

SMAD-6, -7 and -9 are potential molecular biomarkers for the prognosis in human lung cancer

SHUXIAN PAN^{1,2}, GUANGMING ZHOU¹, WENTAO HU¹ and HAILONG PEI¹

¹State Key Laboratory of Radiation Medicine and Protection, School of Radiation Medicine and Protection, Collaborative Innovation Center of Radiological Medicine of Jiangsu Higher Education Institutions, Soochow University, Suzhou, Jiangsu 215123; ²Department of Radiation Oncology, Yancheng Third People's Hospital, Yancheng, Jiangsu 224000, P.R. China

Received December 11, 2019; Accepted May 13, 2020

DOI: 10.3892/ol.2020.11851

Abstract. SMADs, a family of proteins that function as signal transducers and transcriptional regulators to regulate various signaling pathways, including the transforming growth factor- β signaling pathway, are similar to the mothers against decapentaplegic family of genes and the *sm* gene family in *Caenorhabditis elegans*. SMADs generate context-dependent modulation by interacting with various sequence-specific transcription factors, such as E2F4/5, c-Fos, GATA3, YY1 and SRF, which have been found to serve a key role in lung carcinoma oncogenesis and progression. However, the prognostic values of the eight SMADs in lung cancer have not been fully understood. In the present study, the expression levels and survival data of SMADs in patients with lung carcinoma from the Oncomine, Gene Expression Profiling Interactive Analysis, Kaplan-Meier plotter and cBioPortal databases were downloaded and analyzed. It was found that the mRNA expression levels of SMAD-6, -7 and -9 were decreased in lung adenocarcinoma and squamous cell carcinoma compared with that in adjacent normal tissues, while there was no significant difference in SMADs 1-5. Survival analysis revealed that not only were low transcriptional levels of SMAD-6, -7 and -9 associated with low overall survival but they also had prognostic role for progression-free survival and post-progression survival ($P < 0.05$) in patients with lung carcinoma. In conclusion, the present study demonstrated that SMAD-6, -7 and -9 are potential biomarkers for the prognosis of patients with lung carcinoma.

Introduction

Lung carcinoma, the most commonly diagnosed cancer (11.6% of total cases) and the leading cause of cancer-associated mortality (18.4% of total mortalities) worldwide, according to the GLOBOCAN 2018 estimates of cancer incidence and mortality (1), is characterized by a high degree of malignancy, metastasis and high mortality rate (1). Lung carcinoma is the most diagnosed cancer and the leading cause of cancer-associated mortality in men (1), and the third most common cancer and second cause of cancer-associated mortality in women, worldwide (1). Most lung cancer tumors have no obvious symptoms in the early stage and are therefore less likely to be detected; thus, the majority of patients with lung carcinoma are diagnosed at an advanced stage, which reduces the efficacy of treatment and the 5-year survival rate is low (2,3). Therefore, novel potential prognostic biomarkers and drug targets are required to improve the outcome and individualized treatments of lung cancer.

SMADs are a family of genes that encode signal transducers and transcriptional modulators, which mediate various signaling pathways, such as TGF- β /SMAD, BMP/SMAD, ERK/MAPK, Hippo, JAK/STAT and Wnt/ β -catenin (4). In mammals, there are eight SMAD proteins, which are sub-divided into three types: Receptor-regulated SMADs (R-SMADs), common-mediator SMADs and inhibitory SMADs (4-6). SMADs can recognize and bind several sequence-specific and context-dependent transcriptional regulation factors, such as FoxH1, Sp1, YY1 and p53, which have been found to participate in various biological processes, including cell proliferation, apoptosis, differentiation, as well as tumor progression and immune regulation processes (7-9). The majority of signaling pathways regulated by SMADs, such as TGF- β /SMAD, BMP/SMAD, ERK/MAPK, JAK/STAT and Wnt/ β -catenin, are deregulated in various human malignant carcinomas, including lung carcinoma, malignant melanoma, colorectal cancer, kidney cancer, breast cancer, ovarian cancer and prostate cancer (10-17).

A total of seven mammalian SMAD proteins have been reported to participate in the regulation of lung carcinoma tumorigenesis or progression. The *SMAD-1* gene participates in negative regulation of the Akt/GSK3 β pathway to

Correspondence to: Professor Wentao Hu or Professor Hailong Pei, State Key Laboratory of Radiation Medicine and Protection, School of Radiation Medicine and Protection, Collaborative Innovation Center of Radiological Medicine of Jiangsu Higher Education Institutions, Soochow University, 199 Renai Road, Suzhou, Jiangsu 215123, P.R. China
E-mail: wthu@suda.edu.cn
E-mail: hlpei@suda.edu.cn

Key words: SMAD, lung cancer, biomarker, prognosis

maintain the cancer stem cell-like characteristic of cancer stem cells in non-small cell lung carcinoma (NSCLC) (18). Yang *et al* (19) found that SMAD1 knockdown inhibited epithelial-mesenchymal transition (EMT) induced by fine particulate matter (PM_{2.5}) in human lung carcinoma cells. In addition, Tang *et al* (20) reported that transcriptional activation of SMAD-2 and -3 facilitates the growth and metastasis of lung carcinoma through enhanced transforming growth factor (TGF)- β 1-induced EMT and from the generation of the angiogenic factors, such as vascular endothelial growth factor and connective tissue growth factor. SMAD4 is phosphorylated(p) at Tyrosine 95 by an oncogenic tyrosine kinase ALK, preventing it from binding to DNA and eliciting TGF- β -induced tumor suppressing responses during lung cancer tumorigenesis (21). Phosphorylated (p)-SMAD-9 expression levels were found to be markedly enhanced and associated with the metastatic potential of non-small cell lung carcinoma A549 subclones from experimental brain metastases through four rounds of intracardiac injection of A549 cells or its derivatives into athymic nude mice (22). Inhibitory SMADs, including SMAD-6 and -7, have been found to be involved in lung carcinoma by regulating the stability or activity of TGF- β receptors, including the modulation of T β RI ubiquitination and degradation, by which TGF- β signaling is regulated and functions in lung cancer growth and metastasis (6,23). However, the underlying mechanisms by which SMADs are involved in the regulation of lung carcinoma are not fully understood.

At present, the dysregulated mRNA expression levels of SMAD proteins in human lung cancer and their associations with lung carcinoma prognosis have not been investigated. In the present study, bioinformatics analysis was used to investigate the roles of SMAD proteins in human lung carcinoma. The expression patterns and mutations of different SMAD proteins in patients with lung carcinoma were analyzed, from the vast number of gene expression data that has been previously published, to identify SMAD expression patterns and potential prognostic values in human lung carcinoma.

Materials and methods

Oncomine analysis. The Oncomine gene expression array dataset (www.oncomine.org), was used for analyzing the expression levels of SMADs in different carcinomas, including bladder cancer, brain and central nervous system cancer, breast cancer, cervical cancer, colorectal cancer, esophageal cancer, gastric cancer, head and neck cancer, kidney cancer, leukemia, liver cancer, lung cancer, lymphoma, melanoma, myeloma, ovarian cancer, pancreatic cancer, prostate cancer and sarcoma (24). A comparison of *SMAD* mRNA expression levels between clinical tumor specimens and adjacent normal tissues was performed using unpaired two-tailed Student's t-tests (number of samples: GSE19188: 10, GSE10072: 107, GSE32863: 116, GSE31210: 246 and GSE7670: 64). The cut-off values were set as follows, $P < 0.01$ and fold change > 2 , respectively.

Gene expression omnibus (GEO) dataset analysis. GSE19188 (25), GSE10072 (26), GSE32863 (25), GSE31210 (25), GSE7670 (27) were downloaded from the GEO database (www.ncbi.nlm.nih.gov/geo/) to analyze the mRNA expression levels

of all SMAD subtypes in lung neoplasm tissues and adjacent normal lung tissues (28). These datasets were obtained by searching using the following key words: 'lung cancer AND SMAD' or 'lung cancer AND gene'. To avoid generating less reliable results, the 5 datasets were batch normalized in the R computing environment in RStudio (version 1.2.5001) using the *sva* package and merged to reduce the variability (29-31).

Gene expression profiling interactive analysis (GEPIA) dataset. GEPIA is an online cancer database used for analyzing RNA sequences and expression levels for 9,736 lung cancer samples and 8,587 normal samples from The Cancer Genome Atlas (TCGA) and GTEx projects, based on a standard processing pipeline (32). GEPIA can be used to analyze differential expression in tumor and adjacent normal tissues, tumor type or clinical stage, patient survival analysis, correlation, dimensionality reduction (reducing the dimensionality of high dimensional expression datasets while maintaining most of the variances based on principal component analysis) and similar gene detection (32).

Kaplan-Meier plotter. The prognostic value of the SMADs transcriptional levels was assessed using the online database Kaplan-Meier plotter (www.kmplot.com) (33,34), which contains gene expression data and survival information for 2,437 patients with NSCLC. Overall survival (OS), progression-free survival (PFS) and post-progression survival (PPS) were analyzed after the patients had been divided into 2 groups (high and low) based on the median expression value, using Kaplan-Meier curves, with the log rank test to determine any significant difference. The hazard ratio (HR) and 95% confidence intervals (CI) were also calculated. Only SMAD datasets selected using the JetSet best probe package in R were used in Kaplan-Meier analysis.

TCGA data and cBioPortal. TCGA includes sequencing and pathology data for 30 different cancer types, including glioblastoma multiforme, head and neck, kidney clear cell, lung adenocarcinoma, lung squamous cell carcinoma and medulloblastoma (35). Using cBioPortal (36), SMAD analyses were conducted using the provisional lung adenocarcinoma TCGA dataset. Using the Genomic Identification of Significant Targets in Cancer package (version 1.12.0) which can identify mutations, putative copy number alterations, this was included with the z-scores (also known as standard score, it is obtained by dividing the difference between a number and an average by the standard deviation. In statistics, the standard score is the sign number of the standard deviation of the value of an observation or data point higher than the average of the observed value or measured value. Its value is positively correlated with mRNA expression level) (37) of mRNA expression levels [RNA sequencing V2 RSEM (RNA-Seq by Expectation Maximization)] (38) and the protein expression level data (using reverse phase protein array data) to create the genomic profile for 522 patients with lung adenocarcinoma. The co-expression network was plotted using cBioPortal. The functional roles of the target host gene of SMADs were predicted using Gene Ontology (GO) enrichment analysis of three elements: Biological process, cellular composition and molecular function. Kyoto Encyclopedia of Genes and

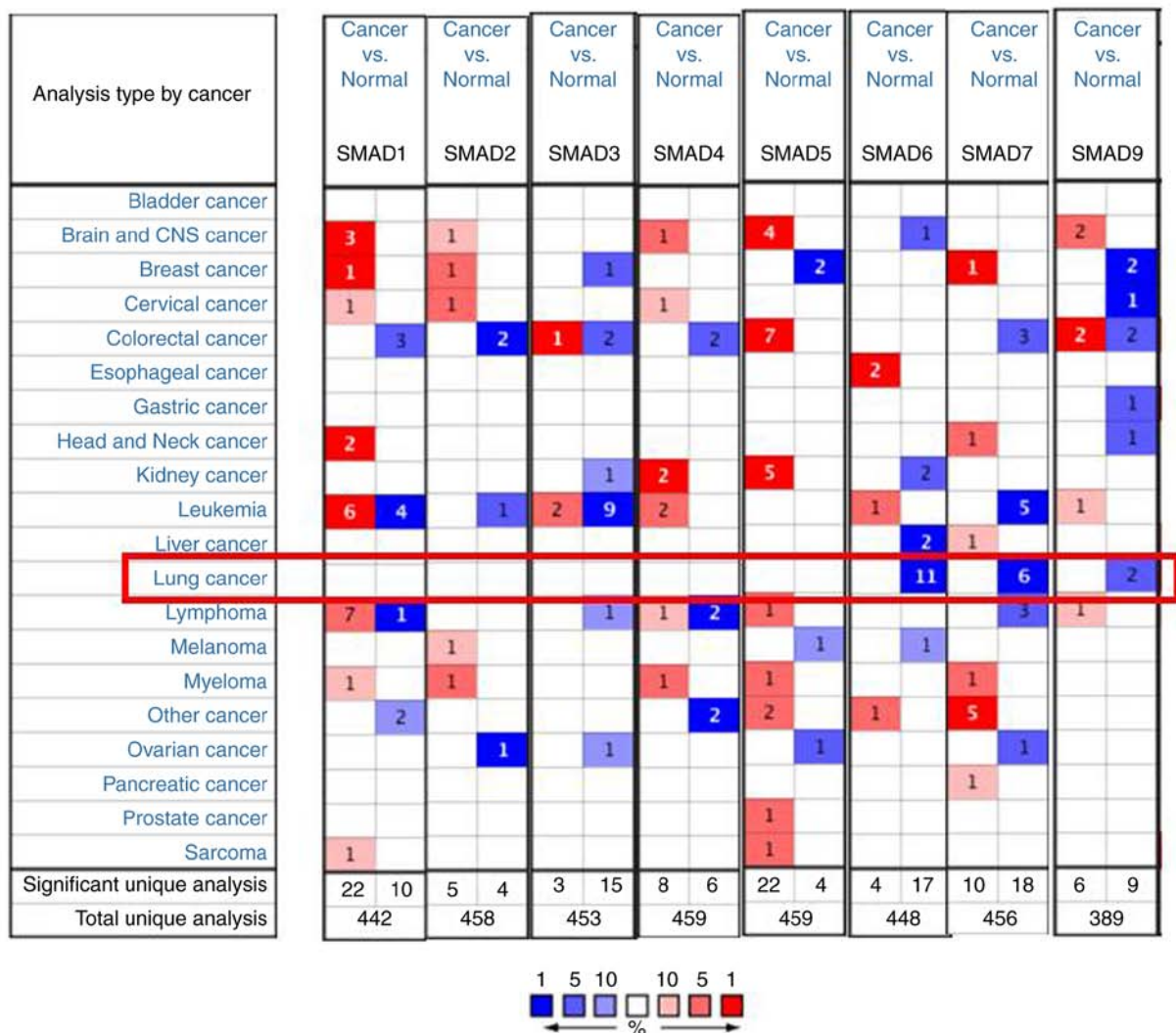


Figure 1. Transcriptional levels of SMAD proteins in different types of cancer. Blue represents low expression, while red represents high expression. The numbers indicate the number of studies meeting the screening criteria.

Genomes (KEGG) function analysis of genes associated with SMAD alterations was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; <https://david.ncifcrf.gov/summary.jsp>).

Statistical analysis. Student's t-tests was used to compare mRNA expression levels in the Oncomine and GEO databases. Kaplan-Meier plotter and Cramér-von Mises tests were used to analyze survival. By defining disease state (tumor or normal) as a variable, GEPIA analysis of variance was performed using one-way ANOVA. Two-tailed Student's t-test was used to compare the expression levels of SMAD mRNA between clinical tumor specimens and adjacent normal tissues. Spearman's correlation analysis was used to evaluate the correlation between gene expression levels in TCGA database. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Variation of SMADs expression levels among different types of lung carcinoma and adjacent normal tissues. The expression levels of the eight SMADs of various cancer types and in

adjacent normal samples were compared using the Oncomine database (Fig. 1). The transcriptional levels of SMAD-6, -7 and -9 were significantly decreased in patients with lung carcinoma ($P < 0.05$). No significant expression difference was observed for SMAD-1/-2/-3/-4/-5 between lung cancer tissues and normal tissues. With respect to lung adenocarcinoma, the expression levels of SMAD-6, -7 and -9 were significantly decreased in cancer tissues compared with that in adjacent normal tissues, with a fold change of -4.622, -3.508 and -2.285, respectively ($P < 0.05$; Table I) (39-41). In particular, only the expression levels of SMAD-7 were decreased compared with that in the adjacent normal samples in all types of lung cancer; SMAD-7 was found to be decreased in squamous cell lung carcinoma by -3.813-fold, in small cell lung carcinoma with a fold change of -4.568 and in lung carcinoid tumor with a fold change of -6.114 (Table I). There were no significant differences in the transcription levels for the remaining SMADs ($P > 0.05$) (25-27).

SMAD mRNA expression levels are associated with the clinicopathological parameters of patients with lung carcinoma. GEPIA and GEO were used to compare the

Table I. Significant changes of SMAD transcriptional levels between different types of lung cancer and normal lung tissues using the Oncomine database.

| Genes | Types of lung cancer vs. normal tissue | Fold change | P-value | t-test value | Author, year | (Refs.) |
|---------------|--|-------------|---------|--------------|-----------------------------------|---------|
| <i>SMAD-1</i> | Lung adenocarcinoma vs. normal | 1.185 | >0.05 | 1.555 | Beer <i>et al</i> , 2002 | (25) |
| <i>SMAD-2</i> | Squamous cell lung cancer vs. normal | 1.205 | >0.05 | 1.525 | Garber <i>et al</i> , 2001 | (26) |
| | Large cell lung carcinoma vs. normal | 1.070 | >0.05 | 0.668 | Garber <i>et al</i> , 2001 | (26) |
| | Large cell lung carcinoma vs. normal | 1.412 | >0.05 | 1.272 | Garber <i>et al</i> , 2001 | (26) |
| | Small cell lung carcinoma vs. normal | -1.069 | >0.05 | -0.427 | Garber <i>et al</i> , 2001 | (26) |
| | Lung adenocarcinoma vs. normal | 1.213 | >0.05 | 1.104 | Beer <i>et al</i> , 2002 | (25) |
| <i>SMAD-3</i> | Lung adenocarcinoma vs. normal | 1.213 | >0.05 | 1.104 | Beer <i>et al</i> , 2002 | (25) |
| <i>SMAD-4</i> | Lung adenocarcinoma vs. normal | -1.110 | >0.05 | -1.405 | Beer <i>et al</i> , 2002 | (25) |
| <i>SMAD-5</i> | Lung adenocarcinoma vs. normal | 1.415 | >0.05 | 1.239 | Yamagata <i>et al</i> , 2003 | (27) |
| | Squamous cell carcinoma vs. normal | 1.302 | >0.05 | 0.962 | Yamagata <i>et al</i> , 2003 | (27) |
| | Large cell carcinoma vs. normal | 1.398 | >0.05 | 1.073 | Yamagata <i>et al</i> , 2003 | (27) |
| <i>SMAD-6</i> | Lung adenocarcinoma vs. normal | -4.622 | <0.0001 | -12.757 | Selamat <i>et al</i> , 2012 | (39) |
| <i>SMAD-7</i> | Lung adenocarcinoma vs. normal | -3.508 | <0.0001 | -5.632 | Bhattacharjee <i>et al</i> , 2001 | (40) |
| | Squamous cell carcinoma vs. normal | -3.813 | <0.0001 | -4.561 | Bhattacharjee <i>et al</i> , 2001 | (40) |
| | Small cell lung carcinoma vs. normal | -4.568 | <0.0001 | -6.228 | Bhattacharjee <i>et al</i> , 2001 | (40) |
| | Lung carcinoid tumor vs. normal | -6.114 | <0.0001 | -7.804 | Bhattacharjee <i>et al</i> , 2001 | (40) |
| <i>SMAD-9</i> | Lung adenocarcinoma vs. normal | -2.285 | <0.0001 | -5.387 | Okayama <i>et al</i> , 2012 | (41) |

mRNA transcriptional levels of SMAD proteins between lung carcinoma tissues and adjacent normal tissues. The results from GEPIA analysis showed that there were significantly lower expression levels of SMAD-6, -7 and -9 in lung adenocarcinoma and squamous cell lung carcinoma tissues compared with normal tissues, while there were no significant differences in the expression levels for SMAD1-5 (Fig. 2A and C). The results from GEO analysis showed similar results, and SMAD-7 showed significantly increased expression in ADC, LCC and SCC samples as compared with normal tissues (Fig. 2B). The transcriptional levels of SMADs in different pathological stages of lung adenocarcinoma and squamous cell carcinoma tissues were also analyzed. The mRNA expression levels of SMAD-6 and -9 were significantly different at stages I-IV, according to the TNM staging system of American Joint Committee on Cancer (AJCC) in 2010 (42), while there were no significant differences in the expression levels of SMAD-1, -2, -3, -4, -5 and -7 (Fig. 3).

Association between decreased mRNA expression levels of SMAD-6, -7 and -9 with the prognosis of patients with lung carcinoma. The association between the mRNA expression levels of SMAD proteins and the survival of patients with NSCLC was analyzed using Kaplan-Meier plotter, and the results revealed that the patients with low mRNA expression levels of SMAD-5, -6, -7 and -9 had poor OS, PFS and PPS rates (from left to right: OS, PFS and PPS) ($P < 0.05$, Fig. 4), suggesting their potential roles as a tumor suppressor, except that SMAD6 is not significantly associated with PPS. SMAD-2, -4 was identified as a tumor suppressor by OS analysis; however, the results for SMAD-3 were ambiguous, which was demonstrated to be a tumor suppressor for PPS but an oncogene for PFS.

Changes in SMAD protein expression levels and their networks in patients with lung carcinoma. Alterations, associations and networks of SMAD proteins were analyzed using the cBioPortal online tool for lung adenocarcinoma. Of the 522 patients with lung adenocarcinoma, 208 (40%) had alterations in SMADs; while >2 changes were detected in 83 samples (Fig. 5A). Among the eight SMADs, SMAD-4 has the most alterations (with 'mRNA low' being the most abundant type) while SMAD-6 has the least changes. Deep deletion was identified in all SMADs except SMAD-1. Missense mutations were identified in SMAD-2/3/4/5/6/9 but not SMAD-1/7. Calculation of the correlations between each of the SMADs in lung adenocarcinoma was performed using cBioPortal online tool based on their mRNA expression levels. The results indicated that SMAD-2 was significantly correlated with SMAD4 ($R=0.55$; $P < 0.001$) and SMAD7 ($R=0.4$; $P < 0.001$), while SMAD-1, -3, -5, -6 and -9 were not significantly associated with any other SMADs (Fig. 5B). The interaction network between SMADs and the 49 most-confirmed altered neighbors was then constructed, demonstrating that cell cycle-associated genes, such as CDK4, BCL-9, MYC, CREBBP and E2F5, were associated with changes to SMAD expression levels (Fig. 5C).

Using the Database for Annotation, Visualization and Integrated Discovery (<https://david.ncifcrf.gov/summary.jsp>), the functions of the SMADs and genes associated with SMAD expression level changes were predicted. The functional roles of the target host genes of SMADs were predicted using Gene Ontology (GO) enrichment analysis of three elements: Biological process, cellular composition and molecular function. It was found that all SMAD mRNA expression changes in lung adenocarcinoma were found to be enriched in cellular metabolic processes, biochemical processes, multicellular progression and biochemical

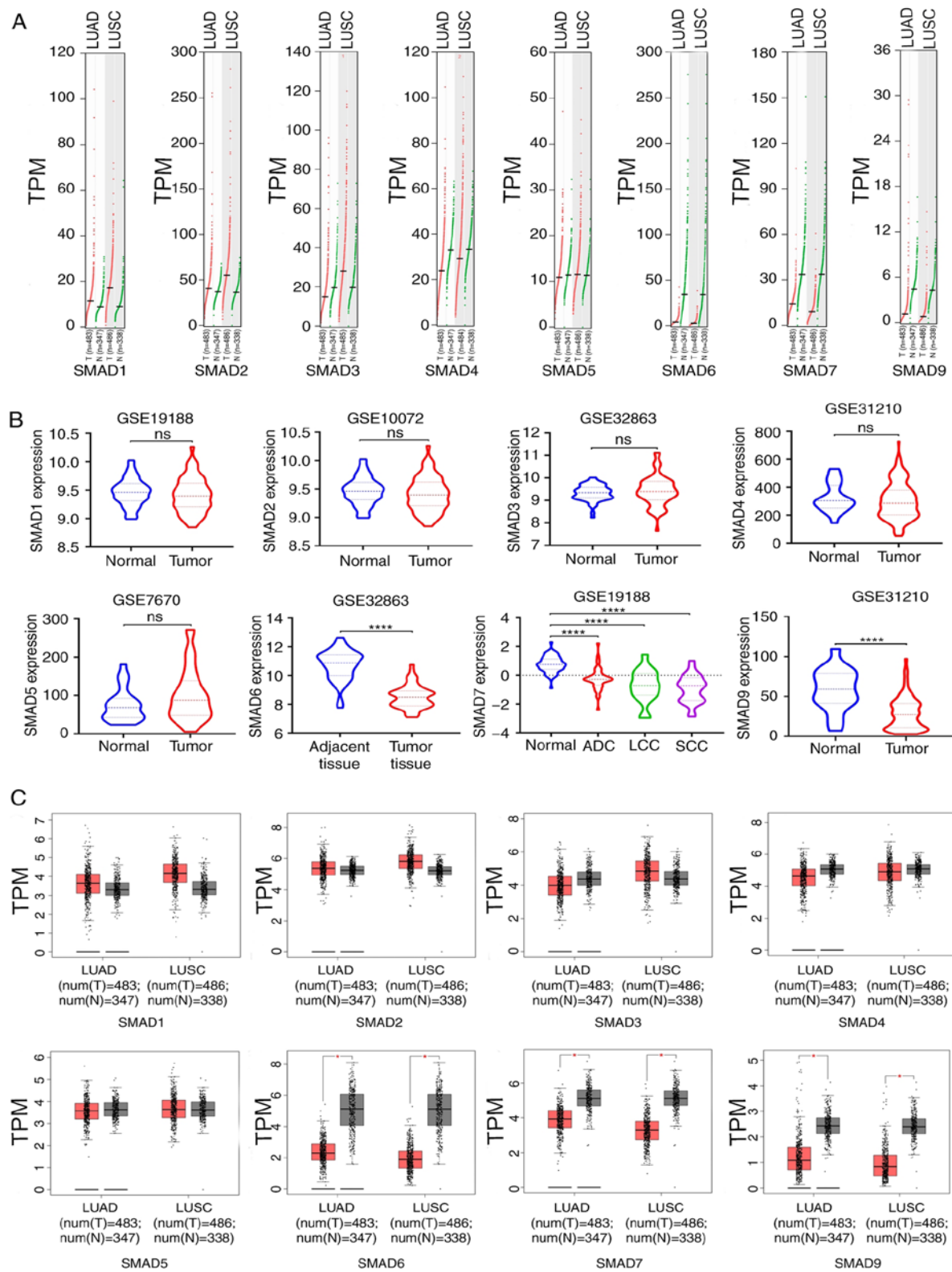


Figure 2. Expression levels of SMADs in lung cancer. (A) Analyses of SMAD expression levels were performed using Gene Expression Profiling Interactive Analysis. (B) Analyses of SMAD expression levels were performed using the Gene Expression Omnibus and the results are depicted as a violin plot. (C) Analyses of SMAD expression levels were performed using Gene Expression Profiling Interactive Analysis and the results are depicted as a box plot. **** $P < 0.0001$ and * $P < 0.05$. ns, not significant; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; T, tumor; N, normal; num, number; ADC, adenocarcinoma; LCC, large cell carcinoma; SCC, squamous cell carcinoma; TPM, transcripts per million.

activity-related proteins, such as stress (Fig. 6A). Among the proteins associated with disease and drug sensitivity, it was found that the SMAD proteins were involved in biological

activities such as 'lung cancer (smoking interaction)', 'severe aortic features in Marfan syndrome' and 'immune response to anthrax vaccine' (Fig. 6B).

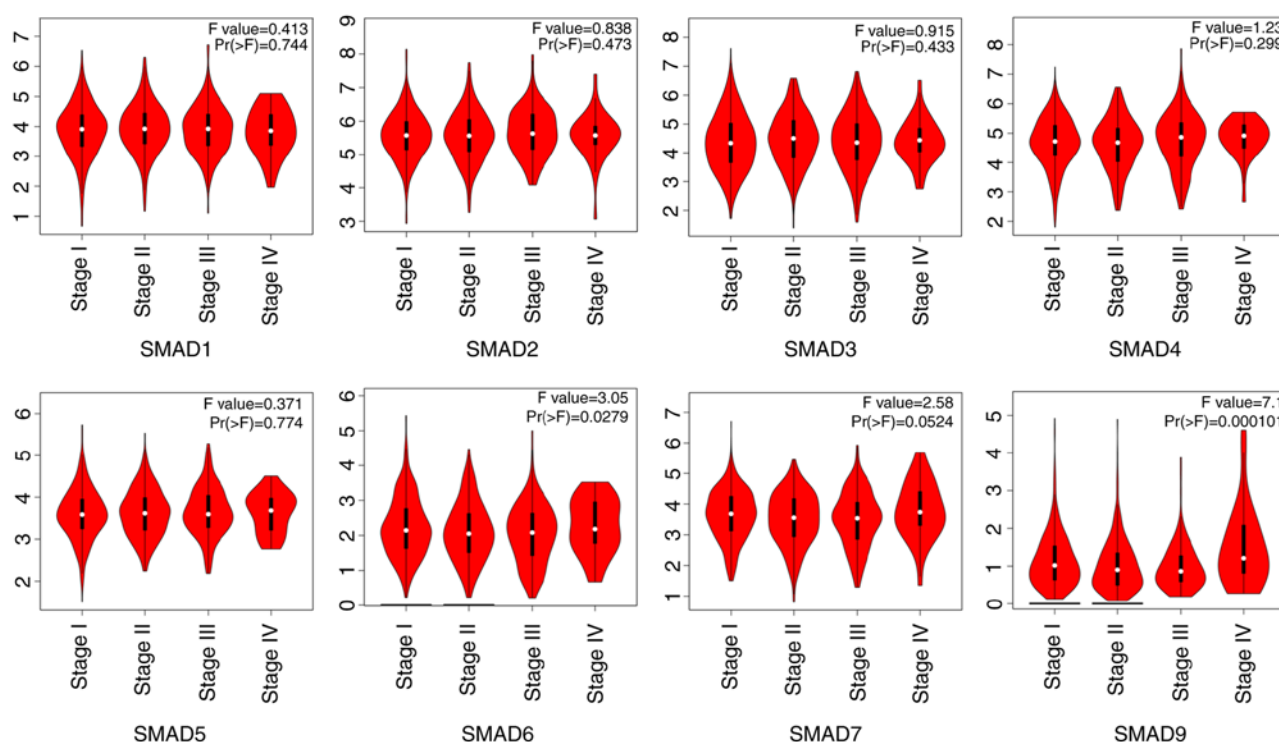


Figure 3. Analysis of SMAD expression levels in different tumor stages of lung adenocarcinoma and squamous cell lung carcinoma tissues using Gene Expression Profiling Interactive Analysis as shown in a violin plot. The white dots represent the median, while the black lines represent the upper and lower quartiles and the ranges.

Discussion

The majority of studies investigating SMAD proteins are related to the TGF- β signaling pathway, which functions in tumorigenesis and tumor progression as well as tumor immunosuppression (11,43-47). Dysregulation of several SMAD proteins has been proved in numerous types of cancer. It has been reported that SMAD1 gene polymorphisms influence colorectal cancer susceptibility (48), while SMAD2 signaling is enhanced in chemoresistant colorectal cancer cells (49). Tone *et al* (50) identified R361G mutation of SMAD4 in primary low-grade serous ovarian carcinoma samples, which is situated within MH2 domain and is speculated to affect the functional specificity and selectivity of SMAD4 protein. SMAD5 was found to be overexpressed in human breast cancer cells and was involved in the induction of cancer stem cell-like phenotype (51). Further, SMAD7 expression is decreased by the overexpression of SETDB1 in human breast cancer, while upregulation of SMAD7 by SETDB1 knockdown inhibits breast cancer metastasis (52). However, bioinformatics analysis of SMAD proteins associated with human lung cancer has not been performed, to the best of our knowledge. The present study reported the mRNA expression levels and survival prediction potential of SMAD proteins in human lung cancer for the first time.

SMAD1 has been found to be involved in both the positive modulation of EMT in human malignant lung neoplasm cells and in the maintenance of stem-like cell traits (18,19). Immunohistochemical analyses was performed to determine the protein expression levels of SMAD1 in 60 cases of lung cancer tissues (lung cancer group), 25 cases of normal alveolus

tissues (alveolus control group) and 29 cases of normal bronchial tissues (bronchial control group), and the results revealed markedly lower expression levels of SMAD1 in the lung cancer group compared with that in normal tissue (53). Furthermore, the expression levels of SMAD-1 protein were significantly associated with lung carcinoma differentiation and lymphatic metastasis (53). However, according to the present analysis, there were no significant differences in the expression levels of SMAD-1 between either lung adenocarcinoma or lung squamous cell carcinoma and adjacent normal tissues. The results from Liu *et al* (18) and Yang *et al* (19) were collected from lung cancer cell lines, which might account for these differences. In addition, the difference between the findings from the present study (based on 650 cases of lung cancer tissues) and with that from Gao *et al* (53) (based on 60 cases of lung cancer tissues) may be caused by the different sample size.

The regulatory functions of SMAD-2 and -3 have been explored extensively in lung carcinoma. It was reported that SMAD-2 and -3 function as key R-SMAD proteins mediating the TGF- β /bone morphogenetic protein signaling pathway to facilitate EMT of lung carcinoma cells, as well as tumorigenesis, invasion and metastasis (20,54-58). Chen *et al* (59) also reported that a higher transcriptional level of p-SMAD-2 in stromal fibroblasts predicted less favorable survival in patients with pathological stage I to IIIA NSCLC, according to TNM staging system of American Joint Committee on Cancer (AJCC) in 2010 (42). SMAD3 was also reported to facilitate the progression of NSCLC by upregulating PAX6 expression (60). However, in the present bioinformatics study there were no significant differences in the expression levels of SMAD-2 and -3 between lung carcinoma and adjacent normal tissues. SMAD-2 was identified

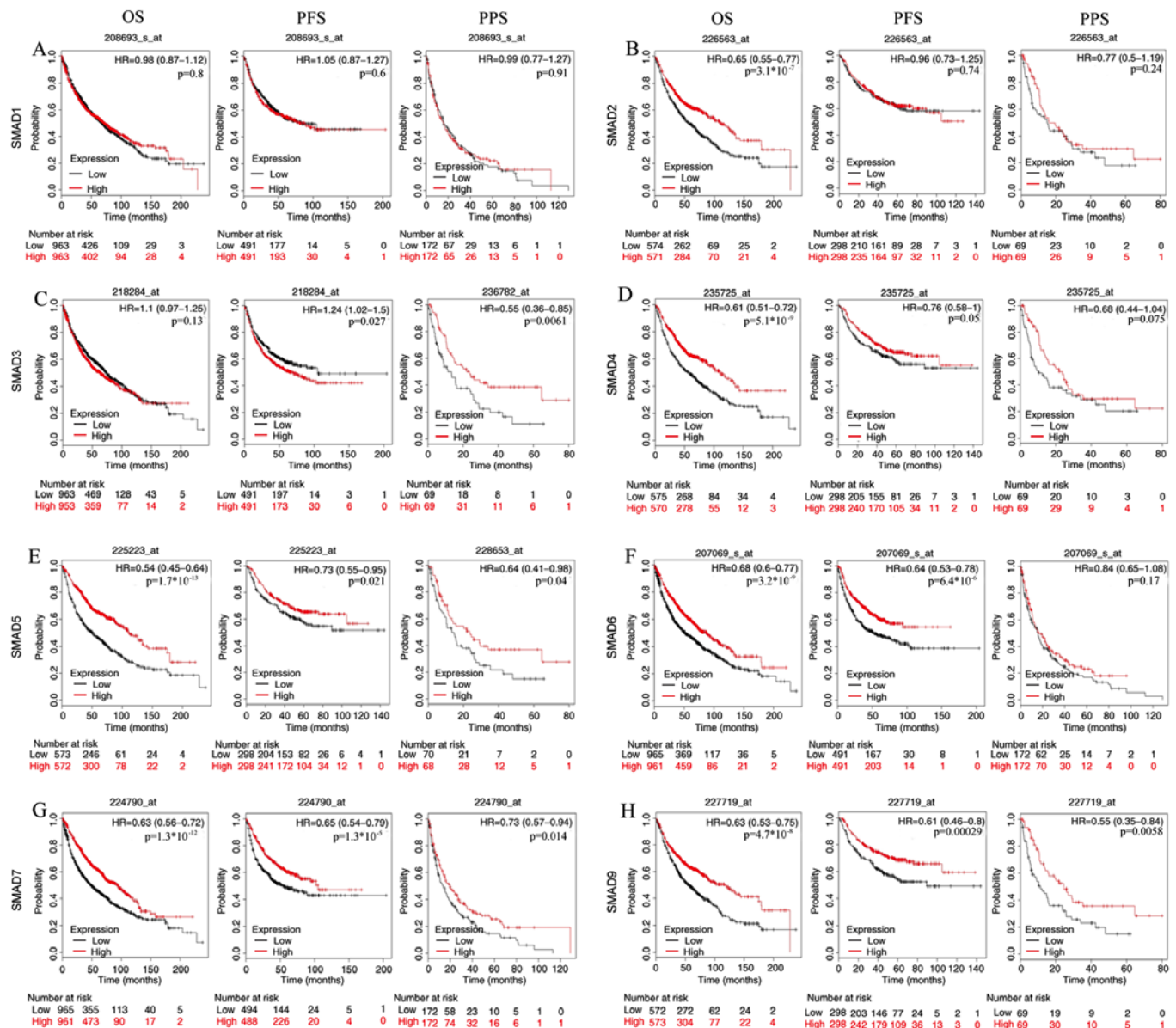


Figure 4. Prognostic value of *SMAD* mRNA expression levels in patients with lung cancer using Kaplan-Meier plotter. OS, PFS and PPS analyses of patients with high and low *SMAD* mRNA expression. (A) *SMAD*-1; (B) *SMAD*-2; (C) *SMAD*-3; (D) *SMAD*-4; (E) *SMAD*-5; (F) *SMAD*-6; (G) *SMAD*-7; (H) *SMAD*-9. The P values were calculated by the log-rank test. OS, overall survival; PFS, progression-free survival; PPS, post-progression survival; HR, hazard ratio.

as a tumor suppressor using OS analysis; however, the results for *SMAD*-3 were ambiguous, as *SMAD*3 was demonstrated to be a tumor suppressor for PPS but an oncogene for PFS.

SMAD-4 binds to other active R-*SMAD* proteins (*SMAD*-1/2/3/5/8) to form homo- and heterotypic complexes that accumulate in the nucleus and regulate transcription of target genes, including *Nanog*, *CDKN1C*, *CDKN2B*, *Nodal*, *PAI-1*, *Lefty1* and *SMAD*7 (6). *SMAD*-4 appears to function as a tumor suppressor in lung carcinoma and is associated with the differentiation status of lung carcinoma tissues (61,62). It was found that the p.R361C mutation in *SMAD*-4 serves a role in downregulating the TGF- β signaling pathway, causing a loss of growth inhibition and transcriptional activation mediated by *SMAD*s, and it is hypothesized that the mutation p.R361C in *SMAD*-4 plays a crucial part in lung oncogenesis (63). It has been reported that the *SMAD*4 protein expression levels in NSCLC tissues are significantly lower compared with that

in a normal tracheal-bronchial epithelium ($P<0.05$) using immunohistochemistry and that *SMAD*4 knockdown initiates and promotes lung carcinoma progression (64). In addition, suppression of *SMAD*4 protein expression has been found to be associated with the promotion of growth and invasion and metastasis of lung cancer (65-67).

In the present study, *SMAD*-4 was shown to be down-regulated in both lung adenocarcinoma and lung squamous cell carcinoma; however, this difference was not significant. OS analysis also demonstrated that *SMAD*-4 functioned as a tumor suppressor, which is consistent with the genetic alteration analysis in lung adenocarcinoma (cBioPortal) shown in Fig. 5A (with both missense and truncating mutations as putative drivers). A possible explanation for the tumor suppressor function of *SMAD*-4 may be due to the missense mutation sites, given that no difference in expression levels was observed between cancer tissues and adjacent normal tissues, similar

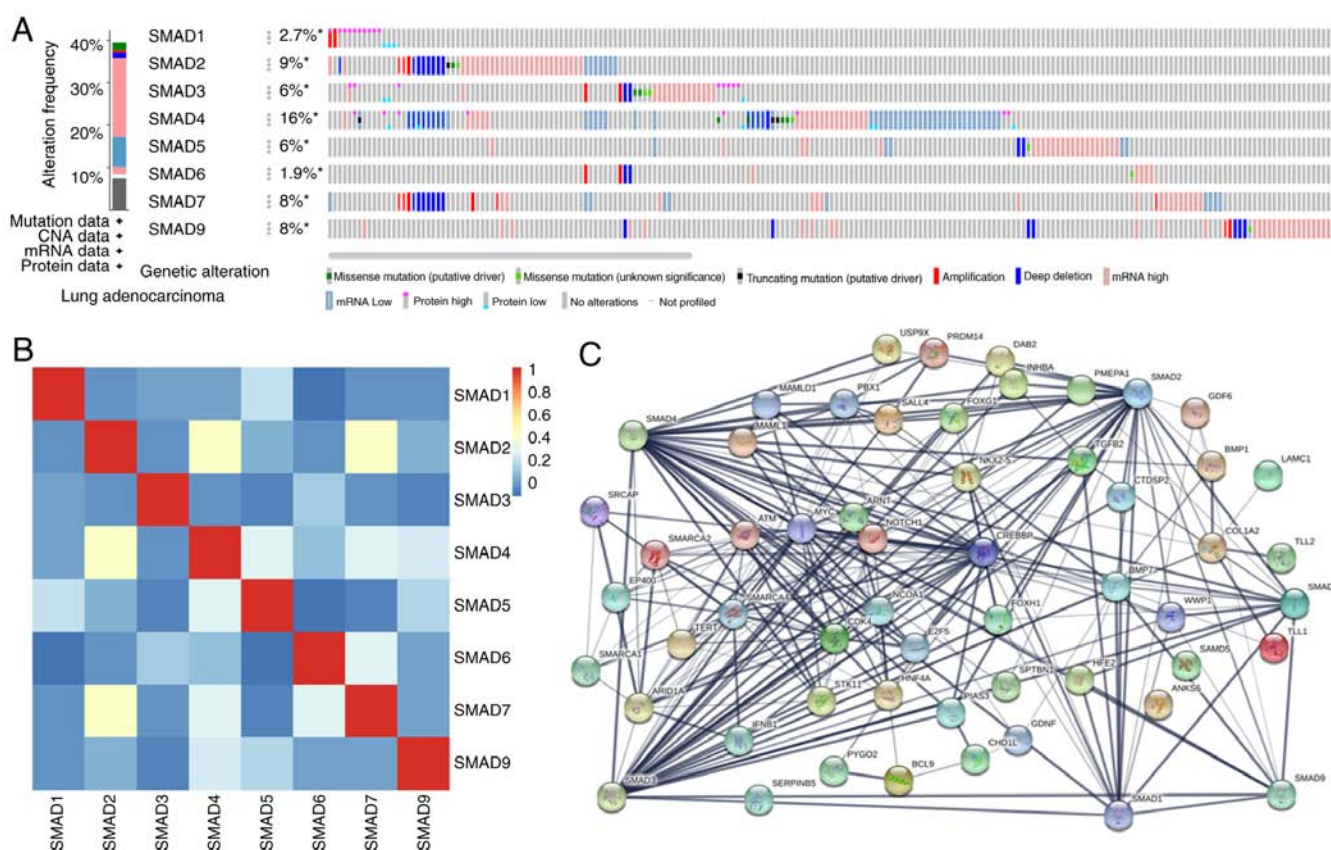


Figure 5. *SMAD* gene expression level and mutation analysis in lung adenocarcinoma using cBioPortal. (A) Genetic alterations in *SMADs* in lung adenocarcinoma. (B) Correlation of *SMADs* with each other. The color scale indicates the correlation coefficient. (C) Network of *SMADs* and the 49 most frequently altered neighboring genes.

with that for SMAD-2. This hypothesis is also in accordance with a previous report indicating that some mutations in SMAD-2 and -4 (D450H, del1434-6 in *SMAD-2* and R420H, R441P in *SMAD-4*) are associated with progression in lung cancer (68), which further supports the present finding that SMAD-2 is significantly correlated with SMAD-4 (Fig. 5B).

It was observed using a two-stage case-control study, with 4,680 cases and controls, that there is a significant association between *SMAD-5* rs12719482 and increased risk of lung carcinoma, suggesting that *SMAD-5* participates in the modulation of lung cancer tumorigenesis. It is speculated that *SMAD-5* rs12719482 is a candidate marker for lung cancer susceptibility, which decreases the expression of SMAD-5 through binding has-miR-1270, hsa-miR-571 or hsa-miR-920 (69). This may also explain why the OS analysis of the present study suggested that SMAD-5 functions as a tumor suppressor despite the fact that there was no significant difference in SMAD-5 expression levels.

With regards to SMAD-9, which is another R-SMAD protein, there have been no previously published studies investigating its function in the regulation of lung cancer to the best of our knowledge. The present findings indicated that no significant mRNA expression level changes were observed for SMAD-5; however, the expression levels of SMAD-9 was found to be significantly decreased in lung adenocarcinoma and lung squamous cell carcinoma tissues compared with that in adjacent normal tissues, which suggests that SMAD-9 may also serve as a tumor suppressor in lung cancer progression. In

addition, the survival analysis also suggested that low expression levels of SMAD-5 and -9 were significantly associated with poor OS, PFS and PPS in all patients with lung carcinoma, which agrees with previous reports demonstrating the tumor suppressor roles of SMAD-5 and -9 (69-71). However, as there was no significant association between SMAD-5 expression levels and the cancer stage of lung carcinoma (Fig. 3), the value of SMAD5 as a potential biomarker is limited.

The two inhibitory SMAD proteins, SMAD-6 and -7, were found to be downregulated in both human lung adenocarcinoma and lung squamous cell carcinoma tissues in the present study. Low expression levels of SMAD6 were associated with poor OS and PFS, but not PPS, in all patients with lung carcinoma, whilst low expression levels of SMAD-7 were associated with poor OS, PFS and PPS. These findings indicate the roles of SMAD-6 and -7 as tumor suppressors in human lung cancer, which agrees with previous published findings regarding these functions in lung cancer (46,72). Moreover, it was found that SMAD7 is markedly correlated with SMAD-2 as shown in Fig. 5B, which is also consistent with the finding that the expression of SMAD-2 is regulated by inhibitory SMAD-7 activity in a negative feedback in cancer cells (73).

As shown in Fig. 5, different deep deletions were found for SMAD-6, -7 and -9 in clinical lung adenocarcinoma samples, which is in accordance with the present survival analysis. Missense mutations were also identified for SMAD-6 and -9 but not SMAD-7, suggesting that both deep deletions and

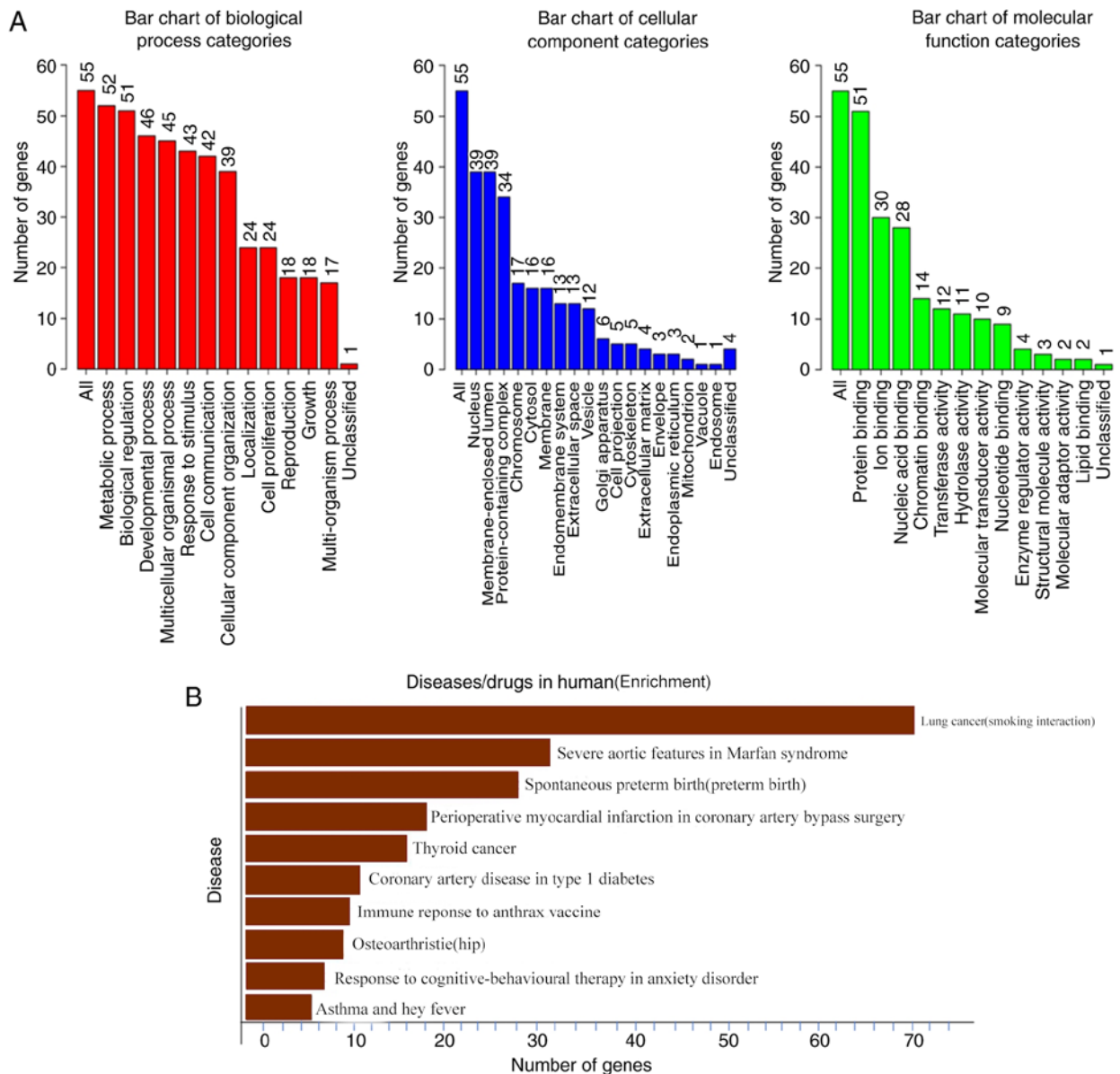


Figure 6. Functional analysis of genes associated with *SMAD* alterations were analyzed using DAVID. (A) The Gene Ontology function analysis and the functional roles of target host genes of *SMAD*s based on three aspects including biological processes, cellular components, and molecular functions. 'all' represents the total number of genes associated with *SMAD* alterations. (B) Kyoto Encyclopedia of Genes and Genomes function analysis of genes associated with *SMAD* alterations were conducted using DAVID. DAVID, Database For Annotation, Visualization and Integrated Discovery.

missense mutations may serve an important role in lung cancer progression.

In conclusion, the expression levels and prognostic values of *SMAD*s in lung cancer were analyzed in the present study, which improved the understanding of the regulation of lung cancer. The results demonstrated that decreased expression levels of *SMAD*-6, -7 and -9 might function in the tumorigenesis and development of lung carcinoma, and that these *SMAD*s may also function as biomarkers to identify patients with a high-risk of lung carcinoma. Nevertheless, there are still some limitations for the present study, for example, all results were obtained by bioinformatics analyses and no experimental validation was performed. It is suggested that both *in vitro* and

in vivo experimental validation is required for the confirmation of the three molecules as *bona fide* biomarkers for lung carcinoma, which should be conducted in future studies. Furthermore, experiments need to be performed to validate the tumor suppressive functions of the three *SMAD*s in both lung cancer cells and transplanted lung tumor models. The experimental validation and the functional deciphering of the three *SMAD*s are of great importance to better understand the functions of TGF- β signaling in human lung cancer.

Acknowledgements

Not applicable.

Funding

This study was supported by The National Key R and D Program of China (grant nos. 2018YFC0115704 and 2018YFC0115705) and The National Natural Science Foundation of China (grant nos. 11405235 and 81602794).

Availability of data and materials

The datasets generated and/or analyzed during the present study are available in the [online] repository, [<http://ualcan.path.uab.edu/analysis.html>], [<https://www.oncomine.org/resource/main.html>], [<https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>], [<https://www.cbioportal.org/>].

Authors' contributions

GZ, WH and HP conceived and designed the study. SP conducted the bioinformatics analyses. WH and SP drafted the initial manuscript. All authors read and approved the final manuscript.

Ethic approval and consent to participate

The present study was conducted according to The Declaration of Helsinki. All the datasets were retrieved from open databases and published literature.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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