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DETECTION OF SQUAMOUS CELL CARCINOMA ANTIGEN (SCCA) IN EARLY AND END STAGE IDIOPATHIC PULMONARY FIBROSIS (IPF): MOLECULAR SUBSTRATES AND CLINICO-PATHOLOGICAL CORRELATIONS

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A Glenda che c'è sempre…

… e ad Alice che verrà

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ABSTRACT

BACKGROUND

Idiopathic pulmonary fibrosis (IPF), morphologically characterized by usual interstitial pneumonia (UIP), represents a progressive disease of unknown aetiology that continues to be associated with poor prognosis. The cardinal pathological features are the epithelial damage/activation, fibroblastic/myofibroblastic foci formation and extracellular matrix remodelling. The current paradigm suggests a pivotal role of the epithelium in the disease pathogenesis. Epithelial injury and subsequent deregulated repair results in profibrogenic cytokines (like TGF-β1) release with consequent abnormal mesenchymal cell activation and proliferation. Therefore, epithelial instability seems a crucial step in the development and progression of the disease, including neoplastic transformation. Few molecular tissue markers have been studied in IPF in order to clarify the pathogenetic mechanisms leading to the disease. Squamous cell carcinoma antigen (SCCA) is a serine protease inhibitor (serpin) physiologically found in the normal squamous epithelium and typically expressed by dysplastic and neoplastic epithelial cells of various origin, more often in squamous cell tumours. No information is actually available on its expression in IPF.

MATERIAL AND METHODS

In this study we analysed SCCA and TGF-β1 expression in surgical open-lung biopsies from 22 IPF patients with early-stage disease (GROUP A), in native lungs from 48 IPF patients with end-stage disease (GROUP B) who underwent to lung transplantation and in 20 control cases (GROUP C, 10 normal lungs from cadaveric donors, 10 lungs from patients with other interstitial diseases).

In vitro study using A549 pneumocytes was also conducted to investigate the relationship between SCCA and TGFβ1 expression. SCCA and TGFβ1 epithelial expression were evaluated by immunohistochemistry and reverse transcription-

polymerase chain reaction (RT-PCR). Time course analysis of TGF-β1 expression in A549 pneumocytes incubated with different SCCA concentrations was assessed by real time RT-PCR. The quantitative immunohistochemical assessment of SCCA and TGF-β1 was undertaken in each IPF sample by counting at least 500 cuboidal cells. A quantitative assessment of different pathological parameters (fibrosis, fibroblast foci and honeycombing changes) have been also considered in each samples. Clinical data including lung function and cardiovascular parameters were correlated to pathological features. In GROUP A lung function tests were re-evaluated at 8-12 months after biopsy.

RESULTS

Alveolar SCCA expression was present in IPF patients, but was not detected in alveolar cells in any of control cases. In GROUP A SCCA was positively correlated with the extension of fibroblastic foci (r=0.49, p=0.02), expression of TGF-β1 (r=0.78, p<0.0001) and with DLCO decline at follow-up (r=0.59, p=0.01). In vitro experiments showed that incubation of cultured cells with SCCA induced TGF-β1 expression, with a peak at 24 hours. In GROUP B SCCA and TGF-β1 values were high and positively correlated (r=0.45, p<0.001), while an inverse correlation was found between SCCA and DLCO (r=-0.43, p=0.005) and TGF-β1 and DLCO (r=-0.42, p=0.04). Interestingly, among metaplastic alveolar epithelial cells, a significant difference in SCCA expression was found between cuboidal, bronchialized and squamous cells, with increased expression for squamous cells that also presented a significant higher grade of dysplasia.

CONCLUSION

The over-expression of SCCA and TGF-β1 in the alveolar epithelium corroborates the hypothesis that disturbed epithelial alveolar regeneration and abnormal secretion of the cytokines are important steps in the pathogenesis of remodelling and fibrosis of IPF.

SCCA could have a double role influencing the epithelial proliferation (autocrine action) and promoting fibroblast proliferation/fibrosis through increased TGF-β1 secretion (paracrine action). SCCA may be considered a potential marker of disease activity being strictly correlated with impairing lung function.

RIASSUNTO

INTRODUZIONE

La fibrosi polmonare idiopatica (FPI), caratterizzata morfologicamente dal correlato patologico di polmonite interstiziale usuale (UIP), rappresenta una malattia ad eziologia sconosciuta, ad andamento progressivo e prognosi infausta.

Gli elementi anatomo-patologici cardine sono il danno/attivazione epiteliale, la formazione di foci fibroblastici/miofibroblastici ed il rimodellamento della matrice extracellulare. La teoria patogenetica più accreditata attribuisce un ruolo determinante alla disfunzione epiteliale alveolare. Il danno epiteliale ed i successivi meccanismi deregolati di riparo portano al rilascio di citochine pro-fibrogenetiche (come il TGF-β1) con conseguente attivazione e proliferazione di cellule mesenchimali. Di conseguenza, l'instabilità epiteliale sembra un elemento cruciale nello sviluppo e progressione della malattia, inclusa la trasformazione neoplastica. Pochi markers molecolari sono stati studiati e descritti fino ad ora nella FPI, per meglio chiarire i meccanismi patogenetici della malattia. Lo squamous cell carcinoma antigen (SCCA) è un inibitore delle serin proteasi (serpine) fisiologicamente presente nell'epitelio squamoso normale e specificamente espresso dalle cellule displastiche e neoplastiche epiteliali di varia origine, più spesso nei tumori a cellule squamose. Non sono ancora disponibili informazioni specifiche sulla sua espressione nella FPI.

MATERIALI E METODI

In questo studio abbiamo analizzato l'espressione di SCCA e TGF-β1 in tessuto polmonare ottenuto da biopsie chirurgiche di 22 pazienti affetti da FPI in stadio clinico iniziale (Gruppo A), in polmoni nativi di 48 pazienti con malattia end-stage e sottoposti a trapianto di polmone (Gruppo B) e in 20 polmoni controllo (Gruppo C, 10 polmoni normali ottenuti da donatori cadaveri, 10 polmoni di pazienti affetti da altre malattie interstiziali).

Abbiamo inoltre condotto uno studio in vitro utilizzando pneumociti della linea A549, per investigare il rapporto tra la produzione di SCCA e l'espressione di TGF-β1. L'espressione di SCCA e TGF-β1 nelle cellule epiteliali è stata valutata con tecniche di immunoistochimica e reazione a catena della polimerasi-trascrizione inversa (RT-PCR). La produzione di TGF-β1 nei pneumoniti A549 incubati a diverse concentrazioni di SCCA è stata verificata con real time PCR. La valutazione quantitativa immunoistochimica di SCCA e TGF-β1 è stata eseguita in ciascun campione contando almeno 500 cellule cuboidali. Una valutazione quantitativa dei differenti parametri patologici (fibrosi, foci fibroblastici e honeycombing) è stata eseguita ugualmente in ciascun campione. I dati clinici, inclusi la funzione respiratoria e i parametri cardiovascolari sono stati correlati ai dati patologici. Nel Gruppo A i test di funzionalità polmonare sono stati ripetuti a 8-12 mesi dalla biopsia.

RISULTATI

L'espressione di SCCA nelle cellule epiteliali alveolari era presente nei pazienti con FPI, mentre era assente nei controlli. Nel Gruppo A l'SCCA era correlato positivamente con l'estensione dei foci fibroblastici (r=0.49, p=0.02), l'espressione di TGF-β1 (r=0.78, p<0.0001) e con il declino della DLCO al follow up (r=0.59, p=0.01). L'esperimento in vitro ha dimostrato che l'incubazione di pneumociti con SCCA induceva l'espressione di TGF-β1, con un picco a 24 ore. Nel Gruppo B i valori di SCCA e TGF-β1 erano elevati e correlati positivamente (r=0.45, p<0.001), mentre vi era una correlazione inversa tra SCCA e DLCO (r=-0.43, p=0.005) e TGF-β1 e DLCO (r=-0.42, p=0.04). Tra le cellule epiteliali alveolari metaplastiche, abbiamo riscontrato una diversa espressione di SCCA tra le cellule cuboidali, bronchializzate e squamose, con una crescente espressione per le squamose che inoltre presentavano un maggior grado di displasia.

CONCLUSIONI

La over-espressione di SCCA e TGF-β1 nell'epitelio alveolare corrobora l'ipotesi che una alterata rigenerazione epiteliale alveolare e una secrezione anormale di citochine sono elementi importanti nella patogenesi del rimodellamento e della fibrosi della FPI. L'SCCA potrebbe avere un duplice ruolo influenzando la proliferazione epiteliale (azione autocrina) e promuovendo la fibrosi/proliferazione dei fibroblasti attraverso lo stimolo alla maggior secrezione di TGF-β1. L'SCCA potrebbe essere considerato un marker potenziale di attività della malattia essendo strettamente correlato con la perdita di funzione polmonare.

BACKGROUND

Definition and classification

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease of unknown aetiology, characterized by progressive and irreversible parenchymal fibrosis and ventilatory restriction, with an unfavourable outcome, leading ultimately to death due to respiratory failure. IPF is also referred to as cryptogenic fibrosing alveolitis (CFA), "lone CFA" (CFA not associated with the presence of collagen vascular disease), and usual interstitial pneumonia (UIP) [1]. To date, no treatment strategies have been effective in modifying the natural course of IPF and its fatal outcome. The median survival time for patients with IPF is less than 3 years and lung transplantation represents the only option for those patients who are refractory to medical therapy [2-5]. The fibrosis in IPF involves the interstitium, that is the space existing between the vascular endothelium and alveolar epithelium. In this critical space, gas is exchanged between red blood cells circulating in blood vessels and alveolar air. When this space is deranged by inflammatory cells or collagen deposition, gas exchange is impaired, and functional alveolar units are reduced [6]. Collectively, diseases that affect this space are called interstitial lung diseases (ILDs) and were initially described by Hamman and Rich in the 1940s [7]. IPF is a confusing term with myriad definitions over the years. This has led to variability in the literature about the specific lung-disease categories being evaluated.

In 1969 Liebow e Carrington first described histological features of chronic interstitial pneumonias classifying 5 forms: usual interstitial pneumonia, interstitial pneumonia with bronchiolitis obliterans and diffuse alveolar damage, desquamative interstitial pneumonia, lymphoid interstitial pneumonia and gigantocellular interstitial pneumonia [8]. In 1997 Katzenstein proposed a new classification in 4 subgroups: usual interstitial pneumonia, desquamative interstitial pneumonia, acute interstitial pneumonia, non
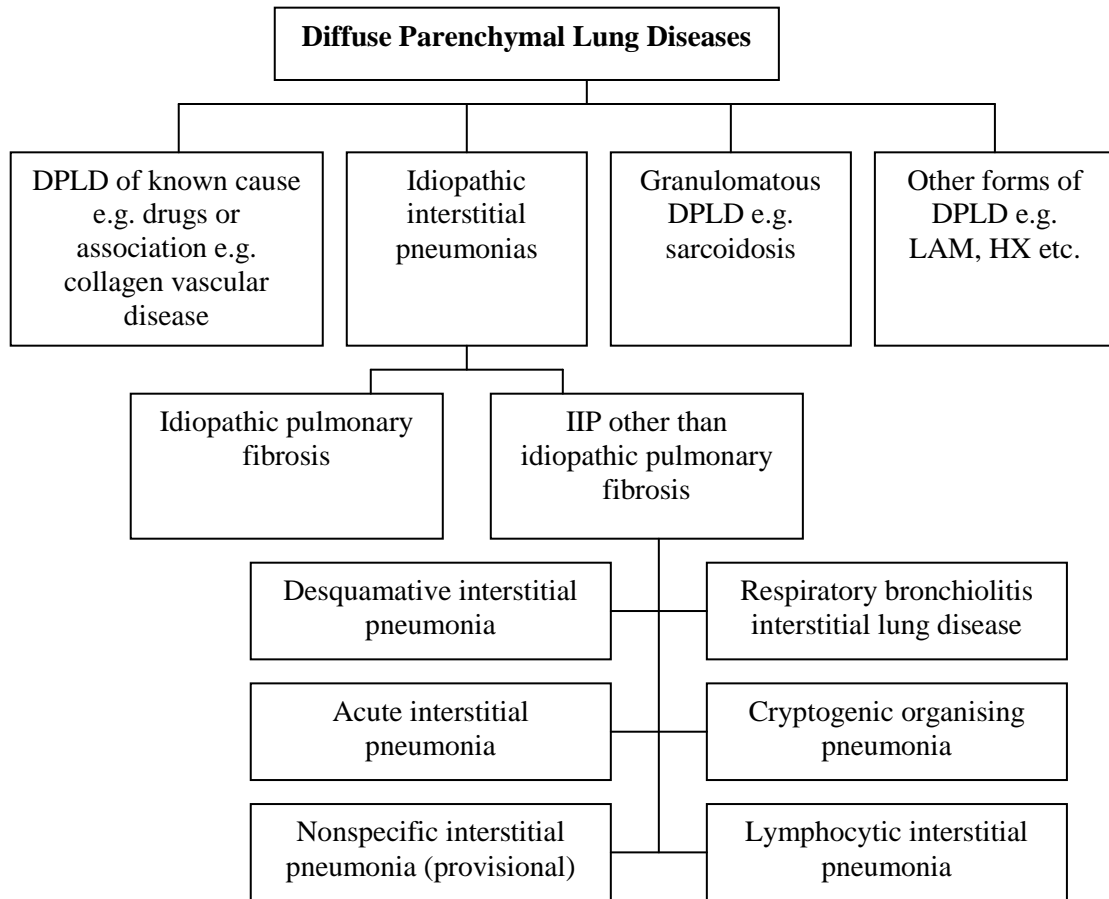
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specific interstitial pneumonia [9]. After that Muller e Colby introduced some modifications at this classification: usual interstitial pneumonia, desquamative interstitial pneumonia, bronchiolitis obliterans with organizing pneumonia, acute interstitial pneumonia, non specific interstitial pneumonia [10].

Recently, an international consensus statement defining the diagnosis, evaluation, and treatment of patients with idiopathic interstitial pneumonias was produced as a collaborative effort by the American Thoracic Society, the European Respiratory Society, and the American College of Chest Physicians [11]. The purpose of the consensus statement was to assist clinicians in the diagnosis and management of idiopathic interstitial pneumonias.

In particular, it has been established that IPF belongs to a family of lung disorders known as the interstitial lung diseases (ILDs) or, more accurately, the diffuse parenchymal lung diseases (DPLDs). Within this broad category of DPLDs, IPF belongs to the subgroup known as idiopathic interstitial pneumonias (IIPs) (Scheme 1).

There are seven distinct IIPs, differentiated by specific clinical features and pathological patterns: nonspecific interstitial pneumonia (NSIP), cryptogenic organizing pneumonia (COP), acute interstitial pneumonia (AIP), respiratory bronchiolitis-associated interstitial lung disease (RB-ILD), desquamative interstitial pneumonia (DIP), lymphoid interstitial pneumonia (LIP), and idiopathic pulmonary fibrosis/usual interstitial pneumonia (IPF/UIP). IPF is the most common of the IIPs, comprising 47-71% of cases [12,13]. The ATS/ERS classification defines IPF as ''a specific form of chronic fibrosing interstitial pneumonia of unknown aetiology, limited to the lung and associated with the histological entity of UIP'' [1]. Thus UIP and IPF are now seen by many as synonymous terms.

Scheme 1: Classification of interstitial lung diseases.

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                    ┌────────────────────────────────────┐
                    │  Diffuse Parenchymal Lung Diseases  │
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| DPLD of known cause e.g. drugs or association e.g. collagen vascular disease | Idiopathic interstitial pneumonias | Granulomatous DPLD e.g. sarcoidosis | Other forms of DPLD e.g. LAM, HX etc. |

| Idiopathic pulmonary fibrosis | IIP other than idiopathic pulmonary fibrosis |

Desquamative interstitial pneumonia	Respiratory bronchiolitis interstitial lung disease
Acute interstitial pneumonia	Cryptogenic organising pneumonia
Nonspecific interstitial pneumonia (provisional)	Lymphocytic interstitial pneumonia

*From: ATS/ERS International Multidisciplinary Consensus Classification of Idiopathic Interstitial Pneumonias. Am J Respir Crit Care Med 2002; 165:277-304.

Epidemiology

The incidence and prevalence of IPF are difficult to determine because uniform diagnostic criteria have only recently been defined [11]. Historical information relating to statistics relied on population studies which utilized diagnostic coding data and death certificates to identify cases. The best available data suggests an incidence of approximately 10.7 per 100,000 persons for men; and 7.4 per 100,000 persons for women. The prevalence of IPF is slightly greater at 20.2 men per 100,000 and 13.2 women per 100,000 [14-16]. The incidence, prevalence, and death rate of IPF increase with age [14, 17, 18]. A large U.S. population based study [14] found that the prevalence of IPF was only 2.7 cases per 100,000 amongst those aged 35 to 44 years-old; meanwhile, 175 cases per 100,000 were found among persons over the age of 75 years. IPF most commonly appears between the fifth and seventh decades of life, with two-thirds of all cases arising in patients over 60 years of age [11, 19]. The mean age at presentation is 66 years old [3, 11, 19].

Familial cases of IPF accounts for 0.5 to 2% of all cases of IPF. Clinical features of familial IPF are indistinguishable from those of the sporadic form, excepting for an earlier age of onset [20]. Data from around the world demonstrates that IPF favours no particular race, ethnic group or social environment. It is estimated that IPF affects at least 5 million persons worldwide. It also appears that, during the last decade, the incidence of IPF was on the rise [21], thus suggesting that IPF should no longer be considered a rarity (so-called orphan disease).

Clinical features and diagnosis

IPF is usually fatal, with an average survival of approximately three years from the time of diagnosis [22-25]. However, the disease course in IPF is variable, with many patients remaining stable for long periods of time while a significant proportion experience exacerbations leading to respiratory failure and death [26]. This variability leads to a need for the early and accurate diagnosis and early referral for lung transplantation. New insight into the natural history of IPF has been gleaned from secondary analysis of the placebo groups assembled for recent multi-center clinical trials [27, 28]. It seems that three potential clinical courses exist: a) slowly progressive disease (the most common); b) disease marked by episodic acute exacerbations; and c) rapidly progressive disease [29]. At present, there are no means for accurately predicting the clinical course.

The clinical manifestations include dyspnoea on exertion, dry cough, and inspiratory crackles, with or without digital clubbing noted on physical examination. Retrospective analysis of IPF patients suggests that symptoms precede diagnosis by a period of 6 months to 2 years [29].

Chest radiography and high-resolution computed tomography (HRCT) typically show patchy, predominantly peripheral, subpleural and in the lower lung zone reticular opacities [11, 30].

HRCT also shows variable but limited ground-glass opacity (usually associated with traction bronchiectasis) and subpleural honeycombing (Figure 1).
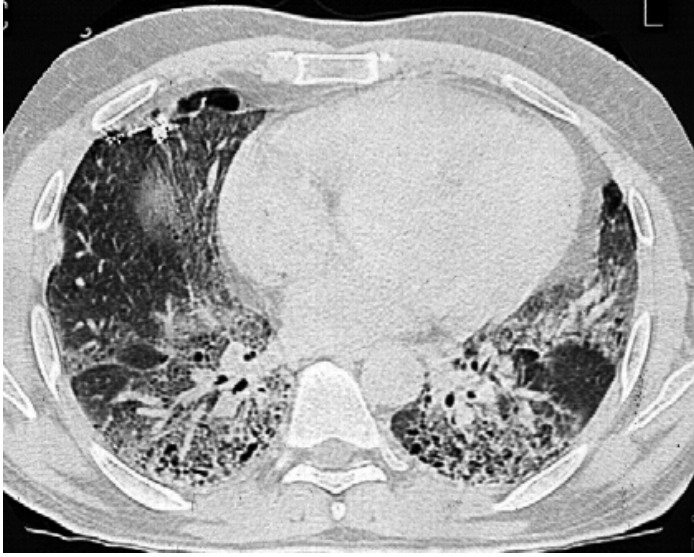
Figure 1. *Chest CT-scan of a patient affected by IPF. Several radiological features are evident: peripheral reticular opacities, associated with ground-glass areas and traction bronchiectases. Small honeycombing areas are also evident.*

Confluent alveolar opacities, evidence of pleural disease, or lymphadenopathy suggest another diagnosis [30, 31]. Routine spirometry reveals decreased values of forced vital capacity (FVC), forced expiratory volume in one second (FEV1) and total lung capacity (TLC). The ratio of FEV1/FVC remains normal in IPF, consistent with restrictive pattern consequence of reduced pulmonary compliance. Gas exchange is impaired in IPF which can be demonstrated by reduced diffusing capacity for carbon monoxide (DLCO), and arterial hypoxemia exaggerated or elicited by exercise [32-35].

The actual "gold standard" diagnosis of IPF consists of clinical-radiological-pathological correlation as defined by the American Thoracic and European Respiratory Societies (ATS/ERS) guidelines [11]. According to the guidelines, the diagnosis of IPF can be considered definitive only in the presence of a surgical (not transbronchial) lung biopsy.

The **definite diagnosis** of IPF requires all of the following:

• Surgical lung biopsy revealing a histologic pattern consistent with UIP

• Exclusion of other known causes of interstitial lung disease (*e.g.*: connective tissue disease, environmental exposure, *etc*.)

• Abnormal pulmonary physiology with evidence of restriction and/or impaired gas exchange (can exist during exercise alone)

• HRCT demonstrating a pattern of "confident" or "possible" IPF.

In the absence of a surgical biopsy, the diagnosis of IPF remains uncertain. Therefore, a set of reproducible clinical criteria were developed to define the **probable diagnosis** of IPF in cases in which a surgical biopsy is not possible. By consensus opinion, it has been established that IPF can be reasonably diagnosed if all four major criteria and three-out-of-four minor criteria are satisfied. They are as follows:

Major criteria

• Exclusion of other known causes for interstitial lung disease (such as drug toxicity, environmental exposure and connective tissue disease)

• Abnormal pulmonary function testing that includes evidence of restriction (reduced VC often with an increased FEV1/FVC ratio) and/or impaired gas exchange (increased A-a gradient or decreased diffusion capacity)

• Bibasilar reticular abnormalities with minimal ground glass opacities on HRCT scans (a "confident" HRCT is preferred)

• Transbronchial lung biopsy or bronchoalveolar lavage (BAL) does not support an alternative diagnosis

Minor criteria

• Age > 50 yr

• Insidious onset of otherwise unexplained dyspnoea on exertion

• Duration of illness ≥ 3 months

• Bibasilar, inspiratory crackles (dry or "Velcro" type in quality)

Pathological features

Usual interstitial pneumonia (UIP) is the classic pathologic description of IPF [1, 11].

The cardinal feature of UIP is a bilateral, heterogeneous process, which predominates in

the peripheral and subpleural regions of the lower lobes [9, 11]. Commonly, the spatial

and temporal heterogeneity of healthy lung, interstitial inflammation (not always

present), fibrosis, and honeycomb change can be observed at low magnification.

Aggregates of proliferating fibroblasts and myofibroblasts within fibroblastic foci are

additional cardinal features of UIP (Figure 2).

Alveolar walls are thickened by collagen, extracellular matrix, and mild-to-moderate

infiltration by lymphocytes, plasma cells, and histocytes. Hyperplasia of type II

pneumocytes is also noted. Honeycomb cysts are composed of cystic, fibrotic airspaces.

These spaces are frequently lined by bronchiolar epithelium and filled with mucin.

Sometimes, other features like organizing pneumonia, diffuse alveolar damage and
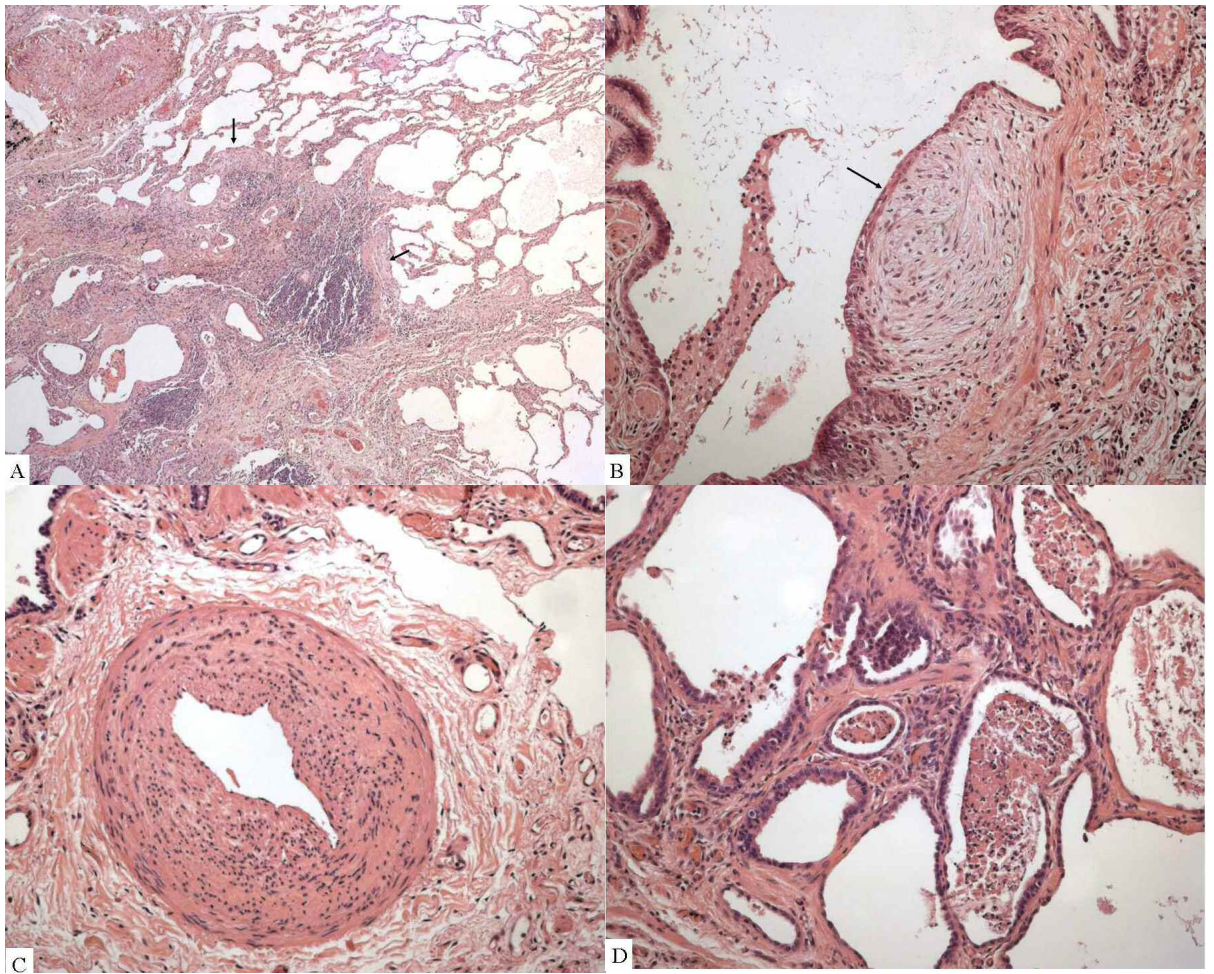
capillary haemorrhages can be detected [36, 37].

Figure 2. Usual Interstitial Pneumonia: histology. **A)** *Low-power microphotograph (original magnification x25, hematoxylin and eosin stain) demonstrates temporal heterogeneity with abrupt transition between normal appearing lung (upper right) and* **B)** *High-power microphotograph (original magnification x 200, hematoxylin and eosin stain) demonstrates fibroblastic focus comprised of plump spindle cells and collagen-poor matrix bulging into an airway lined by hyperplastic/cuboidal cells (arrow).* **C)** *High-power microphotograph (original magnification x 200, hematoxylin and eosin stain) shows secondary vascular remodelling in an area of honeycomb change with marked fibro-intimal hyperplasia and medial hypertrophy of a pulmonary artery resulting in significant narrowing of the vascular lumen.* **D)** *High-power microphotograph (original magnification x 100, hematoxylin and eosin stain) shows subpleural regions of extensively remodelled lung parenchyma with honeycomb cystic changes. The honeycomb cysts are lined by bronchiolar epithelium and filled with inspissated mucus containing acute inflammatory cells and macrophages.*

Etiology

The trigger that initiates the development of fibrosis in IPF still remains unknown. However, there is increasing evidence that a wide range of potentially injurious factors may play a role in the initiation and progression of IPF [38, 39]. Taking into account the presumed long preclinical phase of the disease, it is also possible that a combination of different types of injuries may act on a genetically susceptible individual to trigger the disease.

Oxidative stress, environmental pollutants and dusts have all been implicated as potential causes of IPF [19, 38, 40]. An environmental etiology for IPF is supported by several evidences [40, 41] and it has been demonstrated by case-control studies analogously to known disease, such as asbestosis, in which environmental material is associated with pulmonary fibrosis.

Meanwhile, cigarette smoking is strongly associated with IPF [1]. One study reported a correlation between smoking history (20–40 pack-years) and risk for IPF, with an odds ratio of 2.3 (95% confidence interval, 1.3 to 3.8) for smokers [42]. A recent study of familial pulmonary fibrosis looked at 309 affected individuals [43]. After adjusting for age and sex, this cohort demonstrated a strong association between smoking and IPF (odds ratio [OR], 3.6; 95% confidence interval [CI], 1.3–9.8). A multi-center case-control study conducted in the United States included 248 patients with IPF and 491 matched control subjects [44]. This study demonstrated significant associations between IPF and a) cigarette smoking (OR, 1.6; 95% CI, 1.1–2.4); b) silica exposure (OR, 3.9; 95% CI, 1.2–12.7); and c) exposure to livestock (OR, 2.7; 95% CI, 1.3–5.5).

Studies of viral respiratory tract infection in patients with IPF suggest an increased prevalence of past infection [45-48]. Furthermore, there is some suggestion that IPF patients have suffered infection with specific subtypes of herpesvirus particularly associated with the induction of somatic mutation [46-49]. The positive viruses in IPF

include cytomegalovirus, EBV, and human herpesvirus (HHV)–7 and HHV-8 [46, 49, 50]. However, it is important to note that most patients with IPF are under immunosuppressive therapy prior to biopsy, thus making the results somewhat difficult to interpret. Therefore, the etiologic significance of the viral infection in IPF remains to be determined. Some studies examining the role of gastroesophageal reflux (GER) in individuals with IPF have found a high prevalence of GER compared with normal individuals and patients with other interstitial lung diseases of known cause [51, 52]. These studies suggest that acid-aspiration–induced epithelial injury may contribute to the development of IPF. However, abnormal esophageal acid exposure by GER is frequent in the normal population thus, its putative role in IPF still needs to be systematically studied. Up to 20% of patients with IPF have a family history of the disease, with an autosomal dominant inheritance and variable penetrance, and some candidate genes have been identified [53-56]. Early studies focused on the major histocompatibility complex and study targets have included surfactant proteins, cytokines, chemokines, immunomodulatory factors, extracellular matrix proteins and those involved in the coagulation pathways [57]. Recently, it has been shown that mutant telomerase is associated with familial IPF, and telomere shortening is a process that may contribute to the pathogenesis. Short dysfunctional telomeres activate a DNA-damage response that leds to alveolar cell death and fibrotic lesions [58].

Pathogenesis

Since the mid-1980s, remarkable strides have been made in the understanding of the pathogenesis of IPF. Despite that, at this time the exact pathogenetic mechanisms at the basis of IPF are still unclear. Several hypotheses have been proposed to explain fibrogenesis in IPF [59].

Inflammatory theory

Initial pathogenetic hypotheses were shaped largely by concepts developed from studies of wound models. It was believed that, in fibrosis, an exaggerated and uncontrolled healing response occurs, in which the key initiating features are inflammatory cell influx and release of pro-fibrotic products [60]. This "inflammatory fibrosis" hypothesis asserts that chronic inflammation injures the lung and modulates fibrogenesis, leading to the end-stage fibrotic scar [61].

Several of the key concepts that formed the basis for the inflammatory hypothesis have been demonstrated to be not valid:

- Inflammation is not a prominent histopathologic finding in usual interstitial pneumonia. Careful review of larger numbers of better defined cases showed that the inflammatory component is usually mild, occurs mainly in areas of collagen deposition or honeycomb change, and rarely involves otherwise unaltered alveolar septa [9]. Finally, interstitial lung diseases in which inflammation is a prominent feature of early disease (for example, hypersensitivity pneumonitis) often do not progress to end-stage fibrosis.
  - Inflammation is not required for the development of a fibrotic response.
  - Clinical measurements of inflammation fail to correlate with stage or outcome in idiopathic pulmonary fibrosis.
  - Anti-inflammatory therapy does not improve disease outcome.

Epithelial/mesenchymal cells theory

More recent data suggest that inflammation does not play a major role in inducing the initiation of the disease. A growing body of researchers now argue that fibrosis proceeds independently of inflammatory events. Instead, they suggest that fibrosis develops as the consequence of aberrant epithelial and epithelial–mesenchymal responses to chronic alveolar epithelial injury and activation that provoke the migration, proliferation, and activation of mesenchymal cells with the formation of active fibroblastic/myofibroblastic foci, leading to the exaggerated accumulation of extracellular matrix (ECM) and mirroring abnormal wound repair [62].

Current hypotheses propose that progressive lung fibrosis results from dysregulated function of, and/or communication between, epithelial and mesenchymal cells leading to a "vicious cycle" of epithelial cell injury and mesenchymal cell responses. [62-65]. Myofibroblasts, key effector cells in fibrogenesis, aggregate within fibroblastic foci of UIP, the histopathologic correlate of IPF [66]. These small aggregates of actively proliferating myofibroblasts and fibroblasts constitute many microscopic sites of ongoing acute alveolar epithelial injury and activation associated with evolving fibrosis [9, 66, 67]. The presence/extent of fibroblastic foci correlates with poor outcomes in IPF. [9, 68], Overlying the fibroblastic foci are areas of damaged basement membrane and denuded epithelium [69]. Additionally, alveolar epithelial cells (AECs) that are in close association with myofibroblasts have phenotypic changes that suggest the activation of a stereotypic wound-repair response [70]. Supporting this concept, studies of IPF tissue have reported evidence of AEC proliferation and regenerative hyperplasia, [71] bronchiolar and squamous metaplasia, and apoptosis [72, 73].

The mechanism(s) underlying dysregulated epithelial and mesenchymal cell phenotypes in pulmonary fibrosis remain poorly understood.

Evidence suggests that these complex cellular interactions are mediated by soluble factors acting through autocrine and paracrine mechanisms. Epithelial cells are the primary source of mediators capable of inducing fibroblast migration, proliferation, and activation as well as extracellular (ECM) accumulation in IPF [74, 75].

Additionally, the ECM itself contributes both directly and indirectly to the aberrant cellular phenotypes seen in pulmonary fibrosis. The basement membrane is a complex structure that plays a dynamic role in maintaining the integrity and differentiation of the alveolar epithelium, and its disruption is important in the pathogenesis of lung fibrosis [76, 77]. Migration of fibroblasts and myofibroblasts into the alveolar spaces occurs through partially disrupted and denuded epithelial basement membranes [66, 67, 78]. The disrupted basement membrane may also contribute to the failure of an orderly repair of the damaged alveolar type I epithelial cells (Tables 1 and 2).

Regeneration of a damaged epithelium following injury is required for the reestablishment of normal tissue architecture and function. The alveolar epithelium shows a marked loss of or damage to type I cells, hyperplasia of type II cells, and altered expression of adhesion molecules and MHC antigens [72, 79, 80]. In IPF, the capacity of type II alveolar cells to restore damaged type I cells is seriously altered, resulting in epithelial metaplasia and the presence of transitional reactive phenotypes [79], abnormalities in pulmonary surfactant [81], and alveolar collapse [82]. Thus, cuboidal, bronchiolar and squamous metaplastic cells are usually found lining damaged alveolar structures. Epithelial instability has been largely documented by the presence of frequent evidence of morphological changes through variegated cellular alterations, such as hyperplasia, different degrees of dysplasia and, eventually, carcinoma [83-85]. Failure to establish an intact epithelium may induce a persistent wound-repair response. In the context of pulmonary fibrosis, evidence from human disease, animal models, and

cell-culture studies has identified abnormalities in epithelial cell survival/apoptosis, proliferation, and migration, which likely contribute to disease pathobiology.

Excessive AEC apoptosis may play a key role in microscopic areas of epithelial cell dropout. Uhal et al first reported that apoptosis might be an important mechanism of cell loss in fibrosing lung disease [86]. Since then, there has been rapidly growing and relevant literature on apoptosis in both human and experimental models of lung fibrosis [87]. The activation of this internally encoded suicide program is the result of either extrinsic or intrinsic signals [88] and it has been demonstrated that both bronchiolar and overall AECs, mainly type I AECs, are involved [89]. Different mechanisms have been reported to be involved in apoptotic phenomena from IPF lungs and, in several works, the existence of circulating antibodies to cytokeratins has been demonstrated, reflecting significant epithelial lung injury [90, 91]. Epithelial cell death and necrosis have mainly been detected adjacent to fibroblastic foci, where an irreversible fibrogenesis mechanism starts [92], and it has been hypothesized that type I AECs are more susceptible to myofibroblast-induced apoptosis. A very recent experimental study demonstrated that myofibroblasts from fibrotic lungs possess a cytotoxic phenotype that causes apoptosis of epithelial cell via the Fas-Fas-ligand (Fas-L) pathway [93]. Proliferating fibroblasts seem to contribute to epithelial apoptosis releasing angiotensin (AT) II, which promotes cell death via its AT2 receptor [94]. TGF-β1, an important pro-fibrogenetic factor released by fibroblasts as well as by other different cell types, enhances Fas-mediated apoptosis of epithelial cells through caspase-3 activation and downregulation of p21 [95]. Another important mechanism that may induce apoptotic cell death in IPF is one that promotes cell death by a direct insult on the epithelium. Thus, oxidative and nitrative stress caused by reactive oxygen species (ROS) and nitric oxide synthase (NOS) can induce apoptotic cell death in IPF, as well as in other lung diseases [96]. ROS has been demonstrated to induce apoptotic epithelial cell death

either through upregulation of the Fas-FasL pathway and release of cytochrome c or by activation of the tumor-suppressor gene p53 [97]. In fact, DNA damage and apoptosis are associated with the upregulation of p53 protein in bronchiolar and alveolar epithelial cells in IPF [75].

Alveolar cell fate is an important aspect but is limited to focal areas of the IPF lung. Nonetheless, the high rate of proliferation and migration of epithelial cells is more important. A few tissue markers regarding epithelial instability have recently been described as being overexpressed in lung tissue from patients with IPF. A number of mediators capable of inducing migration and proliferation of AECs have been identified in IPF. The Keratinocyte growth factor (KGF) and the Hepatocyte growth factor (HGF) are important mitogenic factors for type II AECs [94, 95] and HGF receptors are particularly overexpressed on hyperplastic AECs [17]. Recent findings suggest that EGF, as with TGF-β1, may regulate epithelial repair in vivo and in vitro [17]. TGF-β1 is known to induce alveolar cell apoptosis and, if point mutations occur on the TGF-β1 type II receptor, it becomes relevant for the regeneration of type II AECs leading to hyperplasia [97]. Uncontrolled and unstable epithelial proliferation is considered the basis of neoplastic transformations that occur frequently, particularly squamous cell carcinoma type, in patients with IPF. Different point mutations have been described in the K-ras gene and an overexpression of mutated p53 has been detected in type II AECs in patients with lung carcinoma accompanied by IPF [98], leading to an imbalance of different growth factors and, consequently, increased tumorigenesis [73].

Table 1. Main molecular markers driving epithelial remodelling.

Molecular markers	Principal source
Apoptosis	
Fas/Fas-L	Epithelial cells, lymphocytes, granulocytes
FADD/caspase	Epithelial cells
Angiotensin	Fibroblasts
TGF-β1	Fibroblasts
ROS/NOS	Inflammatory cells or myofibroblasts
P53	Upregulated in epithelial cells
Proliferation	
KFG	Fibroblasts
HGF/HGF receptors	Fibroblasts
HDGF	Fibroblasts
TGF-β1 receptor	Mutated in epithelium
K-ras	Mutated in epithelium
P53	Mutated in epithelium

Legend: ***Fas-L****: Fas ligand;* ***FADD****: Fas-associated protein with death domain;* ***HDGF****: Hepatoma-derived growth factor;* ***HGF****: Hepatocyte growth factor;* ***KGF****: Keratinocyte growth factor;* ***NOS****: Nitric oxide synthase;* ***ROS****: Reactive oxygen species*.

Table 2. Main molecular markers driving interstitial remodelling.

Molecular markers	Principal mechanisms
Extracellular matrix deposition with fibroblast/myofibroblast proliferation	
TIMPs	Mesenchymal cell proliferation
TGF-β1	Antimyofibroblast apoptosis
TGF-β1 via SMAD2	EMT
Inflammation	
IL-1	Acute lung injury
TNF	Early inflammation
MCP-1/CCL2 and MCP-1/CCL3	Monocyte recruitment
Vascular remodelling	
VEGF	Neoangiogenesis
CXC chemokines	Aberrant neoangiogenesis

Legend: *CCL: Chemokine ligand; **EMT**: epithelial-mesenchymal transition; **MCP**: Monocyte chemoattractant protein; **MIP**: Macrophage inflammatory protein; **TIMP**: Tissue inhibitor of metalloproteinases.*

Inflammatory and epithelial/mesenchymal cells theory

The importance of alveolar epithelial cell and myofibroblast cross-talk in the pathogenesis of the disease has been confirmed in animal models of lung fibrosis, although the majority of data obtained in animals suggest that the aberrant healing response of pulmonary fibrosis is initiated and keenly regulated by molecules produced during the inflammatory response. Recently, Bringardner et al [99] renowned the interest on inflammation, hypothesizing some possible association with

epithelial/mesenchymal cells alteration through atypical mechanisms that promote fibrogenesis. Various mediators such as cytokines, chemokines and growth factors produced by inflammatory and parenchymal cells have been involved in the recruitment and persistence of inflammatory cells into the alveolar walls and spaces (Table 2).

Authors propose five hypothetical roles for inflammation in producing lung fibrosis:

(**A**) The direct inflammation hypothesis suggests that inflammatory cells directly damage the tissues *via* substances like elastases, as well as cytokines and growth factors, which amplify this process.

(**B**) The matrix hypothesis, in which inflammatory mediators released as a result of a remote injury are trapped in the pulmonary extracellular matrix. This leads to a prolonged and amplified wound-repair mechanism that results in the fibrotic phenotype.

(**C**) The growth factor–receptor hypothesis suggests that some cell types with growth factor receptors proliferate unchecked in this environment, resulting in activation and amplification of the inflammatory cascade. Additionally, these receptors are upregulated in the presence of steroids, suggesting a rationale as to why immunosuppression is not successful in the treatment of IPF.

(**D**) The plasticity hypothesis suggests that numerous cell types can differentiate into other cell types [for example, epithelial cells to mesenchymal cells, neutrophils and monocytes to macrophages], and this differentiation is a result of complex interactions of inflammatory mediators, growth factors, and other unidentified factors. These activated cells then mediate the fibrotic phenotype.

(**E**) The vascular hypothesis suggests that some initial endothelial injury activates the inflammatory cascade with subsequent antibody deposition and resultant fibrosis.

Lung cancer and IPF

The possible association between idiopathic pulmonary fibrosis (IPF) and lung cancer was first theorized in autopsy studies dating back several decades [100, 101]. Several studies have reported incidences of lung cancer associated with IPF ranging from 10.7% to 48% at autopsy [83-85, 102-104]. A small number of epidemiologic reports also found that IPF is an independent risk factor for lung cancer [105]. Moreover, a British retrospective case-control study comparing 890 cases of IPF to 5.884 controls, observed a seven-fold increased risk to develop lung cancer in IPF patients [83]. Age and smoking history can also play a role of cofactors [85, 105]. Adenocarcinomas and squamous cell carcinomas are the most common histological types [85, 106] with a preferential localization of neoplastic transformation in the peripheral area of the lower lobes in the fibrotic area of honeycomb [85, 104, 106] were the atypical regenerative epithelial cells seem more susceptible to carcinogenic agents [104].

A few tissue markers regarding epithelial instability have recently been described to be over expressed in lung tissue from patients with IPF/UIP including the expression of the K-ras gene with point mutation and the presence of multiple mutations of p53 which have been detected in type II alveolar pneumocytes of IPF/UIP lungs [98].

Squamous cell carcinoma antigen (SCCA-SERPIN)

Squamous cell carcinoma antigen (SCCA-SERPIN) was first discovered in uterine cervical squamous cell carcinoma by Kato and Torigoe [107]. Measurement of the serum SCCA level has been used clinically for the diagnosis and management of cancer of the uterine cervix and some other organs (lung, oesophagus and head and neck) [108-110]. SCCA is also expressed in normal tissues: the epithelium of tongue, tonsil, oesophagus, uterine cervix, vagina, the conducting airways, Hassall's corpuscles of the thymus, and some areas of the skin [111]. The physiological roles of SCCA1 (Serpin

B3) and SCCA 2 (Serpin B4) are still poorly understood and previous reports have indicated that SCCA is closely related with cell differentiation of normal squamous epithelium as well as malignant squamous cells [112]. cDNA of the SCCA1 gene was first isolated and reported by Suminami et al [113]. After that, a second SCCA gene, SCCA2, has been identified in the human genome [114, 115]. The genes of SCCA are <10 kb apart, tandemly arrayed in a head-to-tail fashion, and approximately 10 kb in size. Both genes also contain 8 exons and identical intron-exon boundaries. The cDNAs encode for proteins that are 92% identical and 95% similar. Amino acid comparisons show that SCCA1 and SCCA2 are members of the high-molecular weight serine proteinase inhibitor (serpin) family [113]. Physical mapping studies show that the genes reside within the 500-kb region of 18q21.3 that contains at least four other serpin genes (Figure 3 A, B, C). SCCA1 (SERPIN B3) displays inhibitory activities on serine proteinase, e.g. chymotrypsin, and cysteine proteinase, e.g. cathepsin L, K, S and papain, whereas SCCA2 (SERPIN B4) is an inhibitor of serine proteinases such as cathepsin G and human mast cell chymase and cysteine proteinases such as Der p1 and Der f1 [116-118]. These findings suggest that SCCA1 and SCCA2 are capable of regulating proteolytic events involved in both normal (e.g., tissue remodeling, protein processing) and pathologic processes (e.g., tumor progression). Transduction of tumor cells with SCC antigen-1 reveals that SCC antigen-1 inhibits apoptosis of tumor cells induced by anticancer drug, TNF or NK cells. Therefore SCC antigen-1 may work in cancer cells for tumor growth, and in normal squamous epithelium for differentiation by means of the inhibition of apoptosis. Recombinant SCC antigen-2 inhibits cathepsin G and mast cell chymase, suggesting that it protects epithelial cells from the inflammation induced by these proteases.
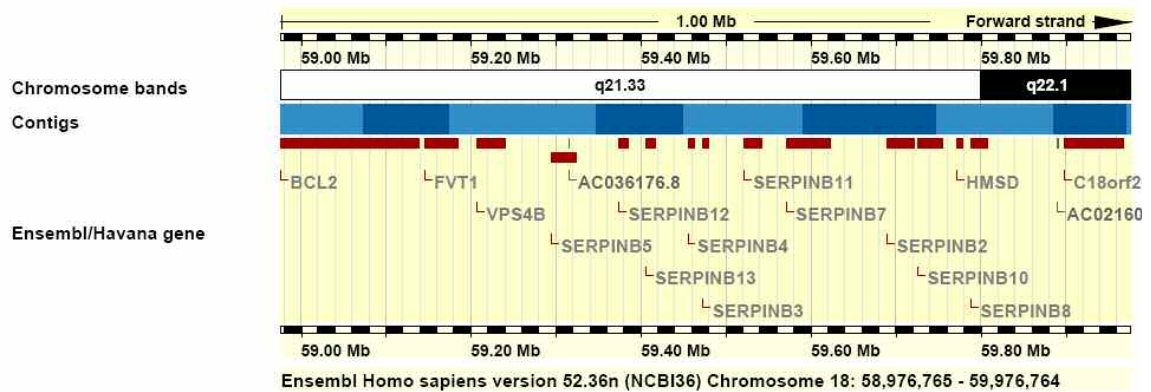
30

Figure 3 A. *Chromosomal localization of SCCA (serpin B3-B4) genes. Available at: http://www.ensembl.org/Homo_sapiens/contigview?panel_top=on;l=18%3A59473412-59480119;h= .*
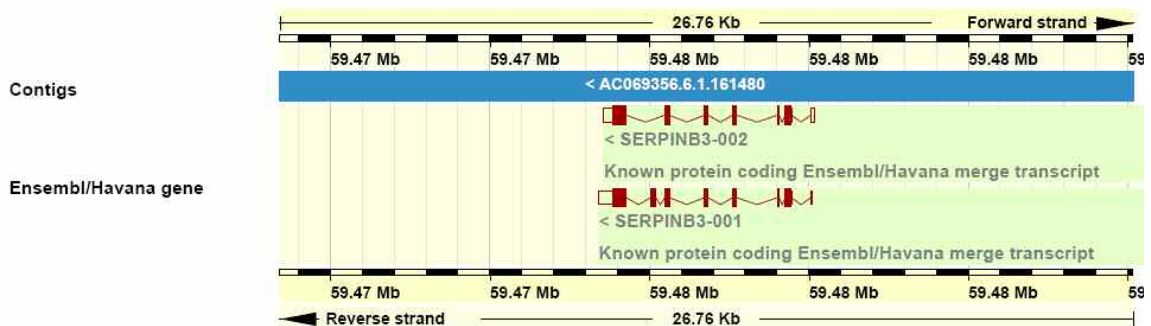

Figure 3 B. *Chromosomal localization of SCCA 1 (serpin B3) gene. Available at: http://www.ensembl.org/Homo_sapiens/geneview?altsplice=%7Censembl_transcript%3Aoff%7Cgenscan%3Aon&db=core&gene=ENSG00000206073.*
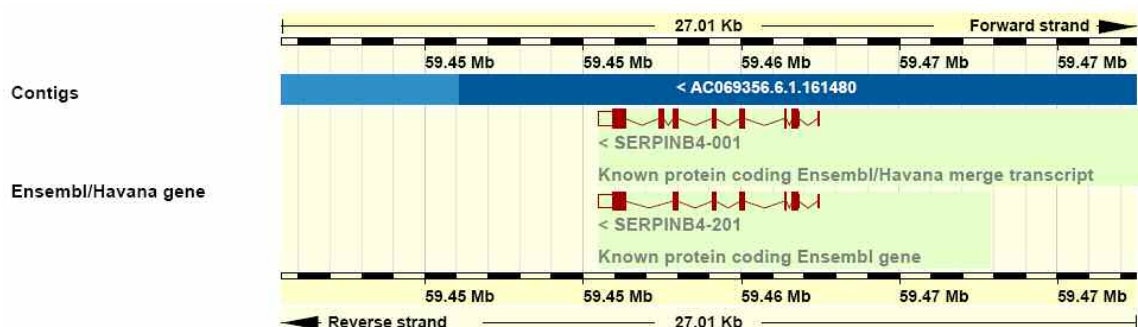

Figure 3 C. *Chromosomal localization of SCCA 2 (serpin B4) gene. Available at: http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000057149;db=core.*

RESEARCH OBJECTIVES

Aims of this research were:

- To assess the expression of SCCA both as mRNA and protein in lung biopsies from IPF patients in early stage disease, in end stage disease and in control patients.

- To understand the biological activity of SCCA and its hypothetical role in pathogenetic mechanisms of IPF.

- To evaluate the possible interaction or activity of SCCA on TGF-β1 expression. For this purpose an in vitro study was conducted.

- To analyse prognostic value of SCCA, the association between its value and different clinical and pathological data.

- To verify the SCCA expression on the basis of the different metaplastic epithelial alveolar cells.

- To study the hypothetical role of SCCA as a biomarker of epithelial instability and/or of increased risk of neoplastic transformation.

MATERIALS AND METHODS

Database

At beginning of the research a multidisciplinary electronic database –website- (Access, Microsoft Office Professional 2007) was built following the privacy rules. The database contains more than 200 items regarding the clinical, radiological,surgical, pathological and molecular data. The data base does no include any personal identification label and is held in secure password protected storage and responsibility of the Unit Coordinators (Prof Rea, Prof.ssa Saetta and Prof Calabrese) in accordance with the requirements of the Health Information.

A specific section for data collection of *in vitro* study was also present. The demographic, clinical and radiological data were collected at the time of recovery of patients at Thoracic Surgery Division for biopsy (early-stage IPF patients) or lung transplantation (end-stage IPF patients and control lungs). Surgical data, including haemodinamic data detected with invasive monitoring (Shwann-Ganz catheter) were collected during lung transplantation or open lung biopsy. Pathological data were recorded during the course of the research. The database was used to collect all data regarding patients entered into the study, and subsequently was used for statistical analysis. Written informed consent was obtained from each patient and the work was approved by the Institutional Ethics Committee.

For each patient blood samples and lung tissue samples have been obtained and preserved in a tissue bank for future studies. The collection and preservation of biological materials followed the guidelines of Regione Veneto and Azienda Ospedaliera di Padova.

Patients with early-stage IPF

22 patients with early-stage IPF (Group A) have been consecutively evaluated during the study period (between 2006 and 2008). The diagnosis of IPF was based on the diagnostic criteria of the American Thoracic Society/European Respiratory Society Consensus Classification System [1]. Samples from IPF patients were obtained from video-assisted thoracoscopic lung biopsies. Histological examination revealed all the major features of UIP, which is a prerequisite for the diagnosis of IPF. Fifteen patients underwent biopsies at two different sites (upper and lower lobes), and the other seven patients were biopsied at three separate sites (upper, middle and lower lobes), giving a total number of 51 biopsies. The majority of the patients were treated after biopsy with a high dose of steroids alone or associated with azathyoprine. The mean age of the patients was 60.2 years (range 44 to 69 years); 17 of the patients were males and five females. The main characteristics of studied subjects are shown in Table 3. All patients underwent routine pulmonary function testing, including spirometry, lung volume measurement, measurement of diffusion capacity of carbon monoxide (DLCO), arterial blood gases at rest and after exercise, chest radiography and high resolution computed tomography (HRCT). Lung function data were recorded less than six weeks before biopsies in all IPF cases and re-evaluated after a median period of 9 months (range 6-11 months). At follow-up, clinical data were completed for 18 patients, two patients died before undergoing the second pulmonary function test and spirometry results were not valuable for two cases. Data were expressed as percentage of values predicted from the subject's age, sex and height.

Table 3: Clinical and pathological characteristics of the study population (22 early-stage IPF patients).

Characteristics		UIP patients	Range
Sex (male:female)		17 : 5	
Status (dead/alive)		2 : 20	
Age at biopsy (years, mean ± SD)		60.2 ± 6.2	44 - 69
Dust exposure (yes/no)		9 : 13	
Smoker (yes/no)		12 : 10	
Smoking (pack-years, mean ± SD)		23.3 ± 19.1	0.45 - 64
Follow-up (months, median)		9	6 - 11
Pathological features			
Fibroblastic foci score (%)	1	4.6	
	2	40.9	
	3	13.6	
	4	22.7	
	5	4.6	
	6	13.6	
Fibroblastic foci (AFF%, median)		8.4	4.3 – 21.8
Fibrosis extension (AFib %, mean ± SD)		35.6 ± 7.4	22 – 51.8
Inflammation score (%)	1	53.8	
	2	38.5	
	3	7.7	
Inflammation (AIC %, mean ± SD)		3.0 ± 1.4	1.2 – 6.6
Spirometry at time of diagnosis			
DLCO (% predicted, mean ± SD)		53.7 ± 12.7	23 - 77
FEV1 (% predicted, mean ± SD)		72.9 ± 15.7	42 - 109
FVC (% predicted, mean ± SD)		69.5 ± 13.6	49 - 97
VC (% predicted, mean ± SD)		71.1 ± 12.7	47 - 94
TLC (% predicted, mean ± SD)		67.1 ± 12.9	48 - 91
RV (% predicted, mean ± SD)		67.7 ±25.5	31 – 125

AFF, fibroblastic foci area; AFib, fibrotic area; AIC, inflammatory cells area; DLCO, Diffusing lung capacity for carbon monoxide; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; VC, vital capacity. For normally distributed quantitative variables, mean ± SD range are shown; for not normally distributed quantitative variables, median range and IQR are shown; for categorical variables percentage distributions are shown.

Patients with end-stage IPF

48 native lungs (Group B) of patients affected by IPF and submitted to lung transplantation between 1995 and 2007 at Division of Thoracic Surgery of Padova, were evaluated. Before lung transplantation, patients were treated with a high dose of steroids alone or associated with azathyoprine. The mean age of the patients was 55.2 years (range 39 to 65 years); 34 of the patients were males and 14 females. The main characteristics of studied subjects are shown in Table 4. All patients underwent pre-transplant pulmonary function testing, including spirometry, lung volume measurement, measurement of diffusion capacity of carbon monoxide (DLCO), arterial blood gases at rest and after exercise, chest radiography and high resolution computed tomography (HRCT), echocardiography and heart catheterization.

Immediately after pneumonectomy at least 3 small lung tissue pieces (1.5 cm^3) were sampled and preserved in RNA later liquid; these were used for molecular investigations. After pneumonectomy, native lungs were sized, weighed and perfused by endobronchial formalin 10% for at least 1 hour at a pressure of 25 cmmH2O. Histological multiple samples were obtained from 3 biopsies for each lobe. All lung samples were then formalin-fixed and paraffin-embedded following standard protocols.

Table 4: Clinical and pathological characteristics of the study population (48 end-stage IPF patients).

Characteristics	UIP patients	Range
Sex (male:female)	34 : 14	
Age at transplantation (years, mean ± SD)	55.2 ± 7.2	39 – 65
Type of transplantation (single:bilateral)	36 : 12	
Smoker (yes/no)	33 : 15	
Smoking (pack-years, mean ± SD)	24.6 ± 23.7	2.5–120
Pathological features		
Fibrosis extension (AFib %, mean ± SD)	34 ± 9	4 – 60.6
Spirometry at time of diagnosis		
DLCO (% predicted, mean ± SD)	22.4 ± 9.5	7 – 43
FEV1 (% predicted, mean ± SD)	44.8 ± 14.2	22 – 73
FVC (% predicted, mean ± SD)	41.9 ± 14.1	21 – 66
VC (% predicted, mean ± SD)	40.4 ± 12.7	21 – 66
TLC (% predicted, mean ± SD)	47.5 ± 10.8	73 – 31
RV (% predicted, mean ± SD)	62.5 ± 24.2	31 – 121

AFib, fibrotic area; DLCO, Diffusing lung capacity for carbon monoxide; FEV, forced expiratory volume in one second; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; VC, vital capacity. For normally distributed quantitative variables, mean ± SD range are shown; for not normally distributed quantitative variables, median range and IQR are shown; for categorical variables percentage distributions are shown.

Control group

Control lungs (Group C) were obtained from non implanted donor lungs (10 cases) and from other forms of interstitial lung diseases (ILDs, 10 cases): two non-specific interstitial pneumonias (NSIP), one desquamative interstitial pneumonia (DIP), one Langerhans-cell histiocytosis (LCH), one lymphangioleiomyomatosis (LAM), two respiratory bronchiolitis interstitial lung diseases (RBILD), one cryptogenic organizing pneumonia (COP), one mixed pneumoconiosis and one hypersensitivity pneumonitis (HP). The donors (5 males and 5 females, mean age 30 ± 17 years, all no smokers) died of cerebral trauma and stayed less than two days in intensive care without evidence of lung infections or other complications. Patients affected by other forms of ILDs (3 males and 7 females; mean age: 45 ± 15 years, all smokers except LAM and HP

patients) were defined by the presence of clinical, radiological and histological evidence of specific ILD. All lung tissues were formalin-fixed and paraffin-embedded following standard protocols.

Histology and morphometry

Fibroblastic foci (FF) and inflammatory cells (IC) were evaluated by a semi quantitative method. In particular, FF were analyzed by using a Brompton score [119], and IC was scored as follows: less than 10% of lung tissue examined (score 1), more than 10% and less than 30% of lung tissue examined (score 2), more than 30% of lung tissue examined (score 3). In all samples from each patient, the extension of fibrosis, inflammation and FF were also measured by computerized morphometric analyses (Image Pro-plus version 5). The extension of fibrosis was quantified on lung sections stained by Azan-Mallory as previously described [120] (Figure 4).
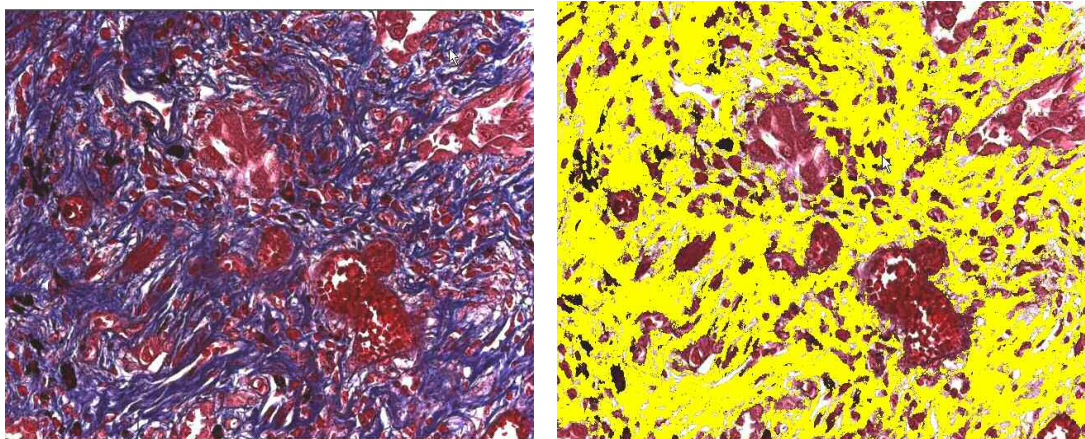
Figure 4. *Lung section stained with Azan- Mallory to evaluate the extension of fibrosis. On right side the same section after evaluation with computerized morphometric analysis: yellow marked areas:70% (Image Pro-plus version 5).*

FF, IC and fibrosis were analysed on ten random fields in the same section of imaged lesions at 50-fold magnification excluding the areas of honeycombing. In each selected field, the ratio of fibroblastic foci, inflammatory cells and fibrotic areas (AFF, AIC, AFIB) were calculated dividing the total AFF, AIC and AFIB by the total tissue area (excluding airspaces) of the section (where n = the number of fields): *FF Ratio = ΣnAFF/Total Area*100, IC Ratio = ΣnAIC/Total Area*100, Fibrosis Ratio = ΣnAFIB/Total Area*100.* For each patient, the ratios obtained from the analysed sections were then averaged and this value was correlated with all pathological and clinical parameters. Differently from the authors recently supporting the value of quantitative analysis of FF [121], we measured all parameters including FF exclusively related to lung tissue (excluding air spaces) to normalize the effect of collapse or expansion of lung tissue during biopsy or tissue fixation. For Group B, dysplasia was evaluated and graded on Haematoxylin&Eosin coloured sections by using a semiquantitative method with a score ranging between 0 (absent), 1 (mild), 2 (moderate), and 3 (severe).

AFIB and AIC were not evaluated as in end-stage IPF these aspects are less representative.

Immunohistochemical analysis

All cases were immunoassayed with a novel polyclonal rabbit antibody anti-SCCA (Hepa-Ab, Xeptagen, Venice, Italy) and mouse monoclonal anti-TGF-β1 (NovoCastra, Newcastle, UK) (Table 5) as previously described in other reports [122, 123]. Sections were incubated with primary antibodies for 30 min, after blocking endogenous peroxidase activity with 3% hydrogen peroxide, heating the slides in 10mM sodium citrate in a microwave oven and blocking nonspecific protein binding in normal goat serum. Biotinylated goat anti-rabbit or horse anti-mouse (Dako, Copenhagen, Denmark)

was then added for 30 min. All samples were then processed using a sensitive avidin-streptavidin peroxidase technique and stained with a mixture of 3,3-diamino-benzidine tetra hydrochloride and hydrogen peroxide. Parallel control slides were prepared either lacking primary antibody or lacking primary and secondary antibodies, or were stained with normal sera to control for background reactivity. Consecutive serial sections immunostained for SCCA and TGF-β1 were evaluated and the quantification was restricted to strongly stained metaplastic epithelial cells (cuboidal, squamous, bronchiolar) A total of 500 metaplastic epithelial cells for each patient were counted in remodelled lung parenchyma (at least two sections) and the value was expressed as a percentage of positive cells/500 for each case. This value was correlated with all pathological and clinical values.

Table 5: Antibodies utilized in the research.

ANTIGEN	ANTIBODY	CLONALITY	DILUTION - TIME - TEMPERATURE	COMPANY
SCCA	Hepa-Ab (rabbit)	polyclonal	1:10 – 1h - T_{env}	Xeptagen
TGF-β1	Anti TGF-β1 (mouse)	monoclonal	1:20 – 1h - T_{env}	NovoCastra

Molecular analysis

Manual tissue dissection

Manual tissue dissection of representative areas, positive for SCCA and TGF-β1, was performed in half of the cases (11 cases) in Group A and in seven cases in Group B (three with IPF and carcinoma) in which metaplastic epithelial aggregates were easily dissected. Briefly, five sequential 5 μm sections (sections were obtained by using a

microtome Leica SM2000R, Milan, Italy) from formalin-fixed paraffin-embedded blocks were placed on non-coated glass slides and coupled with SCCA and TGF-β1 immunostained tissue sections. The areas (at least 1 mm in diameter) carefully marked to easily compare the unstained levels were gently scraped with a sterile scalpel. The procured tissue fragments were then placed in a tube, deparaffinized and washed in xylene and alcohol before nucleic acid extraction. After this procedure, the remaining unselected tissue was stained with hematoxylin-eosin to verify the isolated tissue parts. Areas of normal tissue (negative for SCCA and TGF-β1 immunoassaying) from the same paraffin block and from donor lung were also dissected and processed in the same way.

RT-PCR of SCCA and TGF-β1

Total RNA was extracted by using the modified RNAzol method, as previously described by Chomczynski and Sacchi [124]. The RNA pellet was redissolved in 15 μl sterile DEPC-treated water and incubated with 1 μl of RNAse inhibitor (Applied Biosystems, Milan, Italy) and 20 U of DNAse I (Sigma Aldrich, Milan, Italy) for two hours at 37 C in a total volume of 20 μl. The oligonucleotides used to ascertain the quality of extracted RNA were complementary to the mRNA glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of primers for GAPDH, SCCA and TGF-β1, annealing temperature condition and amplicon sizes are listed in Table 6. The purified RNA was quantified by spectrophotometer (GeneQuant$^{®}$, Amersham Biosciences), At least 1 μg of extracted total RNA was used for the first complementary DNA (cDNA) synthesis (GeneAmp$^{®}$, Applied Biosystems, Milano-Italia) and conventional RT-PCR was used. The PCR mix was made up to a volume of 50 μl using 1X PCR Buffer II, 1mM MgCl2 solution, 200 μM each of dATP, dCTP, dGTP, dUTP, 400 nM of each primer, and 1.25 Units of AmpliTaq Gold. After the initial denaturation at 95°C for 10 min, the cDNA was amplified by 40 three-step cycles (30 sec at 95°C, 30

sec at annealing temperature, 1 min at 72°C). SCCA and TGF-β1 amplicons were both

verified by previously described gene sequencing protocol [122].

Table 6. Oligonucleotide sequences of primers used to amplify GAPDH (housekeeping), TGF- β1 and SCCA.

Primer size	Sequence 5'- 3'	Annealing Temperature	Amplicon
GAPDHFw	GGGCTCTCCAGAACATCATCC	60	130 bp
GAPDH Rv	GTCCACCACTGACACGTTGG		
SCCA Fw	GGCAGCTGCAGCTTCTG	55	80 bp
SCCA Rv	AGCCGCGGTCTCGTGC		
TGF-β1 Fw	GCCCTGGACACCAACTATTGC	60	161 bp
TGF-β1 Rv	AGGCTCCAAATGTAGGGGCAG		

Bp, base pair; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **Fw**, forward; **Rv**, reverse; **SCCA**, squamous cell carcinoma antigen; **TGF-β1**, transforming growth factor- β.

In vitro study

Cell culture – Lung epithelial cell line A549 was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained routinely in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (Gibco, Milan, Italy) and supplemented with 100 mg/ml penicillin G (Gibco, Milan, Italy) and 100 mg/ml streptomycin sulfate (Gibco, Milan, Italy). Cells were then seeded in DMEM with 10% fetal bovine serum at a density of 0.3x106 cells per well in 6-well plates. All the experiments were performed at 100% cell confluence. Time course analysis was carried out to assess the effect of SCCA on TGF-β1 expression. Cells were incubated with SCCA (Xeptagen, Venice, Italy) at increasing concentrations (range: 1-1000 pg/ml) and

checked for TGF-β1 expression at 6-hour intervals (range: 6-48 hours). Cells were collected and lysated immediately with RLT buffer (Qiagen, Milan, Italy) and β-Mercaptoethanol 14.5 M 1% (Sigma Aldrich, Milan, Italy).

Quantitative RT-PCR of TGF-β1

RNA was extracted using RNeasy Mini Kit (QIAGEN, Milan, Italy) according to the manufacturer's instructions. RNA was reverse-transcribed using the Reverse Transcription System (Promega Corporation, Madison, WI) according to the manufacturer's instructions. Real Time PCR was performed using a standard TaqMan® PCR kit protocol on an Applied Biosystems 7000 Sequence Detection System (Applied Biosystems, Milan, Italy). The TaqMan® PCR was carried out in a 96-well microtiter plate format (Applied Biosystems). The PCR mix was made up to a volume of 25 μl using ready-to-use Universal Mastermix containing AmpliTaq DNA polymerase, uracil-Nglycosilase (UNG), dNTPs, KCl, MgCl2 and ROX as passive reference all in optimized concentrations. After UNG treatment at 50° C for 2 min and initial denaturation at 95°C for 10 min, the DNA was amplified by 40 two-step cycles (15 sec at 95°C, 1 min at 57°C). All reactions were run in triplicate. Reactions and cycling were performed as recommended by the manufacturer's instructions. GAPDH was used as a reference gene for the adjustment of relative expression data. Naive cells collected at the same time were used as calibrators. All assays were performed in triplicate to ensure their reproducibility, and a negative control was included in each run. Primers and probe for GAPDH and TGF-β1 were commercially available (4333764 and 4327054, Applied Biosystems).

Statistical analysis

Normality of distribution for quantitative variables was assessed by means of Shapiro-Wilcox statistics. Normally distributed quantitative variables are described as mean value and standard deviation, not normal quantitative variables are expressed as median, range and IQR, while categorical variables are presented as percentage distribution. To evaluate simple linear relationships between quantitative variables, Pearson's or Spearman's correlation coefficients were applied, as necessary. To evaluate the independent association of each factor with the dependent variable, the partial correlation coefficients were estimated. In the analysis of the relationship between SCCA and scores, to allow greater set sizes, foci and inflammation scores were used to group IPF subjects. For foci, the subjects ranked: 1 (score 1 to 2), 2 (score 3 to 4) or 3 (score 5 to 6). For inflammation, the variable was dichotomized (score<=1 , score >1). The mean value of SCCA of the different groups was compared by means of one-way analysis of variance (ANOVA). Statistical analysis was performed using SAS statistical software version 9.1 (SAS Institute, Carry, NC, USA). P values lower than 0.05 were considered statistically significant.

RESULTS

Early-stage disease (Group A)

Pathological findings and clinico-pathological correlations

Median histological score (range, IQR) was 3.0 (1 to 6, 2) and 1.0 (1 to 3, 1) for FF and

for IC, respectively. The mean Afib was $35.6 \pm 7.4\%$ (range $22 - 51.8\%$) (Table 3).

Among all the quantitative variables only fibroblasti foci area (A_{FF}) showed a

distribution different from normality (p = 0.02). Median value of fibroblastic foci area

(A_{FF}) was 8.4% (range 4.3% to 21.8%). Mean value of inflammation area (A_{IC}) and

fibrosis extension were $3.0 \pm 1.4\%$ and $35.6 \pm 7.4\%$, respectively. A direct correlation

was observed between A_{FF} and A_{FIB} (r = 0.56, p = 0.007). A statistically significant

correlation was observed only between A_{FIB} and decline of DLCO at nine months (r =

0.5156, p = 0.0302).

Immunohistochemical findings and correlations with morphological and clinical data

SCCA was expressed in many metaplastic alveolar epithelial cells in all IPF cases. It

varied in the range 9.4 to 44.0% and was normally distributed with mean $24.9 \pm 9.3\%$

(Figure 5. A, B).
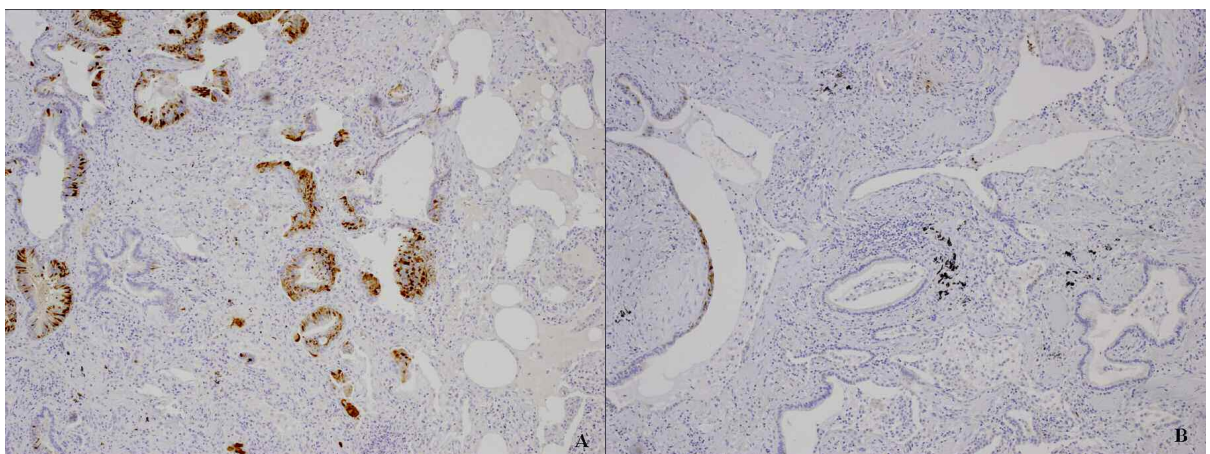
Figure 5: Immunohistochemistry for SCCA.
A: IPF case, female, 56 years old (SCCA mean value: 44%). Many metaplastic bronchiolar and cuboidal cells are strongly marked. Original magnification X 50.
B: IPF case, male, 65 years old (SCCA mean value: 10.5%). A few metaplastic cuboidal and bronchiolar cells are moderately stained. Original magnification X 50.

Immunoassaying was mainly detected in the cytoplasm however in a few cases cytoplasmic and nuclear staining was also observed (Figure 6A).

Cuboidal, flattened metaplastic epithelial cells showed a wide spectrum of staining from strong to weak SCCA positivity (Figure 6B) in contrast to squamous and bronchiolar metaplastic epithelial cells which frequently showed a strong staining (Figure 6C), more often well evident in or close to the honeycomb changes. Interstitial cells were negative for SCCA expression and a weak cytoplasmic staining was observed in some alveolar macrophages.
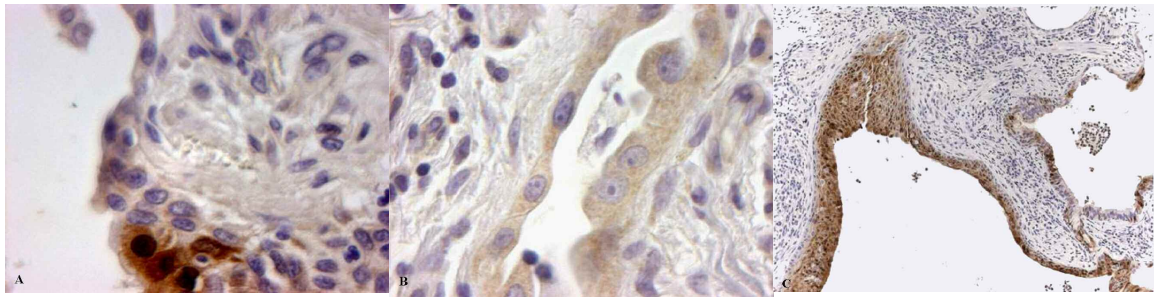
Figure 6. Immunohistochemistry for SCCA.
IPF case, male, 66 years old (SCCA mean value: 32%). Strong nuclear and cytoplasmic staining of metaplastic bronchiolar cells (A), weak cytoplasmic staining of metaplastic cuboidal cells (B), both original magnification X 350, and strong cytoplasmic and nuclear staining of metaplastic squamous cells (C), original magnification X 250.

In all IPF cases TGF-β1, which often marked the same metaplastic epithelial cells positively stained for SCCA (Figure 7A, B), showed a mean value of $29.8 \pm 13.5\%$ (range 7.1 to 55.6%) and was significantly correlated with the expression values of SCCA (r = 0.78, p < 0.0001) (Figure 8).
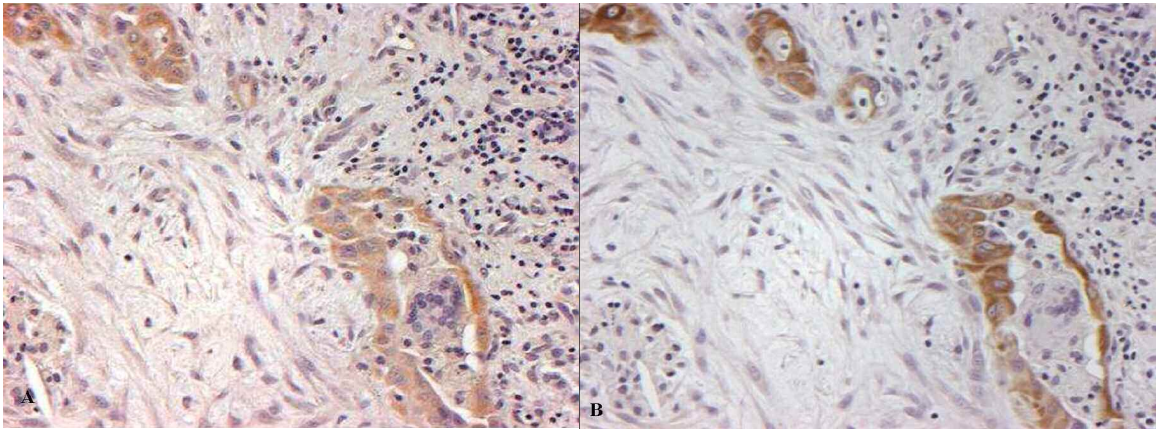
Figure 7: Immunohistochemistry for SCCA and TGF-β1 (A, B).
*IPF case, female, 61 years old. Note the strong staining in the same metaplastic cells of sequential serial sections, original magnification X 300 (**A** and **B**).*
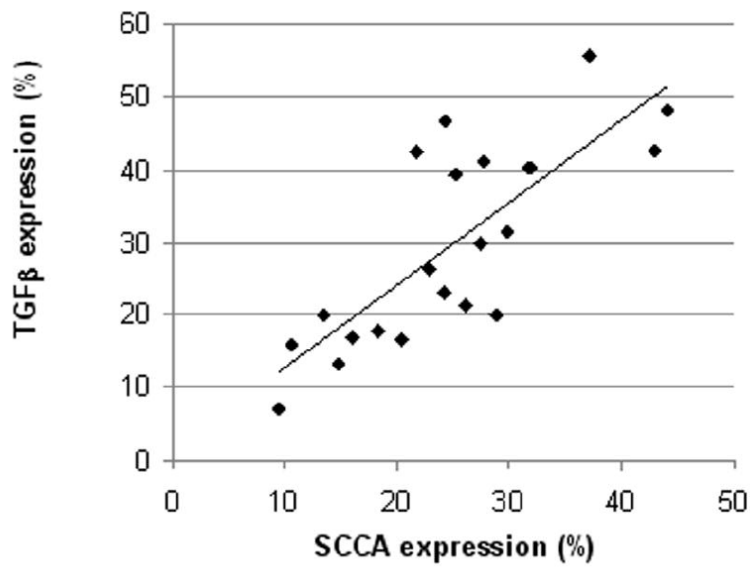

Figure 8. SCCA- TGF-β1 correlation.
Significant correlation between SCCA and TGF-β1 epithelial expression (r = 0.78, p < 0.0001).

A direct correlation was observed between the expression of SCCA and A_{FF} (r = 0.49, p = 0.02) (Figure 9) and between TGF-β1 and AFF (r = 0.44, p = 0.04).
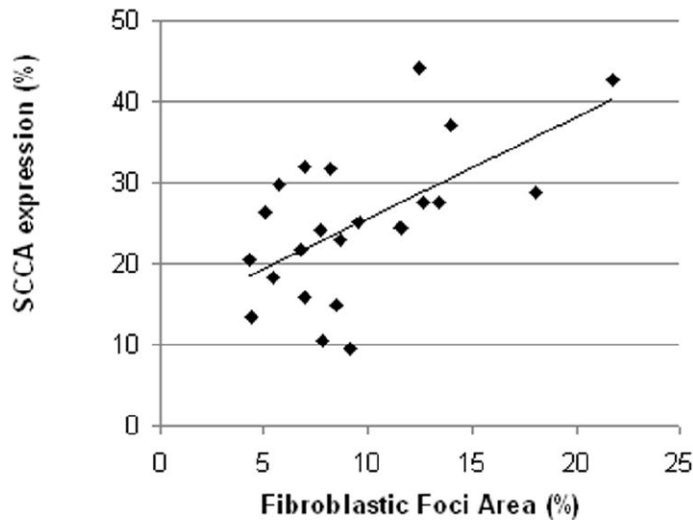
Figure 9. SCCA-AFF correlation
Direct correlation was observed between AFF and SCCA expression (r = 0.49, p = 0.02).

Controlling for SCCA values, the correlation between TGF-β1 and A_{FF} was no longer statistically significant (r = 0.10, p = 0.67). Mean SCCA values did not significantly differ both among foci score groups (F = 0.65 , p = 0.53) and inflammation score groups (F = 0.36, p = 0.56). The only statistically significant relationship between SCCA values and lung clinical data variation during follow-up was observed with decreasing DLCO (ΔDLCO) at nine months (r = 0.38, p = 0.04) (Figure 10).
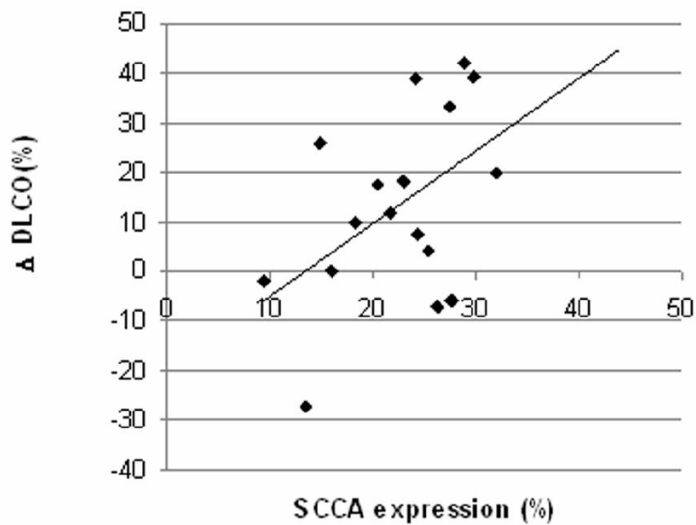
Figure 10. SCCA-ΔDLCO correlation.
Significant correlation was seen between SCCA expression and ΔDLCO (r = 0.38, p = 0.04).

End-stage disease (Group B)

Pathological findings and clinico-pathological correlations

Mean value of AFib was $34 \pm 9\%$ (range 4-60.6%; table 4). Grade of dysplasia was evaluated on metaplastic (cuboidal, bronchiolar and squamous) cells. On five native lungs, foci of neoplastic transformation associated with IPF were detected: these cases were not considered for the evaluation of dysplasia.

The median value of displasia for cuboidal cells was 1 (range 0-2), with grade 0 in 35% of cases, grade 1 in 28%, grade 2 in 37%, grade 3 in 0%.

The median value of displasia for bronchiolar cells was 1 (range 0-2), with grade 0 in 39% of cases, grade 1 in 19%, grade 2 in 42%, grade 3 in 0%.

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The median value of displasia for squamous cells was 2 (range 1-3), with grade 0 in 0% of cases, grade 1 in 33%, grade 2 in 56%, grade 3 in 11%.

A significant difference in mean score of dysplasia was observed between squamous cells and cuboidal (p=0.008) or bronchiolar (p=0.009) cells (Figure 11).
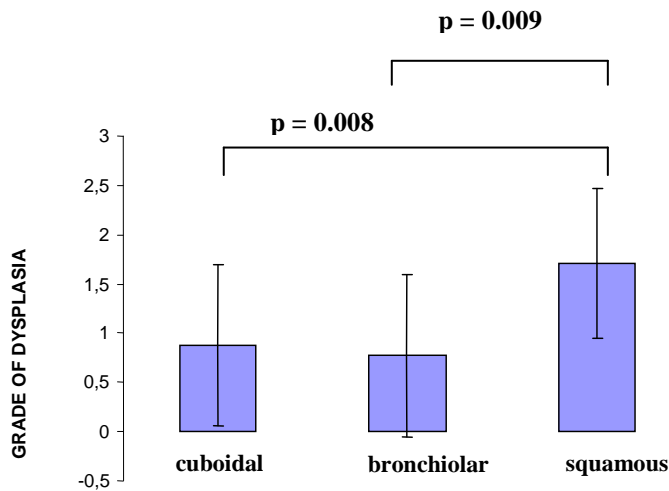

Figure 11. *Different degree of dysplasia in various metaplastic epithelial cells.*

Immunohistochemical findings and correlations with morphological and clinical data
SCCA was expressed in many metaplastic alveolar epithelial cells in all IPF cases. It was normally distributed with mean 34.5 $\pm$ 10.9% (range 8.6-57%). In particular, among metaplastic epithelial cells, cuboidal type showed a mean positivity of 33 $\pm$ 12.2% (range 8.7 - 65.1%), bronchiolar type a mean positivity of 36.1 $\pm$ 12.3% (range 13.6 - 60.9%), squamous type a mean positivity of 75.7 $\pm$ 26.3% (range 24 - 96%). The detection of SCCA in squamous cells was significantly higher in comparison with cuboidal and bronchiolar cells (Figure 12).
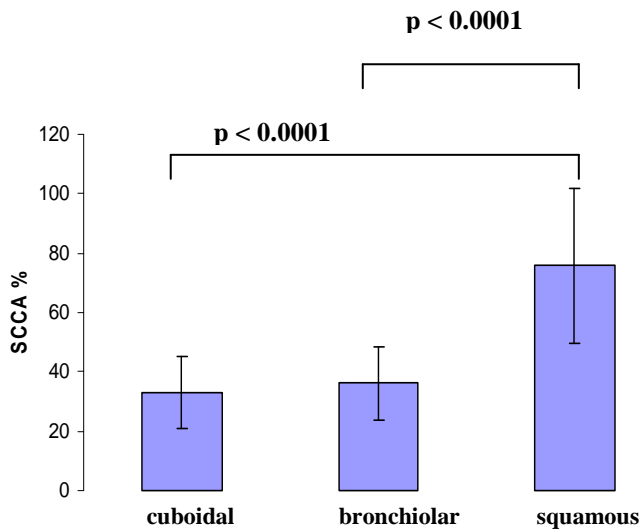
Figure 12. *Different expression of SCCA in the various metaplastic epithelial cells.*

Also in this group, the SCCA positivity was more often well detected in or close to the honeycomb changes. Interstitial cells were negative for SCCA expression and a weak cytoplasmic staining was observed in some alveolar macrophages. In all IPF cases TGF-β1, which often marked the same metaplastic epithelial cells positively stained for SCCA, showed a mean value of 38.6 $\pm$ 8.1% (range 20.5 - 54%) and was significantly correlated with the expression values of SCCA (r = 0.45 ; p < 0.0001) (Figure 13).
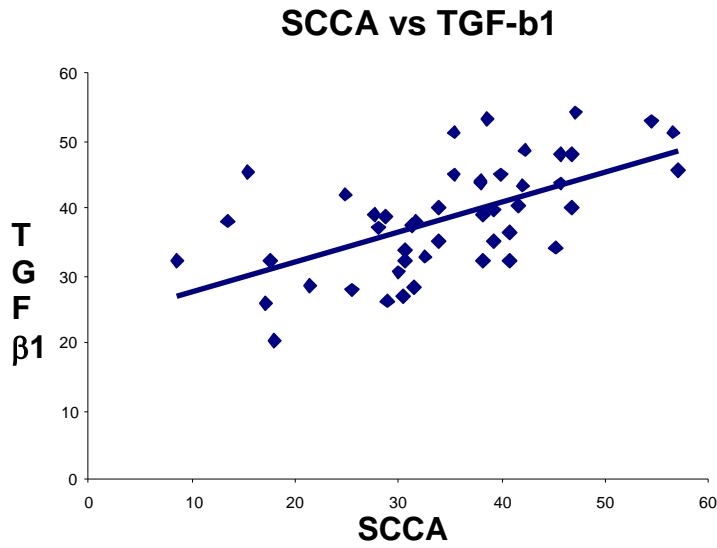
Figure 13. *Correlation between SCCA and TGF-β1 expression.*

An inverse correlation was found whether between pre-transplant DLCO and SCCA values (r = - 0.43; p = 0.005) or between pre-transplant DLCO and SCCA values in cuboidal (r = - 0.32; p = 0.03) and bronchiolar cells (r = - 0.32; p = 0.02) (Figure 14).
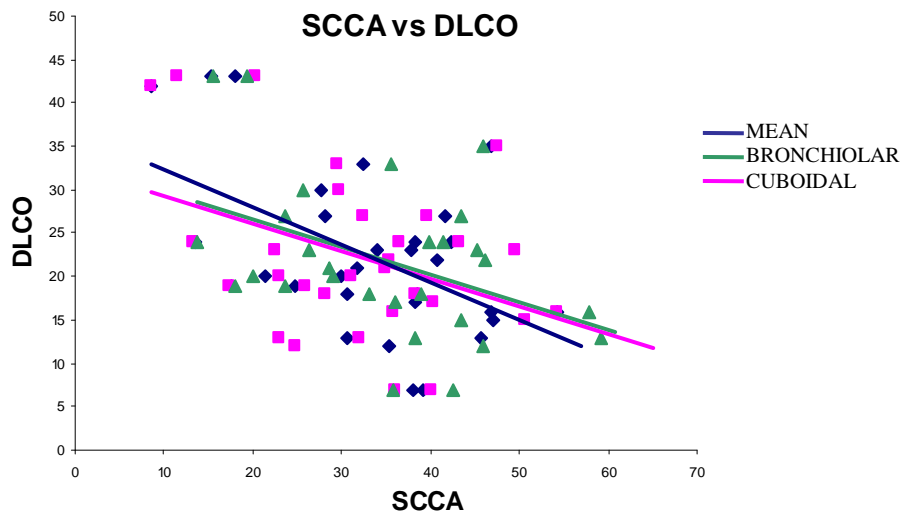

Figure 14. *Inverse correlation between DLCO and SCCA observed in different metaplastic cells.*

A similar inverse correlation was also found between DLCO and TGF-β1 (r = - 0.42; p = 0.046) (Figure 15).

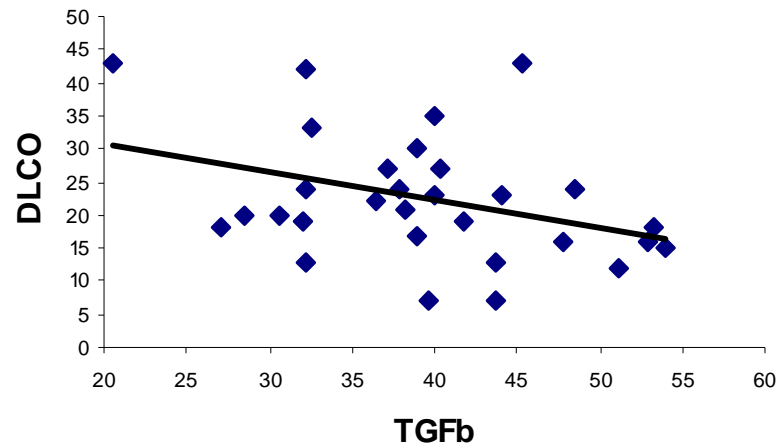**Figure 15.** *Inverse correlation between DLCO and TGF-β1.*

Control Group (Group C)

Metaplastic epithelial SCCA positive staining was seen in only two ILDs different from IPF (NSIP and DIP). The positivity was weak and mainly detected in cytoplasmic areas with a mean of $0.4 \pm 1.3\%$ (from 0 to 5%). The pseudostratified columnar epithelial cells lining the bronchi and bronchioles often exhibited strong cytoplasmic immunoreactivity for SCCA, thus representing a good internal immunostaining control. No alveolar cells were detected in normal lung tissue from donor subjects when only some bronchial cells were marked (Figure 16).

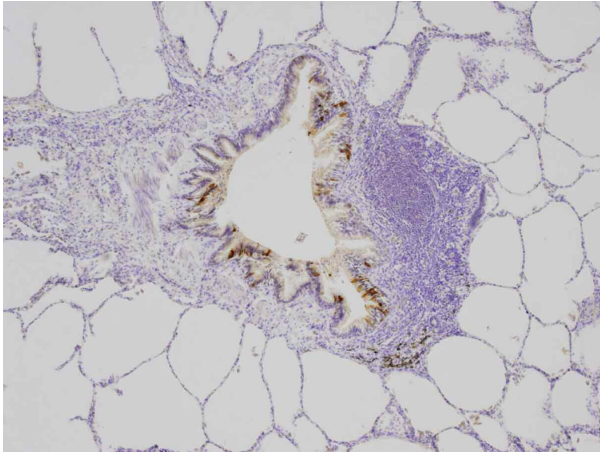TGF-β1 epithelial expression was occasionally seen in control group (up to 2%).

Figure 16: Immunohistochemistry for SCCA.
Control case (non implanted donor lung): positive staining is seen only in epithelial cells of bronchial tract with well developed BALT. Original magnification X 50.

SCCA and TGF-β1 mRNAs

The expected 80 and 161 base pair PCR products of SCCA and TGF-β1, respectively, were detected from all pathological micro dissected lung tissues, confirming epithelial transcription.

TGF-β1 mRNA expression in A549 cells incubated with SCCA

Time course analysis of the effect of SCCA on TGF-β1 transcription in A549 cell lines is reported in Figure 17. The peak of induction of TGF-β1 transcription was achieved at 24 hours, with the highest activity of SCCA at the 100 pg/ml concentration.
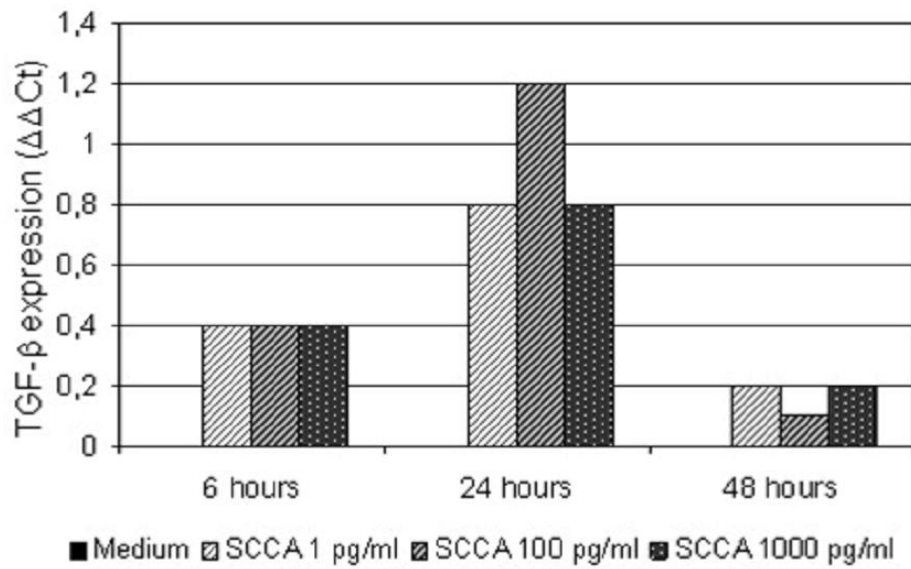
Figure 17. *Effect of SCCA at different time points in A549 cell line. At 24 hours a peak of TGF-β1 RNA transcription was observed and the highest induction was achieved with 100 pg/ml SCCA concentration.*

DISCUSSION

Pulmonary fibrosis is a progressive debilitating restrictive lung disease which, in most cases, is fatal within 3–5 years of diagnosis [2-5]. Over the past decade, it became evident that IPF, a disease more common than previously believed, represents a challenge for clinicians and researchers. The poor understanding of its pathogenesis led to lack of advances in pharmacological therapy or also to experimental use of ineffective or inappropriate therapies [125]. It is now clear that this disease represents a form of abnormal wound healing in the lung that is characterized by fibroblast–myofibroblast migration and proliferation, decreased myofibroblast apoptosis, and increased activity and responses to fibrogenic cytokines (transforming growth factor-β1, tumor necrosis factor-α, platelet-derived growth factor, and insulin-like growth factor). Moreover, an absence of appropriate re-epithelialization and impaired extracellular matrix remodelling (including basement membrane disruption, angiogenesis, and fibrosis) could explain the abnormal repair process [62]. A complete understanding of the sequence of the pathogenic mechanisms as well as the plethora of biological events that control the fibrotic response will improve therapeutic strategies— and ultimately outcome—in patients with IPF.

Today, lung transplantation represents the only effective treatment modality that provides an actuarial survival advantage in this population [126]. Single lung transplantation is the preferred solution, but the better results of bilateral lung transplantation have led clinicians to prefer this solution in the last years [127]. Unfortunately, the progressive nature of this disease and the short interval between diagnosis and death make this therapeutic option available only to a limited number of younger patients. Despite IPF patients have been recognized to be the group that has the best survival gain from transplantation, patients accepted onto the active waiting list will wait on average 12–18 months for a suitable donor organ, consequently, many

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patients never achieve transplantation and die while on the waiting list. While donor organ availability undoubtedly contributes to this, patients with IPF have the highest waiting list mortality of all patients awaiting lung transplantation [128, 129], and the unpredictable natural history of this disease—together with late referral for transplantation assessment—may play an important role in this statistic.

In this line, a better understanding of clinical course of the disease and a search for new more sensitive clinico-pathological markers of disease severity and progression is of paramount importance to optimise the timing of lung transplantation referral for these patients [130]. Aim of this research was to study the expression at the level of alveolar epithelial cells, the hypothetical function and the relationship with clinico-pathological parameters of SCCA, a novel putative marker in IPF. For this purpose we decided to analyse SCCA expression in different clinico-pathological phases of IPF, from early-stage disease to end-stage disease.

SCCA belong to the Serpin superfamily that includes inhibitors of a number of serine proteases with roles in a variety of cellular processes, including fibrinolysis, inflammation, cell migration, adhesion and proliferation [131]. SCCA is transcribed from the tandemly repeated genes, SCCA1 and SCCA2, which have 98% sequence identity at the nucleotide level and 92% identity at the amino-acid level [115]. The products of the two genes have different protease targets; SCCA1 (Serpin B3) inhibits papain-like cysteine proteases (cathepsin S, L and K) [24] and SCCA 2 (Serpin B4) chymotrypsin-like serine proteases (e.g. catepsin G and mast cell chymase) [118]. While some studies have reported increased SCCA expression (mainly in blood serum) in many tumors, particularly those with squamous cell differentiation [132, 133], and in preneoplastic bronchial lesions [134], little is known about the behaviour of this serpin in a nonneoplastic clinical setting.

In the present study we have demonstrated for the first time the overexpression of SCCA in lung tissue of IPF patients compared to other forms of ILDs and normal lungs. In IPF, SCCA was abnormally secreted by metaplastic epithelial cells other than bronchial cells where it is normally expressed. The normal presence of SCCA at this level, as was demonstrated in our control cases, could have a protective function against inflammatory cells and micro organisms because SCCA 1 and 2 are inhibitors of serine and cysteine proteinase. Previous studies have reported that SCCA can have an important influence on epithelial growth through inhibition of different apoptotic pathways [113, 135]. The marked positivity of SCCA in the honeycomb area suggests that this site is more frequently subjected to injury favouring epithelial proliferation, which could be highly unstable when recurrent injuries occur.

Several years ago Meyer and Liebow already showed atypical epithelial lesions in honeycombing pulmonary areas in IPF raising the possibility that atypical epithelial lesions in IPF might be precancerous lesions [136]. Among all metaplastic cells, squamous cells in our samples showed both an higher grade of dysplasia and a frequent and strong staining with SCCA. It is probable that this epithelial setting represents a regeneration epithelial site of high instability at risk of neoplastic transformation. This finding was more evident in end-stage disease in which the fibrosis is highly associated with AECs alterations (e.g. metaplasia or dysplasia). In fact, despite a clinical selection of IPF patients with end-stage respiratory insufficiency candidates to lung transplantation, we found five cases of neoplastic transformation. A recent *in vitro* study has shown an overexpression of SCCA in squamous metaplastic tracheo-bronchial cell lines, increasing with tumor progression [137]. Squamous type carcinoma has in fact been detected in IPF lungs more frequently than other tumour types [85]. The nuclear/cytoplasmic concomitant SCCA immunoreactivity observed in our samples is difficult to interpret. Other works have recently described nuclear-cytoplasmatic

positive staining of serpin (maspin and SCCA) in non small cell lung carcinoma. In this work the nuclear maspin positivity was correlated with a better survival than with cytoplasmic staining [138]. The synthesis of SCCA is supposed to be essentially in the cytoplasm. However when it is overexpressed, nuclear import might occur thus playing a role in influencing the transcription of different growth factor genes such as TGF-β1. A significant overexpression of TGF-β1 was detected in our cases and interestingly it was significantly correlated with SCCA expression. The increased TGF-β1 transcription by SCCA observed in A549 pneumocytes confirms clinical observations detected in patients with IPF. Unfortunately, it is nearly impossible to work with primary alveolar epithelial cells, and we had to resort to the epithelial cell line A549, employed in numerous studies of IPF. There are different sources of TGF-β1 in IPF lungs, and active TGF-β1 transcription was detected in many metaplastic epithelial cells obtained by micro dissection of our samples. Active epithelial secretion of this profibrogenetic cytokine was first reported by Corrin *et al.* [139] and subsequently by other authors [140], thus underlining the important contributing role of epithelial cells in the development/progression of fibrotic process in IPF. The significant correlation between the expression of SCCA and the extension of FF and fibrosis observed in early-stage disease could be mediated through the increased transcription of the fibrotic cytokine TGF-β1, confirming the role of this cytokine as one of the most important inducers of fibrotic processes in IPF. The morphological evaluation confirms the prognostic significance of the quantitative measure of fibrosis and FF (instead of semi quantitative evaluation) in that these are the only pathological findings significantly correlated with DLCO decline and SCCA expression, as above reported. Various authors found low DLCO values alone or in combination with pulmonary hypertension or other clinical parameters, as a negative prognostic factor for IPF [141, 142]. The significant correlation of SCCA with DLCO decline observed in early-stage IPF patients at nine

months of follow-up could be related to progressive fibrosis favoured by increased TGF-β1 transcription. We were unable to correlate the grade of fibrosis with DLCO in end-stage disease, but we confirmed the inverse correlation between DLCO and SCCA values. This finding could be explained by the contributing role of alveolar epithelial dysfunction with impaired alveolar-capillary barrier and consequent progressive impaired gas exchange. In fact the modified metaplastic alveolar epithelium in IPF could change its original characteristics, with loss of gas-exchange function and orientation toward secretion of cytokines and other factors promoting aberrant cellular proliferation and immortalization. Although our findings need to be confirmed in larger case series we consider SCCA an important molecular target in the disease as it could orchestrate two of the most peculiar aspects of the disease: this serpin could act in an autocrine way favouring epithelial proliferation and metaplastic/dysplastic transformation while in a paracrine way it could influence miofibroblast proliferation and collagen synthesis through increased TGF-β1 transcription (Figure 18). The mutual influence of SCCA and TGF-β1 was also sustained in this research by the *in vitro* study. Monitoring of tissue SCCA expression during the clinical course of IPF could be useful for more precise patient stratification. It could be advisable to use a non-invasive approach such as the innovative serological analysis of SCCA-immunoglobulin M complex, a more sensitive and appropriate technique [143] that those previously used.

A final issue that needs to be taken into account is the possible role of SCCA in the development of lung cancer in IPF. In end-stage disease (Group B) we found an overexpression of SCCA in squamous metaplastic cells, that were those transformed epithelial cells with a higher grade of dysplasia. Squamous cell carcinoma or combined adenocarcinoma/squamous cell carcinoma are the most frequently described neoplastic forms detected in IPF [85, 106]. We confirm these data and we reported in our series an

high level of SCCA expression among neoplastic cells. This finding represents an indirect evidence of SCCA oncogenic influence.

Specific experimental models as those regarding transgenic SCCA animal models recently engineered in our lab, could be extremely useful to reinforce these data.
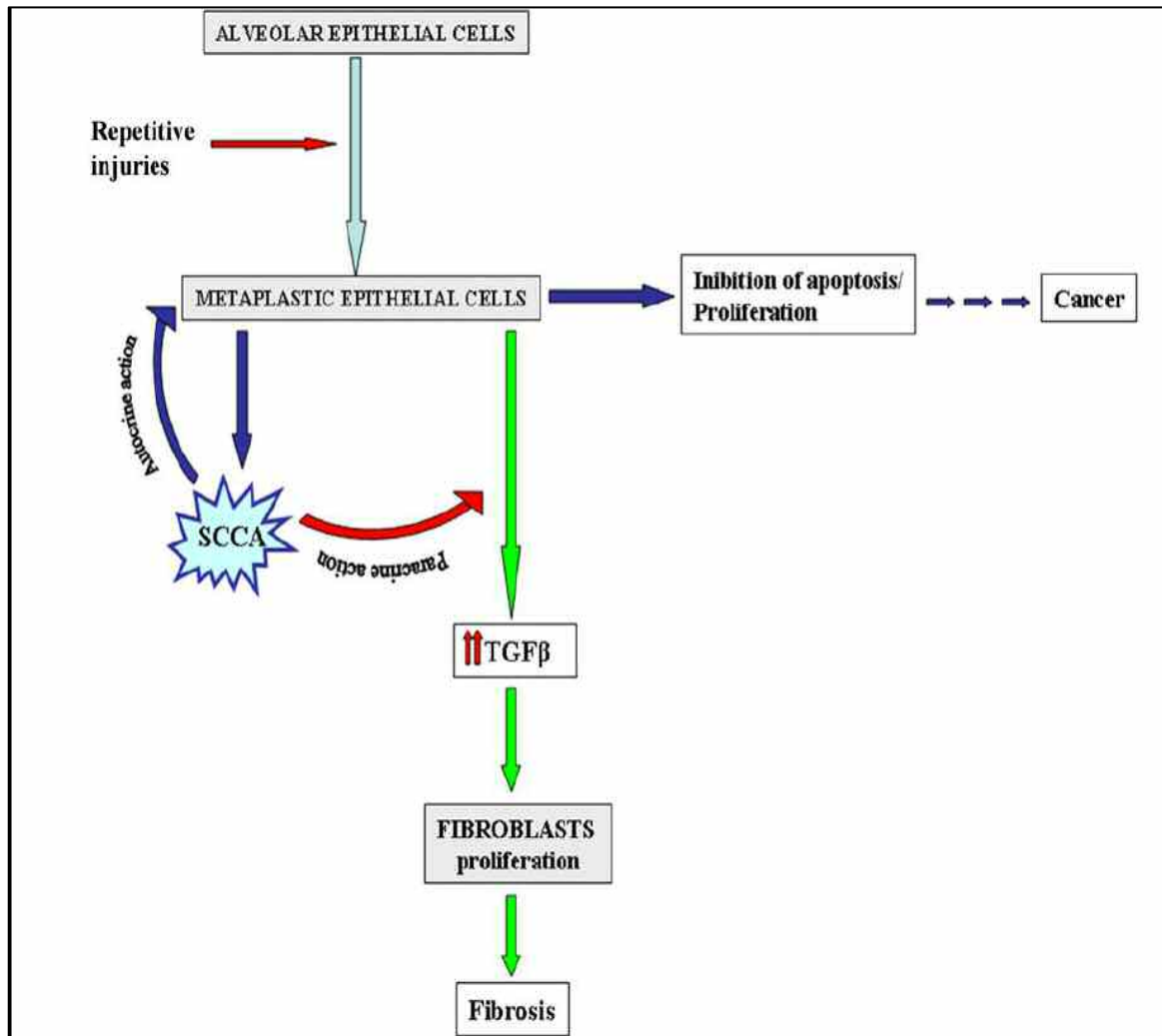

Figure 18. Novel putative schema showing SCCA pathway in IPF.
SCCA could play a crucial role in the development of the disease: influencing epithelial proliferation (autocrine action) or promoting fibroblast proliferation/fibrosis through increased TGF-β1 secretion (paracrine action).

SCIENTIFIC PRODUCTS OF THE PRESENT RESEARCH

Full papers

- Calabrese F, Lunardi F, Giacometti C, Marulli G, Gnoato M, Pontisso P, Saetta M, Valente M, Rea F, Agostini C. Over-expression of Squamous Cell Carcinoma Antigen in Idiopathic Pulmonary Fibrosis: clinico-pathological correlations. Thorax 2008; 63:795-802.

Abstracts

- Calabrese F, Giacometti C, Marulli G, Loy M, Rea F, Agostini C, Saetta M, Valente M. Over-expression of squamous cell carcinoma antigen (SCCA) in idiopathic pulmonary fibrosis. Laboratory Investigation 2006; 86 S1:304.

- Calabrese F, Giacometti C, Marulli G, Loy M, Rea F, Agostini C, Saetta M, Valente M. Over-expression of squamous cell carcinoma antigen (SCCA) in native lung with idiopathic pulmonary fibrosis. J Heart Lung Transplantation 2006; 25(S2):S186.

- Calabrese F, Giacometti, Marulli G, Loy M, Agostini C, Saetta M, Valente M. Epithelial expression of SCCA and TGF-β1 in idiopathic pulmonary fibrosis: clinico-pathological correlation. Proceedings of the ATS 2006; A183.

- Marulli G, Di Chiara F, Loy M, Calabrese F, Sartori F, Rea F. Significato prognostico della pressione polmonare e DLCO in pazienti affetti da fibrosi polmonare idiopatica in lista per trapianto. Abstract SITO 28-30/11/2007.

Oral Presentations (National and International Congress)

- 95th Annual Meeting of USCAP, Atlanta 12-17 February 2006.

- 26th Annual Meeting of the International Society for Heart and Lung Transplantation, Madrid 5-8 April 2006.

- American Thoracic Society (ATS) International Conference, San Diego 19-24 May 2006.

- XXXI Congresso nazionale Società Italiana Trapianti D'organo (SITO). Modena 28-30 November 2007.

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