 7.80	0.871	4.14 ± 7.52	0.679	5.53 ± 7.32	0.167
Female	1.34 ± 1.99		2.05 ± 3.03		3.35 ± 6.95		3.11 ± 5.31	
Age								
<65	1.40 ± 1.94	0.646	1.33 ± 3.44	0.083	4.36 ± 8.05	0.550	5.62 ± 8.24	0.436
>65	1.56 ± 2.20		3.51 ± 10.15		3.59 ± 6.55		4.67 ± 5.31	
Smoking habit								
Never	1.28 ± 1.10	0.630 (1v2)	2.53 ± 3.38	0.945 (1v2)	4.30 ± 7.69	0.947 (1v2)	3.70 ± 6.02	0.474 (1v2)
Former	1.62 ± 2.68	0.540 (2v3)	2.70 ± 9.41	0.608 (2v3)	4.13 ± 9.26	0.872 (2v3)	5.03 ± 6.41	0.639 (2v3)
Current	1.39 ± 1.64	0.813 (1v3)	1.98 ± 6.21	0.738 (1v3)	3.91 ± 5.87	0.826 (1v3)	5.65 ± 7.81	0.364 (1v3)
Performance Status								
0	1.15 ± 1.36	**0.006**	1.29 ± 2.66	**0.019**	3.35 ± 5.60	0.124	4.79 ± 7.33	0.305
1-2	2.16 ± 2.95		4.42 ± 12.09		5.43 ± 9.65		6.11 ± 6.60	
Histology								
SCC	1.91 ± 2.55	**0.011 (1v2)**	2.42 ± 8.39	0.128 (1v2)	5.58 ± 9.34	**0.039 (1v2)**	7.51 ± 8.85	**0.001 (1v2)**
ADC	0.98 ± 1.35	0.367 (2v3)	1.48 ± 2.36	0.331(2v3)	2.79 ± 5.06	0.422 (2v3)	3.24 ± 4.23	0.347 (2v3)
Others	1.32 ± 1.34	0.359 (1v3)	4.30 ± 11.52	0.447 (1v3)	1.74 ± 1.96	**0.003 (1v3)**	2.24 ± 1.89	**<0.001 (1v3)**
Differentiation grade								
Well	1.29 ± 2.22	0.847 (1v2)	1.69 ± 3.10	0.629 (1v2)	4.71 ± 10.20	0.869 (1v2)	5.12 ± 7.80	0.866 (1v2)
Moderate	1.37 ± 1.50	0.497 (2v3)	1.37 ± 2.78	0.147 (2v3)	4.41 ± 6.38	0.179 (2v3)	5.38 ± 6.23	0.343 (2v3)
Poor	1.67 ± 2.74	0.540 (1v3)	4.54 ± 13.00	0.201 (1v3)	2.68 ± 5.45	0.296 (1v3)	4.21 ± 5.21	0.567 (1v3)
Tumor size								
T1a/b	2.00 ± 2.59	0.135 (1v2)	2.61 ± 5.66	0.859 (1v2)	4.67 ± 9.65	0.520 (1v2)	5.88 ± 6.98	0.609 (1v2)
T2a/b	1.31 ± 2.04	0.952 (2v3)	2.32 ± 8.33	0.779 (2v3)	3.61 ± 6.99	0.502 (2v3)	5.07 ± 7.86	0.892 (2v3)
T3	1.29 ± 0.95	0.157 (1v3)	1.80 ± 5.34	0.598 (1v3)	4.68 ± 5.52	0.998 (1v3)	4.84 ± 3.98	0.517 (1v3)
LN involvement								
No	1.58 ± 2.26	0.346	2.84 ± 8.52	0.190	4.12 ± 7.91	0.834	5.55 ± 7.96	0.399
Yes	1.22 ± 1.47		1.03 ± 2.48		3.83 ± 6.23		4.44 ± 4.64	
Stage								
I	1.66 ± 2.48	0.779 (1v2)	3.20 ± 9.57	0.442 (1v2)	3.95 ± 8.57	0.674 (1v2)	5.54 ± 8.33	0.764 (1v2)

II	1.53 ± 1.77	0.099 (2v3)	1.93 ± 4.60	0.128 (2v3)	4.61 ± 6.06	0.457 (2v3)	6.01 ± 6.89	0.061 (2v3)
IIIA	0.97 ± 0.97	0.051 (1v3)	0.68 ± 1.65	0.163 (1v3)	3.48 ± 6.15	0.792 (1v3)	3.46 ± 3.11	0.071 (1v3)
Relapse								
No	1.43 ± 1.65	0.850	1.49 ± 2.79	0.230	3.29 ± 5.05	0.279	4.49 ± 4.82	0.273
Yes	1.50 ± 2.33		3.01 ± 9.62		4.67 ± 8.96		5.81 ± 8.55	
Exitus								
No	1.46 ± 2.23	0.963	1.37 ± 2.98	0.270	3.74 ± 8.55	0.738	4.78 ± 6.14	0.601
Yes	1.47 ± 1.97		2.82 ± 8.80		4.19 ± 6.79		5.44 ± 7.59	

ADC, adenocarcinoma; LN, Lymph nodes; SCC, Squamous Cell Carcinoma; SD, Standard Desviation

Supplementary Table S2. Results from survival analyses based on HH pathway components for the global cohort and the ADC and SCC subcohorts.

	RFS			**OS**		
Gene	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Global cohort						
GLI1	0.927	0.640-1.341	0.687	1.021	0.694-1.503	0.916
PTCH1	0.575	0.395-0.839	**0.004***	0.699	0.473-1.033	0.072
SHH	0.808	0.555-1.175	0.264	0.896	0.607-1.322	0.580
SMO	0.906	0.627-1.310	0.601	0.957	0.651-1.407	0.824
Adenocarcinoma subcohort						
GLI1	0.784	0.420-1.463	0.444	0.933	0.489-1.782	0.834
PTCH1	0.495	0.275-0.889	**0.019***	0.491	0.264-0.913	**0.025***
SHH	1.103	0.588-2.071	0.759	1.158	0.601-2.231	0.662
SMO	0.934	0.525-1.661	0.817	1.019	0.554-1.875	0.952
Squamous cell carcinoma subcohort						
GLI1	0.727	0.435-1.217	0.225	0.754	0.44-1.291	0.304
PTCH1	0.784	0.477-1.290	0.338	0.933	0.553-1.573	0.795
SHH	0.600	0.358-1.008	0.054	0.699	0.409-1.196	0.191
SMO	0.739	0.451-1.209	0.229	0.769	0.461-1.285	0.317

Supplementary Table S3. Results from the multivariate model for OS with genes included in the expression score.

Variable	**Regression coefficient**	**SE**	***p*-value**	**HR**	**95% CI**
PTCH1	-0.170	0.108	0.116	0.844	0.683-1.043
SHH	0.013	0.049	0.795	1.013	0.921-1.114
GLI1	0.074	0.096	0.438	1.030	0.893-1.300

SMO	0.007	0.070	0.916	1.007	0.877-1.157

Supplementary Table S4. Results from the non-parametric tests to determine associations between the relative gene expression of Hedgehog Score and clinicopathological variables.

Hedgehog Score		
	Mean ± SD	*p-value*
Gender		
Male	0.14 ± 0.15	0.732
Female	0.16 ± 0.18	
Age		
<65	0.16 ± 0.18	0.918
>65	0.15 ± 0.17	
Smoking habit		
Never	0.11 ± 0.11	0.305 (1v2)
Former	0.17 ± 0.19	0.574 (2v3)
Current	0.15 ± 0.17	0.432 (1v3)
Performance Status		
0	0.15 ± 0.16	0.898
1-2	0.16 ± 0.21	
Histology		
SCC	0.14 ± 0.19	0.295 (1v2)
ADC	0.17 ± 0.16	0.322 (2v3)
Others	0.13 ± 0.15	0.848 (1v3)
Differentiation grade		
Well	0.13 ± 0.15	0.491 (1v2)
Moderate	0.15 ± 0.17	0.526 (2v3)
Poor	0.18 ± 0.19	0.255 (1v3)
Tumor size		
T1a/b	0.10 ± 0.19	0.053 (1v2)
T2a/b	0.17 ± 0.17	0.830 (2v3)
T3	0.18 ± 0.16	0.096 (1v3)
LN involvement		

No	0.16 ± 0.17	0.444
Yes	0.14 ± 0.18	
Stage		
I	0.13 ± 0.16	0.124 (1v2)
II	0.19 ± 0.20	0.432 (2v3)
IIIA	0.15 ± 0.16	0.604 (1v3)
Relapse		
No	0.15 ± 0.17	0.724
Yes	0.16 ± 0.18	
Exitus		
No	0.12 ± 0.17	0.130
Yes	0.17 ± 0.17	

Supplementary Table S5. Clinicopathological characteristics of the TCGA patients included in the study.

Characteristics	N (860)	%
Age at surgery (median, range)	66 [33-90]	
Gender		
Male	343	39.9
Female	517	60.1
TNM staging		
Stage I	440	51.2
Stage II	233	27.1
Stage III	146	16.9
Stage IV	29	3.4
Not specify	12	1.4
Histology		
ADC	445	51.7
SCC	415	48.3
Smoking status		
Never	83	9.7
Current	218	25.3
Former	540	62.8
Not specify	19	2.2
Relapse		
No	526	61.2
Yes	225	26.2
Not specify	109	12.7
Exitus		
No	532	61.9
Yes	328	38.1

Supplementary Table S6. Results from survival analyses based on HH pathway components for TCGA patients.

	RFS			OS		
Gene	**HR**	**95% CI**	***p-value***	**HR**	**95% CI**	***p-value***
Global cohort						
GLI1	1.040	0.834-1.296	0.727	1.048	0.842-1.304	0.674
PTCH1	0.789	0.632-0.984	**0.035***	0.824	0.661-1.025	0.083
SHH	0.929	0.745-1.158	0.513	0.883	0.709-1.099	0.266
SMO	1.065	0.854-1.329	0.575	1.089	0.874-1.357	0.447
Adenocarcinoma subcohort						
GLI1	0.968	0.705-1.330	0.842	1.040	0.754-1.435	0.809
PTCH1	0.617	0.446-0.852	**0.003***	0.687	0.495-0.952	**0.024***
SHH	0.920	0.670-1.263	0.607	0.924	0.670-1.274	0.629
SMO	1.171	0.852-1.611	0.331	1.234	0.893-1.706	0.203
Squamous cell carcinoma subcohort						
GLI1	0.900	0.662-1.223	0.502	0.874	0.648-1.179	0.379
PTCH1	0.981	0.722-1.332	0.900	0.959	0.712-1.295	0.788
SHH	1.077	0.792-1.332	0.637	1.037	0.769-1.399	0.812
SMO	0.909	0.669-1.235	0.542	0.920	0.682-1.241	0.587