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RESEARCH ARTICLE

Expression analysis of Akt and MAPK signaling pathways in lung tissue of patients with idiopathic pulmonary fibrosis (IPF)

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Abstract

Purpose of the study: Several studies in patients with lung cancer have shown that epidermal growth factor receptor regulates various tumorigenic processes through the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin and Ras/Raf/Mek/Erk (mitogen-activated protein kinase (MAPK)) signalling pathways. The aim of our study is to evaluate whether these pathways are implicated in the pathogenesis of idiopathic pulmonary fibrosis (IPF) and to seek indirect evidence of a common pathogenetic pathway with lung cancer. m-RNA expression of oncogenes participating in these two signaling pathways, as well as the combined m-RNA expression of the suppressor genes R-kip and p53 in lung tissue of patients with IPF were evaluated.

Basic procedures: The study population was composed by two distinct groups. Patients with IPF ($n = 25$) and control subjects who underwent thoracic surgery for reasons other than interstitial lung disease ($n = 10$). Expression analysis of the aforementioned oncogenes and suppressor genes was performed using real-time reverse transcription polymerase chain reaction.

Main findings: We found no difference in the overall m- RNA expression between controls and IPF in both investigated pathways. However, Braf has been overexpressed in IPF samples ($P = 0.01$) in contrast with K-ras that has been found downregulated ($P < 0.001$) in comparison with controls.

Principal conclusions: These findings cannot exclude the hypothesis of involvement of Akt and MAPK signalling pathways in pathogenesis of IPF. However, further investigation is needed in order to verify these data.

Keywords: *Idiopathic pulmonary fibrosis; lung cancer; oncogenes; MAPK; Ras; p53; Akt*

Introduction

Idiopathic pulmonary fibrosis (IPF) is the most frequent form of idiopathic interstitial pneumonias (1,2). Median survival from the time of diagnosis is approximately 3 years and until now there is no effective therapy for this disease (3–5). It is well-known that IPF is a gradually progressive disease with worsening of symptoms, lung function, and gas exchange (6,7). In the past, several studies have shown that IPF can be complicated by lung cancer. Patients with IPF have a higher incidence of lung cancer than the general population with relative risk of 7.3 and 14.1 as reported in two studies and a ratio of 5.3 for prevalence of lung cancer at death as reported in another one (8–10). Moreover, it has been observed that lung carcinomas were peripheral and located in the lower

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lobes which are in accordance to the distribution of the fibrotic lesions in IPF (8–12).

These findings have led to the hypothesis of a common pathogenetic pathway. The ideal study population in order to confirm this hypothesis would be composed by patients with IPF and lung cancer and control patients. Since these patients are difficult to find we thought about an indirect approach. Activation of epidermal growth factor receptor (EGFR) activates the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway early during the process of lung carcinogenesis (13). The signalling pathway including Ras/Raf/Mek oncogenes is also activated by EGFR whereas R-kip, a Raf-1 kinase inhibitor protein can disrupt this pathway (14). Through these pathways EGFR regulates tumorigenic processes such as proliferation, apoptosis, angiogenesis, and invasion and is frequently overexpressed in the development and progression of nonsmall-cell lung carcinoma (NSCLC; 15–18). Activation of the oncosuppressor gene p53 due to irreversible DNA damage induces the expression of proapoptotic members of Bcl-2 family and results in apoptosis (19). Mutation of p53 has been reported in lung adenocarcinoma (15).

Recently our study group has observed that EGFR m-RNA levels were not significantly different between patients with IPF and control subjects (20). So, the purpose of our study is to determine the combined m-RNA expression of oncogenes which participate in downstream signaling pathways in patients with IPF in order to find out a common pathogenetic pathway between IPF and lung cancer. We evaluated the combined m-RNA expression of both PI3K/Akt and Ras/ Raf/ MEK/ERK (mitogen-activated protein kinase (MAPK)) signaling pathways in lung tissue from patients with IPF and control subjects using a real-time polymerase chain reaction (PCR) assay with SYBR-Green I, as previously described (20,21).

Methods

Subjects

The subjects studied consisted of two distinct groups: patient with IPF ($n = 25$) and control subjects who underwent thoracic surgery for reasons other than interstitial lung disease ($n = 10$). Tissue specimens were obtained from consenting individuals in accordance with institutional review board approval. All patients had clinical and radiographic findings consistent with the diagnosis of IPF, and all had pathologic confirmation of the diagnosis of usual interstitial pneumonia made by open lung biopsy. None of the patients in either the IPF or control group had been previously treated or were currently being treated with corticosteroids or other immunosuppressive agents. The control lung tissue specimens were from subjects undergoing thoracic surgery for either clinical Stage I or II NSCLC. The lung tissue was obtained from a site as distant as possible from the primary tumor, and was histologically free of neoplasm (Table 1).

RNA extraction and reverse transcription

Total RNA was extracted form each specimen using a power homogenizer and the TRIzol® reagent (Invitrogen, Carlsband, CA) according to the manufacturer's instructions. cDNA was synthesized using the Strascript reverse transcriptase kit (Stratagene, La Jolla, CA) as previously described (20,21).

Real-time RT-PCR

Oncogenes and tumor suppressor genes m-RNA expression was measured using a real-time reverse transcription polymerase chain reaction (RT-PCR) assay with SYBR-Green I. Primers were designed to span introns (20,21). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control, in order to normalize Akt 1-2-3, b-Raf, R-kip, and p53 expression levels (Table 2). Specifically, 1 μL cDNA from pathological or control samples was amplified in a PCR reaction containing 2X Brilliant SYBR-Green I QPCR Master Mix, 300 nM of each primer and 30 μM ROX passive reference dye, in a final volume of 20 μL. After an initial denaturation at 95°C for 10 min, the samples were subjected to 40 cycles of amplification, comprized of denaturation at 95°C for 30 s, annealing at appropriate temperature for each primer pair for 30 sec and elongation at 72°C for 30 sec, followed by a melt curve analysis, in which the temperature was increased from 55°C to 95°C at a linear rate of 0.2°C/sec. Data collection was performed both during annealing and extension, with two measurements at each step, and at all times during melt curve analysis. In each PCR reaction two

Table 1. Demographic and spirometric characteristics of patients with idiopathic pulmonary fibrosis (IPF) and control subjects.

Characteristics	Control subjects	IPF patients
Number	10	25
Sex: male/female	5/5	19/7
Age, median (year)	49 (32–65)	68 (40–75)*
Smokers/nonsmokers	6/4	17/8
FVC, (% pred)	103 ± 14	77.3 ± 13.0*
TLC, (% pred)	101 ± 19	67.4 ± 14.2*
TLCO, (% pred)	96 ± 6	60.3 ± 17.8*
PαO2, (mm Hg)	—	80.3 ± 10.0

Values are expressed as mean ± SD, and age as median (range).
*Statistical significance between IPF patients and healthy controls ($P < 0.05$)
FVC, forced vital capacity, PαO2, arterial partial pressure of oxygen; pred, predicted; TLC, total lung capacity, TLCO, diffusing capacity for carbon monoxide.

Table 2. Primer sequences used for quantitative real-time RT-PCR.

Gene	Primer pair sequence (5'-3')	Annealing temperature
Akt-1	For: CTATGGCGCTGAGATTGTG Rev: CTTAATGTGCCCGTCCTTGT	58°C
Akt-2	For: TGAAAACCTTCTGTGGGACC Rev: TGGTCCTGGTTGTAGAAGGG	60°C
Akt-3	For: GGCGAGCTGTTTTTCCATTTG Rev: GGCCATCTTTGTCCAGCATTG	58°C
K-ras	For: GGGGAGGGCTTTCTTTGTGTA Rev: GTCCTGAGCCTGTTTTGTGTC	60°C
H-ras	For: GGGGCAGTCGCGCCTGTGAA Rev: CCGGCGCCCACCACCACCAG	65°C
N-ras	For: GGGGCAGTCGCGCCTGTGAA Rev: CCGGCGCCCACCACCACCAG	45°C
p53	For: GTGAGCGCTTCGAGATGTTC Rev: ATGGCGGGAGGTAGACTGAC	60°C
Braf	For: AGAAAGCACTGATGATGAGAG Rev: GGAAATATCAGTGTCCCAACA	53°C
R-kip	For: AGACCCACCAGCATTTCGTG Rev: GCTGATGTCATTGCCCTTCA	55°C
GAPDH	For: GGAAGGTGAAGGTCGGAGTCA Rev: GTCATTGATGGCAACAATATCCACT	60°C

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription polymerase chain reaction.

nontemplate controls were included. All PCR experiments were conducted on the Mx3000P real-time PCR thermal cycler using the software version 2.00, (Stratagene, La Jolla, CA). To verify the results of the melt curve analysis, PCR products were analyzed by electrophoresis in 2% agarose gels, stained with ethidium bromide and photographed on an ultraviolet light transilluminator. Primer sequences, annealing temperatures and PCR products length for all the growth factors analyzed, as well as for GAPDH, are described in Table 2.

All reactions were run in triplicates, and peptide growth factor transcript levels were calculated and normalized to each specimen's house keeping gene m-RNA (GAPDH) as well as the appropriate calibrators, using the ΔΔCt method for relative quantification. Specifically, after amplification, standard curves were constructed from samples used in a series of consecutive dilutions, for both the gene of interest (GF) and the internal control (GAPDH). Growth factor and GAPDH amplification efficiencies were the same, reaching 100%. IPF and control data were first normalized against variation in sample quality and quantity. Normalized values to GAPDH, ΔCts, were initially calculated using the following equation:

$$\Delta Ct_{sample} = Ct_{GENE} - Ct_{GAPDH}$$

The ΔΔCt was then determined using the formula:

$$\Delta\Delta Ct = Ct_{IPF} - Ct_{Control}$$

And the expression of the normalized (to GAPDH) genes in IPF compared to the mean of the control samples as a calibrator equals $= 2^{-\Delta\Delta Ct}$. Twofold increased (a value ≥ 2) or decreased (a value ≤ 0.5) value was considered m-RNA overexpression or downregulation respectively, in that IPF sample.

DNA extraction and PCR amplification

Genomic DNA was extracted using proteinase K followed by phenol extraction and ethanol precipitation according to standard procedures. PCR reactions were performed using Go Taq Flexi DNA Polymerase (Promega, WI, USA). Primer pairs and amplification conditions were as previously described (21).

DNA sequencing

Direct DNA sequencing was used to identify mutations in our amplified sequence of *Kras*. The sequencing reactions were carried out using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) in a 10 μL volume containing purified PCR product. Primers are shown in Table 2. The reaction products were precipitated with 2-propanol, washed with 75% ethanol, re-suspended in 25 μL water and loaded onto ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Sequencing data were analyzed using sequence analysis software (Sequence Analysis 3.7; Applied Biosystems).

Statistical analysis

Oncogenes and tumor suppressor genes m-RNA levels were first evaluated by the one-sample Kolmogorov-Smirnov

goodness of fit test, in order to determine whether they follow a normal distribution pattern. Based on the results, the nonparametric Spearman rank correlation was used to examine their relation pair-wise. The Mann–Whitney U and Kruskal–Wallis H test, used when indicated by the analysis, were used to examine Akt 1-2-3, b-Raf, R-kip, p53 and EGFR expression status after stratification for normal or IPF samples. All statistical analyses were performed with SPSS 11.5 (SPSS, Chicago, IL). Statistical significance was set at the 95% level (P-value < 0.05).

Results

m-RNA expression of Akt 1-2-3 oncogenes

Akt-1, Akt-2, Akt-3 m-RNA expression was not statistically significant between patients with IPF and control subjects. In addition, all IPF and controls samples have expressed the aforementioned genes.

More in detail, in IPF group, Akt-1 oncogene was overexpressed in 8% (2/25), underexpressed in 88% (22/25) and had the same expression in 4% (1/25) of the cases. Akt-2 oncogene was overexpressed in 24% (6/25), underexpressed in 56% (14/25) and had the same expression in 12% (3/25), while absence of expression was observed in 8% (2/25) of the samples. Akt-3 oncogene was not expressed in either IPF or control patients.

m-RNA expression of Braf and R-kip oncogenes

Braf and R-kip m-RNA expression was not statistically significantly different between patients with IPF and control subjects. Braf oncogene has been expressed in 16 of 25 IPF patients (64%) and in two of 10 controls samples (P = 0.01; Table 3). R-kip m-RNA expression has been detected in all IPF and controls samples.

In IPF group, Braf oncogene was underexpressed in 36% (9/25), whereas there was no expression in 64% (16/25), R-kip oncogene was overexpressed in 8% (2/25), and underexpressed in 92% (23/25).

m-RNA expression of suppressor oncogene p53

We found no m-RNA expression of suppressor oncogene p53 in both IPF and control subjects.

Table 3. *Ras and Braf* genes m-RNA expression in IPF and normal specimens.

m-RNA expression	IPF patients	Control subjects	P (x^2 test)
K-ras	4% (1/25)	60% (6/10)	<0.001*
H-ras	4% (1/25)	10% (1/10)	0.490
N-ras	28% (7/25)	60% (6/10)	0.077
BRAF	64% (16/25)	20% (2/10)	0.019*

*P < 0.050 is considered statistically significant.
IPF, idiopathic pulmonary fibrosis.

m-RNA expression of K-ras, H-ras and N-ras

K-ras m-RNA was barely expressed in the IPF (4%, 1/25 cases) as opposed to the control group where expression was 60% (6/10 cases). This K-ras downregulation in IPF is statistically significant (Table 3). This lack of m-RNA expression in IPF is in accordance with our finding of lack of K-ras activating mutations in codons 12, 13 based on the sequencing analysis carried out.

H-ras exhibited m-RNA expression only in one patient and one control (4% and 10% respectively. Finally N-ras transcript levels were detectable in 7/25 IPF patients (28%) and 6 controls (60%; Table 3). Both H-ras and N-ras genes have been expressed in all controls samples at the m-RNA level.

Mutation analysis

Sequencing analysis did not detect any mutations in the amplified sequence of *Kras* gene.

Pair-wise correlations of oncogenes

A significant co-expression was observed between EGFR and Akt-1 in the IPF group (P = 0.001; Figure 1). Moreover, in IPF group we found significant pair-wise correlations between Braf and R-kip oncogenes (P = 0.002 Spearman correlation; Table 4; Figure 2).

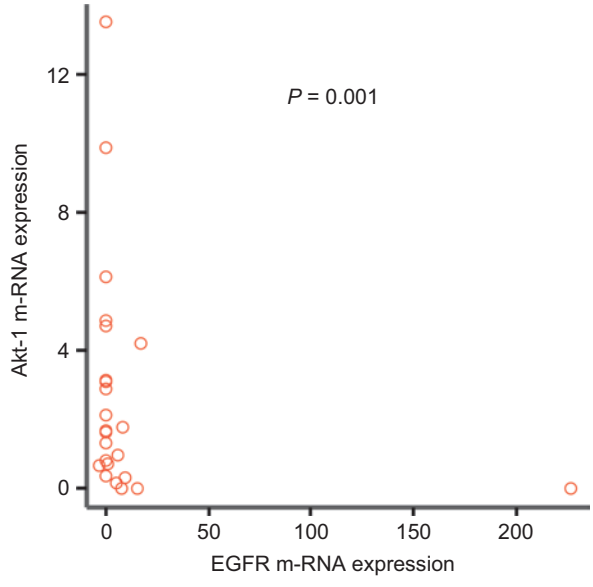

Figure 1. Akt-1 m-RNA is significantly correlated with epidermal growth factor receptor (EGFR) m-RNA in the idiopathic pulmonary fibrosis group (P = 0.001 Spearman). Note: for the shake of clarity of the figure the marks that represent the same values are depicted horizontally on the left of 'point zero' of the x-axis.

Table 4. Spearman Co-expression analysis in the group of control samples.

		Akt-1	Akt-2	Braf	R-kip
Control samples					
Akt-1	CC	1.000			
	P				
	CC	0.067	1.000		
Akt-2	P	0.865			
	CC	−0.143	0.327	1.000	
Braf	P	0.693	0.391		
	CC	0.350	0.262	0.634	1.000
R-kip	P	0.356	0.531	0.067	
IPF					
Akt-1	CC	1.000			
	P				
	CC	0.023	1.000		
Akt-2	P	0.913			
	CC	−0.183	0.191	1.000	
Braf	P	0.380	0.360		
	CC	0.038	0.230	0.586 (**)	1.000
R-kip	P	0.855	0.269	**0.002**	

Statistically significant correlations are highlighted in bold.
*CC, correlation coefficient, Spearman ρ.
**Correlation is significant at the 0.01 level (two-tailed).
IPF, idiopathic pulmonary fibrosis.

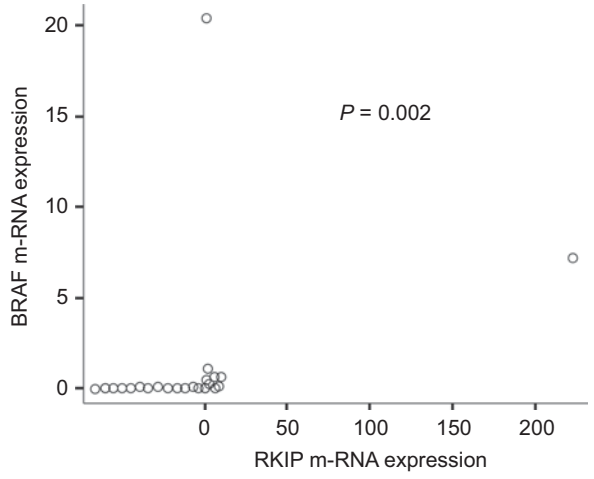

Figure 2. Significant pair-wise correlation between Braf and R-kip oncogenes ($P = 0.002$ Spearman). Note: for the shake of clarity of the figure the marks that represent the same values are depicted horizontally on the left of 'point zero' of the x-axis.

Discussion

The goal of this study was to identify the expression profile of cardinal oncogenes involved in MAPK and Akt signalling pathways in lung tissue of patients with IPF. Our major finding is that we have not detected any difference regarding the overall expression in the investigated pathways. However, Braf has been overexpressed in IPF samples in contrast with K-ras that has been found downregulated in comparison with controls. Moreover, we evaluated K-ras expression, confirming m-RNA lack of expression with sequencing analysis. Furthermore, a significant correlation has been found between Braf and R-kip, as well as between EGFR and Akt-1 gene in patients' samples.

The possibility that pulmonary fibrosis and lung cancer are associated has been recognized, but it remains unclear whether fibrosis precedes lung cancer of *vice versa* (22). Genetic alterations, response to growth and inhibitory signals, resistance to apoptosis, myofibroblast origin and behavior, altered cellular communications, and intracellular signalling pathways are cardinal pathogenetic hallmarks of IPF and cancer (23).

More in detail, the PI3K and MAPK signaling pathways are central regulators of oncogenic transformation and tumor maintenance (24,25) and mediate prosurvival/antiapoptotic signaling by several pathways (26). The acquisition of an apoptosis-resistant phenotype of myofibroblasts induced by transforming growth factor (TGF) β1 is, at least in part, due to the autocrine secretion of a soluble growth factor that activates the PI3K–Akt pathway (27). On the other hand, it is interesting to note that the activation of the PI3K/PTEN-Akt signaling pathway is a fundamental event for many cancers where the overexpression of phosphorylated Akt is linked to a poor prognosis (23,28). Novel research is implicating MAPK and Akt pathways in lung cancer (25,26). Therapeutic inhibition of these activated oncoproteins can induce massive apoptosis of tumor cells, leading to sometimes dramatic tumor regressions in patients. These oncogenes have been recently investigated in different malignancies and various positive or negative associations have been reported (21,22,29) by our study group.

Activating mutation in RAS is found in approximately 30% of human cancer (30). RAS plays an essential role in tumor maintenance and is therefore an appropriate target for anticancer therapy. Several of these new therapeutic agents are showing promising result in the clinic and many more are on the way (30). Moreover, *in vivo* studies suggest that inhibitors of PI3K-mTOR pathway may be active in cancers with PIK3CA (PI3K α catalytic subunit) mutations and, when combined with MEK inhibitors, may effectively treat K-ras mutated lung cancers (31). However, we found an increased expression of Braf in IPF population in contrast with a downregulation of K-ras in those samples. In alignment with the aforementioned finding, it has been strongly supported that Braf/Kras activating mutations might be alternative genetic events in the pathogenetic cascade of cancer (32).

Furthermore, it has been suggested that Braf mutagenesis might be strongly influenced by extrinsic, micro-environmental genomic mechanisms, like oxidative stress (29), in agreement with IPF pathogenesis (23,26,33).

More in detail, activated carcinogens, such as those found in tobacco smoke, might interact with genes,

such as p53, k-ras, and fragile histidine triad leading to genetic or epigenetic alterations which deregulate proliferative control at the molecular level (23). Furthermore, the Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt pathways also interact with the p53 pathway (31). It has been also found that expression of ras protein in type II alveolar pneumocytes and mutation in the codon 12 of K-ras gene in lung tissue may contribute to the induction of lung carcinoma in patients with IPF (31). Furthermore, the presence of multiple mutations in the p53 gene may explain the high incidence of lung carcinoma in patients with IPF (25). Microsatellite instability and loss of heterozygosity are usually correlated with a high rate of mutation and DNA repair while such alterations in different related genes have been demonstrated in ~50% of IPF patients, reported by our study group (34). This genomic mechanism suggests that IPF, independently of lung cancer, exhibits a high mutational frequency which may well affect other genes leading to deregulated control of cell cycle and apoptosis (23,26). More recently, other mutations so far exclusively related to carcinogenesis, such as telomere shortening and telomerase expression, have been described in IPF (35). However, it is rather unsafe to confront these findings with our results, as the evaluation was performed with different methodology and in a different study population.

We also found a significant co-expression between EGFR and Akt-1 in the IPF studied population. EGFR is involved in the development and progression of NSCLC through activation of Akt and MAPK pathways (13–18). Somatic mutations in the tyrosine kinase (TK) domain of the EGFR gene are frequent in patients with lung cancer who have never smoked and are associated to sensitivity of tumor to treatment with TK inhibitors like gefitinib and erlotinib (36). EGFR and its ligands, TGF-α, and epidermal growth factor play an important role in the pathogenesis of pulmonary fibrosis (20,37). Therefore, inhibition of the EGFR signal by an EGFR TK inhibitor (EGFR-TKI) may prevent the development of pulmonary fibrosis. Two elegant studies have shown conflicting data regarding the effect of imatinib mesylate when implicated in treatment of fibrotic diseases (38,39). More recently, imatinib did not affect survival or lung function in the first randomized, placebo-controlled trial of patients with mild to moderate IPF followed for 96 weeks (40).

A significant co-expression was also observed between Braf and R-kip oncogenes in the IPF group. R-kip, a Raf-1 kinase inhibitor, can negatively regulate the Ras/Raf/Mek pathway (14). Several studies suggest that reduced RKIP-1 function may influence metastasis, angiogenesis, resistance to apoptosis, and genome integrity (41).

The current study does not lack limitations. First of all, we have studied the m-RNA expression of these oncogenes and not the protein level of the encoded proteins. Since it is well-known that these signalling proteins are activated via phosphorylation (26), a deficit during this process or a decreased expression at the protein level could better explain our results. Real-time RT-PCR is an established technique for quantifying m-RNA expression in biological samples. Benefits of this procedure over conventional methods include its sensitivity, large dynamic range, and the potential for high as well as accurate quantification output. However, the regulation of oncogenesis is a complex process requiring transcriptional and post-transcriptional mechanisms. The investigation of m-RNA expression of these genes in a nontumorigenic process like IPF is only a first step of our study design.

Secondly, but not less importantly, lung digests used in this study, when negative, do not exclude differences in the pathways in question, as cell-type and spatial heterogeneity may confound the analysis. Akt activation in myofibroblasts has been suggested to play a role in fibrogenesis, while bronchogenic carcinomas arise from epithelium. It would be more informative to investigate signatures of carcinomas that arise in patients with IPF with signatures present within the fibroblastic foci of these patients.

Furthermore, the control lung tissue specimens were from subjects undergoing thoracic surgery for either clinical Stage I or II NSCLC. The lung tissue was obtained from a site as distant as possible from the primary tumor, and was histologically free of neoplasm. However, this methodology is a 'well-known' limitation, used in different, ours (20), and other studies (42).

An additional limitation of the current study is that the detected co-expression may be coincidental rather than causal as the majority of the studied factors have putative pathophysiologic actions other than oncogenesis (angiogenesis, fibroblast proliferation, and activation, etc).

The pathobiology and mortality of IPF and cancer have several similarities (23). Telomerase abnormalities, telomere shortening, and age-related methylation changes have recently suggested in both disorders (35,43). Many techniques have been developed to analyze the genome-wide methylation content. Novel methodology either examines only one specific DNA sequence at a time, or provide limited genomic information on the screened sequences (44). New techniques will serve as an efficient tool in understanding the nature of epigenetic changes and their significance to the aging process in both cancer and fibrosis development.

In conclusion, our findings cannot exclude the hypothesis of involvement of Akt and MAPK signaling pathways in pathogenesis of IPF. However, further investigation is needed in order to verify these data.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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