

Activation of Oncogenic Pathways in Idiopathic Pulmonary Fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) is defined as a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown cause. The most recent hypotheses on IPF pathogenesis suggest a central role of epithelial cell damage, followed by a dysregulated molecular cross talk between epithelial cells and fibroblasts. Thus, IPF progression has often been assimilated to that of cancer, and several signaling patterns appear to be disrupted in both diseases. Here, we analyze the expression in an IPF series of a panel of molecules, which are known to play a role in tumorigenic process. Our findings, although preliminary, reveal that IPF landscape is enriched in neoplastic potential expressed in a context of complex genomic polyclonality and cellular heterogeneity. These results provide a rationale for further investigations aimed to exploit—in a similar fashion to cancer—targeted therapies for a “precision medicine” approach to IPF.

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive, fatal lung disease of unknown etiology that is still lacking of effective therapy. IPF is associated to lung cancer onset with a prevalence that is ranging from 4% to 48% [1]. IPF progression has often been assimilated to that of a neoplastic disease, and several signaling patterns appear to be disrupted in both conditions [1]. For the past decades, comprehensive sequencing programs have led to define cancer as, in essence, a genetic disease [2]. Cancer cells accumulate somatic DNA alterations that are responsible for oncogene activation or tumor suppressor gene silencing. Among cancer genes, the protein tyrosine kinase (TK) family plays a central role, and several of these enzymes have been found to be altered in cancers by a variety of molecular mechanisms. Kinases and their inhibitors, phosphatases, are key regulators of several cellular functions, and their appropriate activity is required for the cellular homeostasis; on the contrary, their aberrant activation is crucial in driving oncogenesis. The concept that cancer-mutated kinases molecularly mark “druggable” targets has resulted in intensive efforts to survey the kinome across a wide spectrum of human tumor types for mutations and to the development of several targeted inhibitors [3].

On this basis, we reasoned that, as in malignant proliferations, TK activation could play a role in IPF, although few data about molecular mechanisms involved in disease onset and progression are available. A confirmation of a role of TK activation pathways in IPF would make them actionable with specific molecules, in a similar fashion to cancer-targeted therapy.

Materials and Methods

We selected and analyzed 17 consecutive IPF samples derived from medical thoracoscopy cases from a cohort of patients aged ≥ 18 years who referred to our Institution for diagnosis and therapy. In all patients with IPF, the histopathologic examination revealed all of the major features of usual interstitial pneumonia (UIP) [temporally and

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Table 1. Clinical Characteristics of the Analyzed UIP and NSCLC Samples

ID Patient	Gender	Biopsy	Age at Diagnosis (Years)	Histology	Smoking Habit	Mutational profile	
						EGFR	KRAS
1	M	Thoracoscopy	59	UIP	Never smoker	wt	wt
2	M	Thoracoscopy	54	UIP	Past smoker	L861Q	wt
3	M	Thoracoscopy	66	UIP	Past smoker	wt	wt
4	M	Thoracoscopy	51	UIP	Current smoker	wt	wt
5	F	Thoracoscopy	64	UIP	Current smoker	wt	wt
6	F	Thoracoscopy	58	UIP	Current smoker	wt	wt
7	M	Thoracoscopy	63	UIP	Never smoker	wt	wt
8	M	Thoracoscopy	62	UIP	Current smoker	wt	wt
9	M	Thoracoscopy	71	UIP	Never smoker	wt	wt
10	M	Thoracoscopy	48	UIP	N.A.	wt	wt
11	M	Thoracoscopy	60	UIP	Never smoker	wt	wt
12	M	Thoracoscopy	55	UIP	N.A.	wt	wt
13	M	Thoracoscopy	65	UIP	Past smoker	wt	wt
14	M	Thoracoscopy	64	UIP	N.A.	wt	wt
15	M	Thoracoscopy	80	UIP	Past smoker	L858R	wt
16	M	Thoracoscopy	77	UIP	N.A.	wt	wt
17	M	Thoracoscopy	63	UIP	Past smoker	wt	wt
2	F	Transbronchial	70	ADC	Past smoker	L858R	wt
3	M	Endobronchial	65	ADC	Past smoker	wt	wt
4	M	Transbronchial	59	ADC	Never smoker	wt	wt
5	F	Transbronchial	67	ADC	Never smoker	del ex 19	wt
6	M	Transbronchial	74	ADC	Current smoker	wt	G12D
7	M	Transbronchial	75	ADC	Never smoker	del ex 19	wt
8	F	Transbronchial	66	ADC	Past smoker	G863D	wt
9	F	Endobronchial	58	ADC	Past smoker	wt	wt
10	M	Endobronchial	50	ADC	Past smoker	wt	wt
11	M	Transthacic	67	ADC	Past smoker	wt	wt
12	M	Transthacic	74	ADC	Current smoker	wt	wt
13	F	Transthacic	72	ADC	Current smoker	wt	G13D
14	M	Endobronchial	62	ADC	Never smoker	wt	wt
15	F	Endobronchial	79	ADC	Never smoker	wt	wt
16	F	Endobronchial	56	ADC	Never smoker	wt	wt
17	M	Endobronchial	64	ADC	Current smoker	wt	G12D
18	M	Endobronchial	73	ADC	Past smoker	wt	wt
19	M	Transthacic	78	ADC	Past smoker	del ex 19	wt
20	F	Transthacic	68	ADC	Past smoker	wt	wt

architecturally heterogeneous interstitial fibrosis, with fibroblast foci (FF), microscopic honeycombing, subpleural and periseptal accentuation, and absence of histologic features specific of other diseases], which is a prerequisite for the diagnosis of IPF. The final diagnosis of IPF was based on the diagnostic criteria of the American Thoracic Society/European Respiratory Society Consensus Classification System after evaluation of all clinical, laboratory, and instrumental data [4,5]. We also checked 40 non-small cell lung cancer (NSCLC) samples [20 adenocarcinoma (ADC) and 20 from squamous cell cancer] obtained through endobronchial, transbronchial, or transthacic biopsy. Clinical characteristics of cases analyzed are reported in Table 1.

Immunohistochemical (IHC) analysis was performed with antibodies against phospho-mammalian target of rapamycin (P-mTOR) (1:100, rabbit monoclonal, clone 49 F9; Cell Signaling Technology, Danvers, MA), phosphatase and tensin homolog (PTEN) (1:400, mouse monoclonal, clone 6H2.1; Dako, Cernusco sul Naviglio, MI, Italy), phospho-MET (P-met) (1:100, rabbit monoclonal, clone D26; Cell Signaling Technology), and phospho-ezrin/radixin/moesin (P-ERM) (1:300, rabbit monoclonal, clone 41A3; Cell Signaling Technology) on 4-µm-thick paraffin sections. Tissue sections were incubated at 60°C overnight and then deparaffinized. The slides underwent 40 minutes of heat-mediated antigen retrieval in citrate buffer (pH 6) and incubated for 20 minutes with ready-to-use normal horse blocking serum. Primary antibody was incubated overnight at +4°C. After inhibition of endogenous peroxidase, the reactions were revealed with ImmPRESS

Anti-Goat Ig detection system (Vector Laboratories, Burlingame, CA), using DAB tetrahydrochloride as chromogen substrate (Dako). Finally, all slides were counterstained with Harris hematoxylin to visualize the nuclei. Each reaction set included a negative control obtained with substitution of the primary antibody with dilution buffer and positive controls as suggested by the manufacturer. Immunostained slides were examined to identify the cell types expressing antigen and to semiquantitatively score the amount of protein present in the lung.

For each case, genomic DNA was manually microdissected from fibrotic areas highlighted on hematoxylin and eosin-stained sections and processed for mutational analysis. Normal DNA was extracted from healthy areas adjacent to fibrotic lesions and normal tissues from lobectomies and used as control. The expression the mTOR and MET kinases of the PTEN phosphatase and of ERM proteins was assessed with IHC stains; the stained slides were reviewed by the study pathologist (P.M.), and the results were classified as positive when strong immunostain was observed and negative in absence of immunostain. The presence of faint but specific (i.e., negative background) immunostain was also recorded. *Epidermal growth factor receptor (EGFR)* and *KRAS* mutational status was analyzed by real-time polymerase chain reaction as previously described [6]. Results were properly compared to a series of NSCLC samples (ADC) and squamous cell cancer as well as to normal lung tissue.

Results

Here, we report the results of a preliminary screening performed on a series of IPF and lung cancer cases aimed at comparing the expression of a panel of key molecules whose pathways are known to drive NSCLC onset and progression [3]. In detail, we checked the status of the EGFR and MET receptors together with that of the downstream transducer KRAS and of intracytoplasmic signaling molecules as the mTOR, the PTEN, and the ERM protein complex. Molecular pathways in study are described in detail in Figure 1A.

Our preliminary data in IPF samples showed strong phospho-mTOR immunoreactivity and scarce PTEN expression in activated type II pneumocytes lining FF. Phospho-ERM was expressed on the luminal and lateral cytoplasmic membranes of these cells. MET was expressed in both epithelial and stromal cells, whereas PTEN was exclusively expressed in myofibroblasts of FF. A similar immunoprofile in both epithelial and stromal cells was demonstrated in cancers, whereas in normal lungs, only m-TOR and PTEN were expressed at low levels exclusively in bronchiolar epithelia. Immunophenotypes found are illustrated in Figure 1B. We then moved to check the *EGFR* and *KRAS* mutational profile of each analyzed sample. Two of the 15 analyzed samples carried an *EGFR* mutation, in both cases affecting the exon 21. The somatic origin of the mutations found was confirmed by processing, together with IPF DNA, normal matched DNA: mutations were found only in IPF specimens, whereas adjacent normal areas showed wild-type *EGFR*. No changes were documented in the hot-spot encoding region of the *KRAS* gene. Results are summarized in Figure 1C.

Discussion

Although preliminary and limited, our findings allow drawing some relevant considerations. Increased mRNA and protein levels of EGFR have recently been described in patients with IPF [7]. Notably, we are reporting for the first time in IPF the presence of activating mutations in the exon 21 of EGFR coding sequence, which in NSCLC are known to be associated to sensitivity to targeted inhibitors [3]. EGFR mutational incidence in IPF seems to be high (13%), comparable to that occurring in NSCLC.

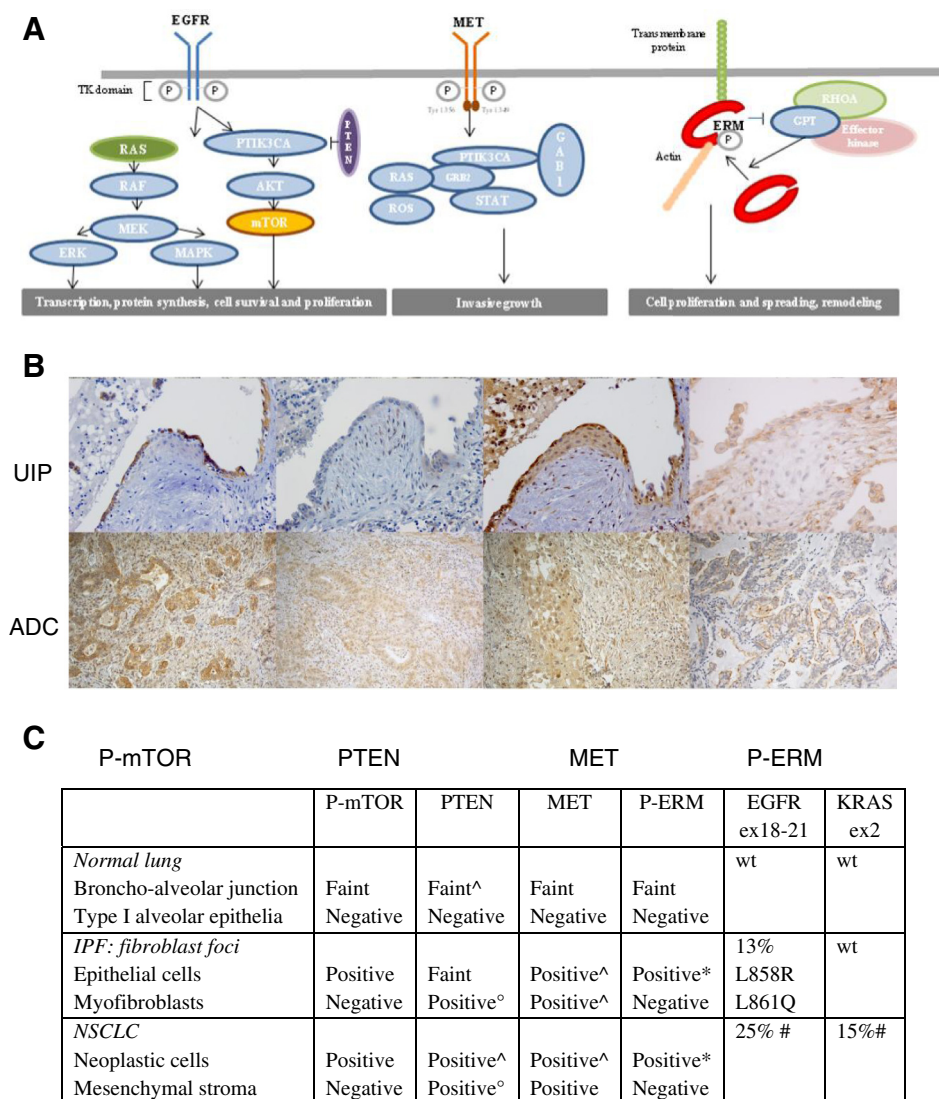


Figure 1. Oncogenic activation in IPF. (A) The molecular oncogenic pathways analyzed. EGFR activation triggers the following two main signaling pathways: KRAS-BRAF-MEK pathway, which, by activating ERK and the mitogen-activated protein kinase, sustains cell proliferation; and PIK3CA-AKT-mTOR axis, which is mainly involved in cell survival and motility. Indeed, mTOR regulates cell growth by controlling mRNA translation, ribosome biogenesis, autophagy, and metabolism. MET receptor is the key player of invasive growth (or EMT), a physiological process that occurs during embryonic development and postnatal organ regeneration and that is usurped by cancer cells. MET activation induces intramolecular phosphorylation of two tyrosine residues (Tyr¹³⁴⁹ and Tyr¹³⁵⁶); the latter creates a unique docking site responsible for the recruitment of a large spectrum of downstream signal transducers, which ultimately activate the invasive growth program (scattering, migration, proliferation, survival, and differentiation). It behaves as a sensor of adverse microenvironmental conditions (hypoxia and radiation), drives cell invasion and metastasis, and mediates adaptive resistance through the transcriptional activation of a set of genes that control blood coagulation [1]. Phosphorylation of ERM leads to weakened self-association and translocation to membrane-cytoskeleton interface. Active ERM interacts directly with actin and with several transmembrane- or membrane-associated partners, thus contributing to tumor development and metastasis by causing cell depolarization, loss of contact-dependent inhibition of proliferation, and increased motility and invasiveness. Activation of GTPase and RHOA leads to activation of ERM through the activation of an effector kinase. (B) Comparative sections of oncogenic immunostaining in UIP and lung cancer (ADC). Immunohistochemistry analysis of phosphorylated (P) oncomarkers expression in UIP compared to lung ADC. (C) Overall expression of oncogenes and oncosuppressors. Schematic distribution of oncogenic activation in UIP compared to NSCLC and normal lung. *, apical membrane; ^, nuclear and cytoplasmic; °, nuclear; when not otherwise specified, the immunostain was cytoplasmic. #, mutational frequencies were comparable to already available data from COSMIC database (website at <http://www.cancerxgene.org>). [1] Bocaccio C, Comoglio PM (2006). Invasive growth: a MET-driven genetic programme for cancer and stem cells. BRAF; v-RAF murine sarcoma viral oncogene homolog B1, MEK; also known as MAPKK, mitogen activated protein kinase (MAPK) kinase, ERK; extracellular signal-regulated kinase, PIK3CA; phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha, RhoA; Ras homolog gene family, member A, COSMIC; Catalogue Of Somatic Mutations In Cancer. *Nat Rev Cancer* 6, 637–645.

It should be noted that there are many similarities between the pathogenesis of lung cancer and IPF. Smoking is strongly associated with IPF and is a strong negative predictive factor for tumors with

EGFR mutations according to previous reports. The issue of *EGFR* mutation incidence and smoking habit focuses on the following two points: the frequency of mutation detection in smokers on one

hand and the effects of cigarette smoking on mutated EGFR tumors on the other. Cigarette smoking is the major cause of lung cancer (about 75% of cases) that, in turn, is the leading cause of death in the Western world [3]. Although early studies reported *EGFR* activating mutations in ADC aroused in female patients with East Asian ethnicity and never or light smokers [8], it is now known that mutations can be also found in ADC specimens from men and people who smoke cigarettes [9,10]. In IPF, the prevalence of tobacco use ranges from 41% to 83% [11,12]; whereas no data are available, to our knowledge, about *EGFR* mutational incidence in IPF. Within the limits of the cohort analyzed in the present study, both the two patients with mutated IPF were previous smokers (<30 pack-years), but also patients with *EGFR*-mutated cancer had a history of cigarette smoking (Table 1). The second point is that cigarette smoking dosage of ≥ 30 pack-years has been reported to be an independent negative predictive factor of EGFR-TK inhibitor (TKI) treatment outcome in patients with lung ADC with activating *EGFR* mutations [10]. Potential explanation for this correlation has been related to the fact that cigarette smoking not only activates EGFR but also stabilizes the EGFR protein by preventing from ubiquitination and degradation, remaining membrane bound or trafficked to perinuclear region. Thus, exposure to cigarette smoke results in prolonged signaling by the EGFR and may contribute to uncontrolled lung cell growth [13]. Moreover, preclinical investigation conducted by Filosto et al. also suggested that cigarette smoking induces conformational change of EGFR, resulting in downstream activation through c-Src and caveolin 1 binding [14]. We should only hypothesize that smoking could act on mutated EGFR aroused in IPF in a similar fashion to that in cancer, but experimental data are clearly required, and this point goes beyond the scope of this paper.

EGFR mutation detection in IPF is unlikely to predict sensitivity to specific agents. It should be underlined that, because real-time polymerase chain reaction sensitivity enables the identification of mutations in samples containing less than 30% mutated cells, which can be otherwise missed by direct sequencing [15], we probably identified an emerging clone of *EGFR*-mutated cells in a genetically heterogeneous population, whose role in the progression of lung fibrosis or, possibly, in oncogenesis needs further investigation. Indeed, due to the multiclonal FF cellularity, the emergence of an oncogenic phenotype in IPF is unlikely to be read in a context of “oncogenic addiction” [16], which is considered the driving force of malignant proliferation.

Nevertheless, it should be underlined that a relation subsists between NSCLC associated with ILD and *EGFR* mutations [17–19]. Indeed, it has been reported that *EGFR* mutation is rare in Asian patients with ILD and lung cancer. In particular, an inverse association has been reported between occurrence of ILD and tumors with *EGFR* mutations in patients with lung ADC [19]. From this perspective, the finding of *EGFR*-mutated cells in the fibrotic area points out some relevant considerations. First of all, it is well documented that treatment with EGFR TKIs gefitinib and erlotinib is associated with a significant increase in the risk of developing both all-grade and fatal ILD events in advanced *EGFR*-mutated NSCLC [20]. In those settings, the occurrence of ILD is a secondary—iatrogenic—event, although the bimolecular mechanisms of ILD induction have not been yet clarified. A different question is that associating ILD and lung cancer and two different links may be identified. The first is that, within respect to IPF, growing evidence suggests that this process is driven by pathogenic events very similar to cancer, including epigenetic and genetic changes, altered response to

regulatory signals, abnormal expression of microRNAs, and activation of specific signaling pathways [1]. IPF also resembles cancer with regard to its poor response to medical treatment and prognosis. The other is that ILD, and mainly IPF, most often coexists with cancer as concomitant disease. In this scenario, ILD seems to be inversely associated to the occurrence of *EGFR* mutation in lung cancer.

The EGFR is a member of the EGFR receptor family TKs that represent both key regulators of normal cellular development and critical players in a variety of pathophysiological phenomena, among which is cancer [21]. In NSCLC, EGFR inappropriate activation is mainly due to the occurrence of somatic mutations affecting the sequence encoding for receptor TK domain. Mutation detection has been found to be closely linked with favorable response to the anti-EGFR TKIs gefitinib and erlotinib, according to the “oncogenic shock” model [22].

Although very preliminary and performed on a limited cohort of cases, our findings reported the occurrence of *EGFR* mutations in IPF areas, with an incidence that seems not to be trivial. Notably, the fibrotic *EGFR*-mutated samples analyzed here are not aroused after an anti-EGFR therapy nor are associated to a synchronous carcinogenic process. It is well known that, in normal airway, EGFR expression is low and only transiently increased during repair [23]. The EGFR pathway has been implicated in lung fibrosis pathogenesis through the activation of an EGFR-dependent paracrine loop between epithelial and fibroblast cells, resulting in excessive collagen production and deposition [24]. From this perspective, clonal heterogeneity that characterizes FF—in contrast to monoclonality that is a hallmark of cancer—brings into question the role of EGFR activation by mutation in lung fibrogenetic process and if it could be therapeutically exploited in a similar way of cancer-targeted therapies. On the basis of the biologic functions of the receptor of EGF [25,26], we could hypothesize that its activation is required in FF to induce cell proliferation and also to prevent apoptosis in a context of cross talk between pneumocytes and myofibroblasts. It is unlikely that fibroblasts may rely (or “be addicted to”) on this sustained EGFR activity for growth and proliferation. Nevertheless, there are no elements to exclude that the *EGFR*-mutated cellular fraction could represent an early marker of malignant transformation arising inside the fibrotic landscape, because mutation of the TK domain of EGFR is an early event in the pathogenesis of lung ADCs [27]. Further experimental data are required to validate our very preliminary findings and to clarify the many questions that remain open on the role played by EGFR in fibrogenesis.

Quite unexpectedly in such a heterogeneous context, the analyzed kinases seem to be distributed according to a spatial gradient, throughout the cell layers of the FF [28]. Interestingly, a similar profile of expression was observed at the interface between epithelial neoplastic cells and tumor stroma in most NSCLCs. As discussed above, it could be hypothesized that IPF fibroblasts may rely on TK activation for their inappropriate proliferation and that the specific TK phosphorylation could be a consequence rather than the cause of the proliferating phenotype, or that fibroblast proliferation is driven through abnormal signaling by epithelial cells, in a similar fashion as that observed in stromal proliferation in epithelial tumors [29]. The mTOR is an intracellular serine/threonine protein kinase that has been identified as a major link in the cellular processes that contribute to the development and progression of cancer [30]. As in cancer, in IPF, mTOR expression may directly impact the translational capacity of the epithelial cells, thus sustaining their proliferation. As far as ERM is concerned, it is active in organizing the cell cortex and in

regulating cell polarity during epithelial morphogenesis, a process that is often disrupted in tumorigenesis [31]. Due to its function as scaffold in supporting cell growth and promoting the proliferative frontline, we hypothesized that ERM could potentially be implicated in IPF proliferative processes. However, we did not document a significant activation of phospho-ERM in cells of the FF or in NSCLC. The profile of PTEN expression is more puzzling. We observed clear and strong nuclear PTEN reactivity in FF mesenchymal cells. This finding is at odds with reported data and with the knowledge on PTEN function: its loss of function rather than overexpression has been associated with cancer progression and pulmonary fibrosis through reduced apoptosis, and previous studies reported the absence of IHC PTEN expression in IPF myofibroblasts [32]. Given the complex mechanisms of PTEN regulation, protein expression does not necessarily imply increased activity; thus, this aspect also needs further clarification. Finally, we demonstrated that both myofibroblasts and epithelial cells of FF harbor MET, the TK receptor for scatter factor/hepatocyte growth factor (HGF) [3] in its activated form. It has been suggested that low levels of HGF in the fibrotic lung may contribute to the development of lung fibrosis by inhibiting epithelial-to-mesenchymal transition (EMT) [33]; however, several evidences point toward a role of EMT in the formation of FF in IPF [34]. We have now shown that the HGF receptor MET is specifically and strongly expressed in FF cells, thus suggesting that, besides the reported dysregulation of cadherins [35], the activation of MET could have a role in the inappropriate activation of EMT in IPF.

Overall, these data reveal that IPF landscape is enriched in neoplastic potential expressed in a context of complex genomic polyclonality and cellular heterogeneity. Rather than being a driving mechanism conferring clonal growth advantage, TK activation may represent a tactic exploited in IPF to promote continued and diffuse cell growth and proliferation. On this perspective, pharmacological targeting of oncogenic molecules in IPF may represent an approach to hamper progression rather than to affect cell growth and survival (addiction).

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