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UNIVERSITY OF OULU,
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MEDICAL RESEARCH CENTER OULU;
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NHLRC2 AND EXTRACELLULAR MATRIX PROTEINS IN IDIOPATHIC PULMONARY FIBROSIS AND LUNG CANCER

Academic dissertation to be presented with the assent of the Doctoral Programme Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 10 of Oulu University Hospital, on 18 August 2023, at 12 noon

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Abstract

Idiopathic pulmonary fibrosis (IPF) and lung cancer share poor prognosis, similar genetic and epigenetic alterations, and common risk factors such as smoking. Myofibroblasts, which are often referred to as cancer-associated fibroblasts in tumors, are believed to act as principal pathogenetic cell types in both IPF and lung cancer.

Certain variants of NHL repeat-containing protein 2 (*NHLRC2*) gene are associated with fibrotic interstitial lung disease in early childhood. However, its cell type-specific expression in human lung tissues is unknown.

The aim of this study was to examine the gene expression profiles of cultured fibroblasts derived from patients with IPF and lung adenocarcinoma (ADC) as well as from normal lung by microarray analysis. Additionally, NHLRC2 expression was evaluated in lung cell and tissue samples from patients with IPF, ADC, and squamous cell carcinoma (SCC).

The microarray analysis revealed that 20 genes were similarly dysregulated in IPF and ADC compared to control. However, most of the altered genes, including several extracellular matrix genes, in IPF and ADC were differently expressed. Collagen α1(IV) chain as well as matrix metalloproteinases-1 and -3 were differentially expressed in IPF compared to control and ADC. Periostin expression was higher in both IPF and ADC in comparison to control.

NHLRC2 was expressed mainly in alveolar and bronchiolar epithelium and macrophages in normal lung, hyperplastic alveolar epithelial cells in IPF, and in cancer cells and inflammatory cells in lung cancer tumors. Additionally, the immunohistochemical expression of NHLRC2 determined by digital image analysis method was higher in IPF, ADC and SCC than in control lung. Furthermore, NHLRC2 expression was higher in ADC than in SCC. NHLRC2 expression was associated with smoking in IPF patients and with survival and mitotic activity in ADC patients. Further studies are needed to confirm the connections between IPF and lung cancer and to clarify the pathogenetic role of NHLRC2 in IPF and lung cancer.

Keywords: extracellular matrix, fibroblast focus, hyperplastic alveolar epithelium, idiopathic pulmonary fibrosis, lung adenocarcinoma, lung cancer, lung squamous cell carcinoma, NHLRC2, smoking

Kreus, Mervi, NHLRC2 ja soluvälitilan proteiinit idiopaattisessa keuhkofibroosissa ja keuhkosyövässä.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu; Oulun yliopistollinen sairaala

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Tiivistelmä

Sekä idiopaattisella keuhkofibroosilla (IPF) että keuhkosyövällä on huono ennuste, samantyyppiset solu- ja molekyylibiologiset muutokset sekä samoja riskitekijöitä, kuten tupakointi. Molemmissa sairauksissa esiintyy aktiivisia sidekudossoluja, joita kutsutaan myofibroblasteiksi tai syövän tapauksessa syöpään liittyviksi fibroblasteiksi.

NHLRC2 (NHL repeat containing protein 2) -geenin eräiden varianttien on havaittu olevan yhteydessä vakavaan monielinsairauteen, johon liittyy fibrotisoiva keuhkosairaus nuorilla lapsilla. NHLRC2:n soluspesifistä ilmentymistä keuhkokudoksessa ei kuitenkaan ole aiemmin julkaistu.

Tämän tutkimuksen tavoitteena oli verrata mikroarray-menetelmän avulla geenien ilmentymistä fibroblasteissa, jotka on kerätty IPF:ää ja keuhkosyöpää sairastavilta potilailta sekä normaalirakenteisesta keuhkosta. Lisäksi tavoitteena oli selvittää, missä solutyypeissä NHLRC2 ilmenee keuhkokudoksessa, verrata sen ilmentymistä IPF:ssä tai keuhkosyövässä ja normaalirakenteisessa keuhkossa, sekä selvittää, onko sillä yhteyttä IPF ja keuhkosyöpäpotilaiden kliinisiin tietoihin, kuten elinaikaan ja tupakointitaustaan, tai histologisiin tietoihin.

Mikroarray-menetelmällä saatujen tulosten mukaan kahdenkymmenen geenin ilmentyminen oli IPF:ssä ja keuhkosyövässä samalla tavalla korkeampi tai matalampi kontrolliin verrattuna. Useimpien geenien, joihin sisältyy soluvälitilan proteiineihin liittyviä tekijöitä, ilmentyminen kuitenkin erosi IPF:ssä ja keuhkosyövässä. Tyypin IV kollageenin α1-ketjun sekä matriksin metalloproteaasien 1 ja 3 ilmentyminen erosi IPF:n ja keuhkosyövän välillä. Periostiinin ilmentyminen oli puolestaan kohonnut sekä IPF:ssä että keuhkosyövässä kontrolliin verrattuna.

NHLRC2 ilmentyi pääasiassa alveoliepiteelissä, pienten ilmateiden epiteelissä sekä makrofageissa normaalissa keuhkossa, hyperplastisessa alveoliepiteelissä IPF:ssä, ja syöpäsoluissa sekä tulehdussoluissa keuhkosyövässä. NHLRC2:n määrä oli sekä IPF:ssä että keuhkosyövissä korkeampi kuin kontrollissa. Lisäksi NHLRC2:n määrä oli korkeampi keuhkon adenokarsinoomassa kuin levyepiteelikarsinoomassa. NHLRC2:n ilmentyminen oli yhteydessä tupakointiin IPF-potilailla sekä elinaikaan ja syöpäsolujen mitoottiseen aktiivisuuteen adenokarsinoomapotilailla. Lisätutkimuksia tarvitaan IPF:n ja keuhkosyövän yhteyden selvittämiseksi sekä selventämään NHLRC2:n roolia IPF:n ja keuhkosyövän patogeneesissa.

Asiasanat: adenokarsinooma, fibroblastifokus, hyperplastinen alveoliepiteeli, idiopaattinen keuhkofibroosi, keuhkosyöpä, levyepiteelikarsinooma, NHLRC2, soluvälitilan proteiinit, tupakointi

To my family

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Oulu, May 2023 Mervi Kreus

Abbreviations

ADAM	a disintegrin and metalloproteinase
ADAMT	ADAM with a thrombospondin motif
ADC	adenocarcinoma
AE	acute exacerbation
ALK	anaplastic lymphoma kinase
α-SMA	α-smooth muscle actin
ATCC	American type culture collection
ATS	American Thoracic Society
BALF	bronchoalveolar lavage fluid
CAF	cancer associated fibroblast
COPD	chronic obstructive pulmonary disease
CD	cluster of differentiation
CPI	composite physiology index
CT	computed tomography
DAB	3,3'-diaminobenzidine
DAD	diffuse alveolar damage
DLCO	diffusion capacity for carbon monoxide
DSS	disease-specific survival
ECM	extracellular matrix
EDA	extra type III domain A
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
ERS	European Respiratory Society
FEV1	forced expiratory volume in one second
FF	fibroblast focus
FGF	fibroblast growth factor
FINCA	fibrosis, neurodegeneration and cerebral angiomatosis
FVC	forced vital capacity
GAP	gender-age-physiology index
HRCT	high-resolution computed tomography
ILD	interstitial lung disease
IIP	idiopathic interstitial pneumonia
IPF	idiopathic pulmonary fibrosis
IQR	interquartile range
MMP	matrix metalloproteinase

MUC	mucin
NAC	N-acetylcysteine
NSCLC	non-small cell lung cancer
NHLRC2	NHL repeat-containing protein 2
OS	overall survival
PBTE	primary bronchial/tracheal epithelial cells
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PD-L1	programmed cell death ligand 1
ROS	reactive oxygen species
RT-qPCR	real-time quantitative reverse-transcription polymerase chain reaction
SAEC	small airway epithelial cells
SD	standard deviation
SLB	surgical lung biopsy
TGF-β	transforming growth factor β
Trx	thioredoxin
TTF-1	thyroid transcription factor-1
TNM	tumor-node-metastasis staging system
UIP	usual interstitial pneumonia
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
VEGF	vascular endothelial growth factor
WHO	World Health Organization

List of original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:

I Kreus, M., Lehtonen, S., Skarp, S., & Kaarteenaho, R. (2021). Extracellular matrix proteins produced by stromal cells in idiopathic pulmonary fibrosis and lung adenocarcinoma. *PloS One*, *16*(4), Article e0250109. https://doi.org/10.1371/journal.pone.0250109

II Kreus, M., Lehtonen, S., Hinttala, R., Salonen, J., Porvari, K., & Kaarteenaho, R. (2022). NHLRC2 expression is increased in idiopathic pulmonary fibrosis. *Respiratory Research*, *23*, Article 206. https://doi.org/10.1186/s12931-022-02129-z

III Kreus, M., Lehtonen, S., Mäkinen, J., Lappi-Blanco, E., Laitakari, K., Johnson, S., Hinttala, R., & Kaarteenaho, R. (2023). High NHLRC2 expression is associated with shortened survival in lung adenocarcinoma. *Manuscript accepted for publication*.

Contents

1 Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease (ILD) (Raghu et al., 2022). It is considered to result from a failure of alveolar epithelial cell repair after injury, which leads to increased expression of profibrotic mediators, e.g., transforming growth factor (TGF)-β1, and activation of fibroblasts and abnormal wound healing responses (Fernandez & Eickelberg, 2012b; Moss et al., 2022). The histological pattern of IPF, referred to as usual interstitial pneumonia (UIP), is characterized by heterogeneous lesions with dense fibrosis, fibroblast foci (FF) consisting of fibroblasts, myofibroblasts and extracellular matrix proteins, hyperplastic and metaplastic epithelial cells lining the alveoli and re-epithelialized air spaces, i.e., honeycomb cysts (Raghu et al., 2022).

The clinical course of IPF varies widely, with some patients experiencing acute exacerbations (AE), defined as acute, clinically significant respiratory deteriorations (Collard et al., 2016). The most common histopathological finding of AE-IPF is diffuse alveolar damage superimposed on underlying UIP (Oda et al., 2014). The molecular mechanisms underlying AE-IPF are poorly understood.

Lung cancer is the leading cause of cancer-related deaths and comprises a heterogeneous group of tumors (Dyba et al., 2021). The most common histological types of lung cancer are squamous cell carcinoma (SCC) and adenocarcinoma (ADC), which are further divided into several subtypes (WHO Classification of Tumours Editorial Board, 2021). SCC and ADC originate from different cells and have differences in molecular characteristics, biological patterns, and therapeutic strategies. SCCs are believed to arise from the epithelial cells of large airways and are most commonly associated with smoking, while ADCs, which are mainly located at the peripheral lung, are the most common type of lung cancer observed in nonsmokers (Pesch et al., 2012).

Genetic and epigenetic alterations, uncontrolled proliferation, and appearance of hyperplastic and metaplastic epithelial cells have been shown to occur in IPF as well as in cancer (Ballester et al., 2019). Lung cancer and IPF also share common risk factors, such as smoking, and patients with IPF have been reported to be at greater risk of developing lung cancer compared to general population (le Jeune et al., 2007). In addition, a tyrosine kinase inhibitor, namely nintedanib, is used in the treatment of both lung cancer and IPF (Reck et al., 2014; Richeldi et al., 2015). Myofibroblasts, which are often referred to as cancer-associated fibroblasts (CAFs) in tumors, are thought to act as principal pathogenetic cell types in both IPF and lung cancer and are the main cell type producing high levels of extracellular matrix

proteins (Moss et al., 2022; Wong et al., 2022). Despite the many similarities between IPF and cancer further studies are needed to show possible connections between these two diseases at the molecular level.

The gene expressions of IPF and lung cancer have previously been evaluated mainly individually with microarray studies performed on RNA isolated from whole lung tissues. A few studies have used cultured stromal cells derived from IPF and normal lung tissues (Hsu et al., 2011; Lee et al., 2017; Lindahl et al., 2013; Peng et al., 2013; Rodriguez et al., 2018; Vuga et al., 2009). Stromal cells from lung cancer have been investigated in only one microarray analysis (Navab et al., 2011). Fibrotic and tumor tissues from patients with both lung cancer and IPF have been studied with a microarray-based analysis (Takenaka et al., 2009). However, differences in gene expression profiles between cultured stromal cells derived from IPF and lung ADC have not been investigated previously in the same study.

Certain variants of NHL repeat-containing protein 2 (NHLRC2, gene name *NHLRC2*) have been associated with a multiorgan disease in early childhood called FINCA (fibrosis, neurodegeneration, and cerebral angiomatosis) (Brodsky et al., 2020; Rapp et al., 2021; Uusimaa et al., 2018). Histopathological findings in the lungs of some FINCA patients resembled those of fibrotic non-specific interstitial pneumonia (Rapp et al., 2021; Uusimaa et al., 2018). *NHLRC2* has been identified as a differentially expressed gene between rapidly progressing and relatively stable IPF patients in one genome-wide expression study performed on lung tissue samples (Boon et al., 2009). High *NHLRC2* gene expression in lung tumor samples combined with the expression profiles of one long non-coding RNA and two other protein coding genes has been associated with a long survival time in lung ADC patients in a study utilizing publicly available gene expression datasets (Ye et al., 2019). However, its cell type-specific expression in human lung tissues has not been published previously.

In this study, mRNA expressions in stromal cell lines derived from ADC from tumor and corresponding normal tissue as well as from patients with IPF were studied by microarray analysis. The mRNA and protein expression of extracellular matrix proteins periostin, collagen α1(IV) chain, matrix metalloproteinase 1 (MMP-1), and MMP-3 in fibroblasts was further studied in cell lines and lung tissue samples derived from IPF and lung cancer due to their previously shown association with pulmonary fibrosis (Okamoto et al., 2011; Urushiyama et al., 2015; Yamashita et al., 2011). Additionally, the expression pattern of NHLRC2 in lung tissue samples derived from patients with IPF, ADC and SCC was described, and its expression levels were associated with clinical and histological information of

the IPF and lung cancer patients. Furthermore, the effect TGF-β1 on NHLRC2 mRNA and protein levels in primary lung stromal or epithelial cell lines was investigated *in vitro*.

2 Review of literature

2.1 Human lung and pulmonary cells

The human respiratory system is divided into the proximal conducting airways, including the trachea and large airways, i.e., bronchi, and the distal respiratory airways, including small airways, i.e., bronchioles, and alveolar parenchyma (Figure 1). The bronchi consist of epithelium, smooth muscle, cartilage, and submucosal glands (Garusi et al., 1964; Miller, 1906). Bronchioles lack cartilage, and depending on their diameter, they can be further divided into conducting bronchioles, respiratory bronchioles, and alveolar ducts (Garusi et al., 1964).

Single-cell RNA sequencing studies of normal and diseased lung tissues have revealed huge cellular heterogeneity within the airways (Carraro et al., 2020; Deprez et al., 2020; Travaglini et al., 2020). The structure and cell type composition of the airway epithelium changes with increasing airway generations. The airway epithelium is pseudostratified in the large airways, and columnar and cuboidal in the small airways. The major cell types of the airways are multiciliated cells, goblet cells, club cells (previously known as Clara cells), and basal cells (Davis & Wypych, 2021; Tata & Rajagopal, 2017). They also include rare cells such as ionocytes, neuroendocrine cells, brush cells (also known as tuft cells), hillock cells, and microfold cells (Davis & Wypych, 2021). The basal cells are more abundant in the bronchi than in the bronchioles (Boers 1998). They are the main airway stem cells as they show self-renewal capacities and the ability to differentiate into multiciliated, club and goblet cells (Davis & Wypych, 2021). The number of goblet cells decreases from bronchi towards bronchioles, and they are absent in the respiratory bronchioles (Boers et al., 1999). In contrast, the number of club cells increases in the small airways, being highest in respiratory bronchioles (Boers et al., 1999).

Alveoli make up the functional tissue of the lungs known as the lung parenchyma. The interalveolar septum provides the structural basis for oxygen and carbon dioxide exchange by separating the alveolar and capillary lumen (Reid, 1959). Alveoli are lined by a continuous epithelium composed of alveolar epithelial type I and type II cells, i.e., type I and type II pneumocytes. Type I pneumocytes are thin and flat, with multiple branches spread over a large area of the alveolar epithelial basal lamina. Thus, although they represent around 40% of the alveolar cellular population, they cover 95% of the alveolar surface and are the primary

mediators of gas exchange (Knudsen & Ochs, 2018). In contrast, the small, cuboidal type II pneumocytes cover the remaining 5% of the alveolar surface area (Knudsen & Ochs, 2018). Type II pneumocytes produce surfactant to reduce the surface tension and function as the primary progenitors for injured type I pneumocytes (Knudsen & Ochs, 2018). Alveolar epithelial cells and endothelial cells are attached to basement membranes. Basement membranes are sheet-like specialized extracellular matrix (ECM) structures that function as an interface between epithelial cells and underlying connective tissue (Jayadev & Sherwood, 2017). The space between the epithelial and endothelial basement membranes of peripheral lung tissue is called interstitium (Knudsen & Ochs, 2018).

The interstitium contains cells and an extracellular network of elastic fibers and bundles of collagen fibrils (Knudsen & Ochs, 2018). The cells within the connective tissue include resident fibroblasts, myofibroblasts, pericytes, and mesenchymal stem cells. Fibroblasts and myofibroblasts are the main cells in the production, maintenance and repair of ECM. Heterogeneity of fibroblasts, defects in their repair functions, and activation of myofibroblasts may contribute to the pathogenesis of fibrotic interstitial lung diseases (ILD) and lung cancer (Kuhn & McDonald, 1991; Wong et al., 2022). The pathogenetic mechanism of idiopathic pulmonary fibrosis (IPF) and lung cancer is discussed later in more detail.

The lungs also contain innate and adaptive immunity cells including alveolar and interstitial macrophages, innate lymphoid cells, dendritic cells, lymphocytes, and inflammatory cells that participate in defenses against respiratory infections, toxins, and carcinogens (Ardain et al., 2020). Macrophages are active, phagocytic cells differentiated from a certain type of leukocytes called monocytes (Gordon & Read, 2002). They are located in different lung compartments (Gordon & Read, 2002).

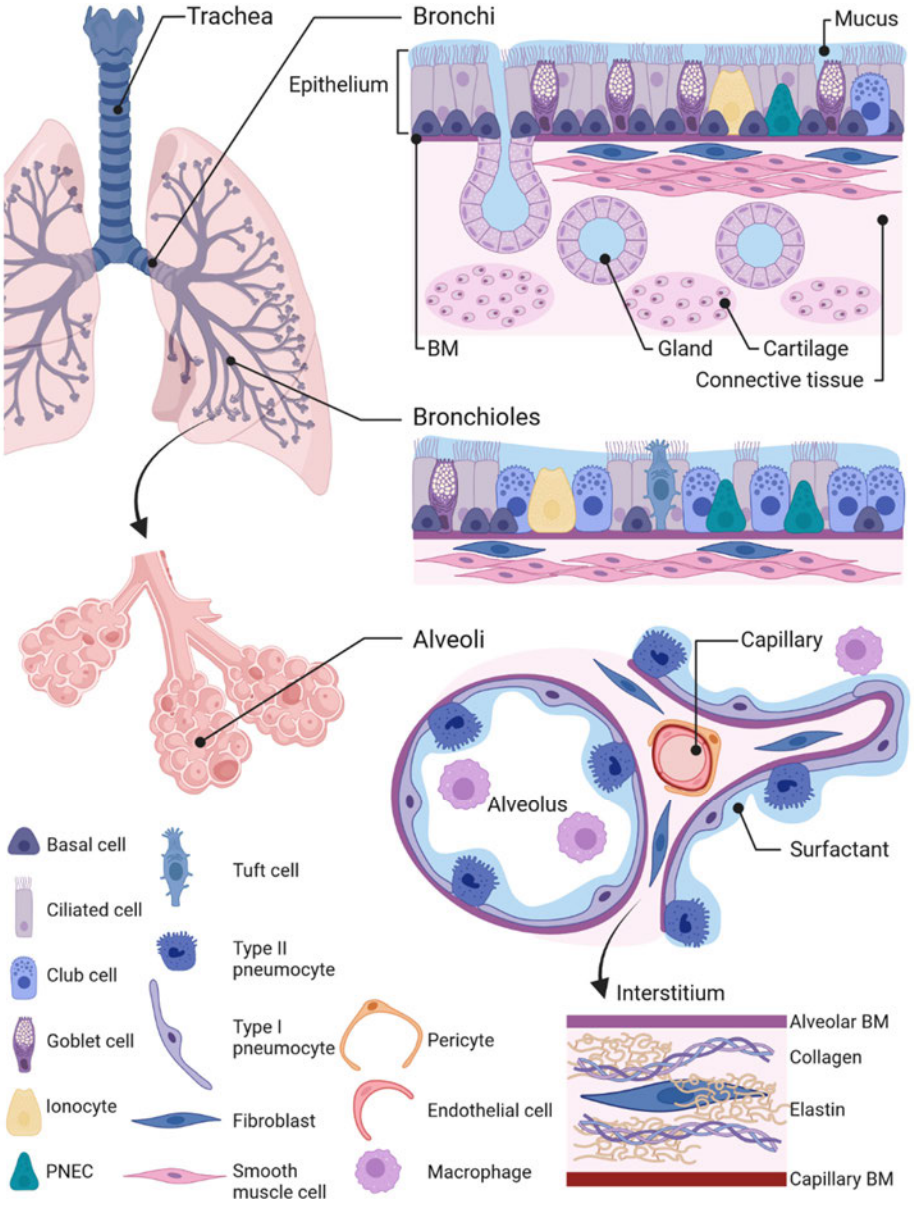

Fig. 1. Structure and cell types of the lungs. The human respiratory system consists of trachea, large airways i.e, bronchi, small airways, i.e., bronchioles and alveoli. The distinct lung compartments have different structures and epithelial and stromal cell populations (Davis & Wypych, 2021; Tata & Rajagopal, 2017; Travaglini et al., 2020).

Lung interstitium, i.e., the space between the epithelial and endothelial basement membranes, contains cells such as fibroblasts and extracellular matrix proteins like elastin and collagen (Knudsen & Ochs, 2018). BM, basement membrane; PNEC, pulmonary neuroendocrine cell. Created with BioRender.com

2.1.1 Fibroblasts and myofibroblasts

Lung stromal cells include fibroblasts, myofibroblasts, muscle cells, endothelial cells, and pericytes (Travaglini et al., 2020). They not only provide structural support, but also participate in maintaining homeostasis by participating in the synthesis and degradation of ECM and by interacting with immune cells. Fibroblasts are a heterogeneous group of different cell types, and different subtypes are located in different compartments of the lungs (Tsukui et al., 2020). Compared to normal lung, different lung diseases have different types of fibroblast populations (Lambrechts et al., 2018; Tsukui et al., 2020). Morphologically, fibroblasts are described as spindle-shaped connective tissue cells that are surrounded by fibrous ECM. After activation, for example, during wound repair, fibroblasts can differentiate into myofibroblasts. Fibroblasts first differentiate into proto-myofibroblasts, which form cytoplasmic actin-containing stress fibers, express and organize cellular fibronectin at the cell surface, and can generate contractile force (Tomasek et al., 2002). TGF-β1 increases the expression of ED-A fibronectin, which in the presence of mechanical stress promotes the differentiation of proto-myofibroblasts into myofibroblasts (Tomasek et al., 2002). Myofibroblasts exhibit an increased contractile capacity, which is caused by the presence of alpha-smooth muscle actin (α-SMA)-positive filaments (Darby et al., 1990). Myofibroblasts produce increased amounts of ECM proteins, such as collagen I and III, tenascin-C and EDA-fibronectin (Kaarteenaho-Wiik et al., 2005; Kuhn & McDonald, 1991; Muro et al., 2008).

Fibroblasts and myofibroblasts are essential cell types in the pathogenesis of IPF and lung cancer. Myofibroblasts are accumulated in IPF and together with aggregated ECM proteins, they form structures called FF within the lung interstitium (Kuhn & McDonald, 1991). In lung cancer, fibroblasts and myofibroblasts are often called CAFs, and they secrete various chemokines, cytokines, growth factors and ECM proteins that are involved in cancer cell proliferation, invasion, and metastasis (Wang et al., 2017). The most commonly used marker for myofibroblasts is α-SMA (encoded by ACTA2 gene), although it

is not specific for them since it is also expressed in other cells such as smooth muscle cells and pericytes (Tsukui et al., 2020).

Based on electron microscopic analyses, fibroblastic cells cultured from tissue samples and bronchoalveolar lavage fluid from patients with different lung diseases, including IPF and lung cancer, are mixtures of fibroblasts and myofibroblasts (Karvonen et al., 2012, 2014). The ultrastructure of cultured stromal cells, especially myofibroblasts, differs between ILD, lung cancer and normal lung (Karvonen et al., 2012, 2014). The cells derived from ILDs have shown to have more actin filaments and express more α-SMA expression than cells derived from normal lung (Karvonen et al., 2012). Cells derived from IPF patients also have higher invasive and migration capacity than cells derived from normal lung (Karvonen et al., 2012; Suganuma et al., 1995). CAFs are shown to exhibit more contraction in collagen gel than the fibroblastic cells of normal peripheral lung (Horie et al., 2012; Karvonen et al., 2014). Fibroblastic cells cultured from lung tissues of smokers have been reported to display less myofibroblastic ultrastructural features, e.g., less actin filaments, than those of non-smokers (Karvonen et al., 2014; Lehtonen et al., 2021). The fibroblastic cells have been shown to maintain their phenotype at least partially through several passages (Karvonen et al., 2012). However, the gene expression profile of cells can change during the culture (Rodriguez et al., 2018).

2.1.2 Extracellular matrix proteins

Within all tissues, ECM and ECM-associated proteins, together referred to as the matrisome, make up a non-cellular component that consists of a complex network of highly cross-linked proteins, water, and polysaccharides (Burgstaller et al., 2017; Naba et al., 2012). ECM is a dynamic structure that is constantly remodeled, either enzymatically or non-enzymatically, and its components are subjected to various post-translational modifications. ECM provides architectural, mechanical, and biochemical support for cells and organs (Burgstaller et al., 2017). ECM functions as a reservoir for several growth factors and cytokines and it is essential for cell signaling. Matrisome proteins can be divided into core matrisome and matrisome-associated proteins (Naba et al., 2012). The core matrisome consists of collagens, glycoproteins, and proteoglycans. Matrisome-associated proteins are further divided into ECM regulators, ECM-affiliated proteins, and secreted factors (Naba et al., 2012). ECM-affiliated proteins either share some structural similarities with ECM proteins or are associated with ECM proteins.

As in other organs, ECM in the lungs is organized into two different structural types: basement membranes and interstitial matrixes. Basement membranes are thin sheets that line the basal side of endothelia and epithelia and surround muscle, peripheral nerve, and fat cells (Pozzi et al., 2017). One of the major components of the basement membranes is type IV collagen, which belongs to non-fibrillar collagens (Pozzi et al., 2017). Collagen IV is made up of six distinct alpha chains, α1-6(IV), that form the heterotrimers α1α1α2, α3α4α5, and α5α5α6, which are selectively expressed in the mammalian basement membranes (Hudson et al., 1993). These heterotrimers form mesh-like networks and integrate with laminins, nidogens, and the heparan sulphate proteoglycans perlecan and agrin to form basement membranes (Jayadev & Sherwood, 2017).

Collagens are a major structural component of ECM. Disruption of collagen homeostasis is an important contributor to different lung diseases, including pulmonary fibrosis. Type I and III collagens are the most common components in the alveolar interstitium (Suki et al., 2005). Structurally, they belong to fibrillar collagens and contribute to the architecture of the lung with their great tensile strength (Suki et al., 2005). In contrast to collagens, elastic fibers have low tensile strength and high elasticity, and provide the lung with its necessary compliance and elastic recoil (Knudsen & Ochs, 2018). The core of elastic fibers is composed of elastin whereas the outer periphery contains microfibrils. Large glycoproteins (proteins containing oligosaccharide chains, i.e., glycans) called fibrillins are the main components of microfibrils (Suki et al., 2005).

The degradation of ECM is an essential event in tissue remodeling, and metalloproteinases play an important role in it (Burgstaller et al., 2017; Lu et al., 2011). The superfamily of metalloproteinases comprises the MMPs and adamalysins. They are calcium- and zinc-dependent endopeptidases that together degrade all components of the ECM and basement membranes. In addition, they can cleave many bioactive mediators such as cytokines, growth factors, and chemokines. MMPs shed a wide range of ECM proteins. MMP-1 and MMP-8, for example, target collagens III and I (Lu et al., 2011). Adamalysins include a disintegrin and metalloproteinase (ADAM) family and ADAMs with a thrombospondin motif (ADAMTS). ADAMs can shed cytokines and growth factors while ADAMTs mainly participate in degradation of ECM components (Lu et al., 2011). Factors that can inhibit metalloproteinases include tissue inhibitors of metalloproteinases (Lu et al., 2011).

Changes in ECM are associated with many chronic lung diseases, including IPF and lung cancer. In a fibrotic lung, an imbalance exists in the turnover of ECM,

causing excessive production and deposition of ECM proteins and resulting in a disease-specific composition and organization of the matrix (Burgstaller et al., 2017). The ECM and its remodeling by cancer, stromal and immune cells can promote the growth, survival, and metastasis of cancer cells (Wong et al., 2022).

2.2 NHLRC2

NHLRC2, encoded by the *NHLRC2* gene, contains an N-terminal Trx-like domain, a six-bladed NHL repeat (named after *NCL-1*, *HT2A*, and *LIN-41* genes)-containing β-propeller domain, and a C-terminal β-stranded domain (Biterova et al., 2018). NHLRC2 is cleaved by caspase-8 in reactive oxygen species (ROS)-induced apoptosis in colon cancer cell line, and loss of NHLRC2 resulted in an increased susceptibility of those cells to apoptosis (Nishi et al., 2017). Thus, it has been suggested to have a role in the regulation of ROS-induced apoptosis (Nishi et al., 2017). It also has a role in vesicle transport, cytoskeleton organization, and fibroblast differentiation (Paakkola et al., 2018). NHLRC2 has been shown to have a role in phagocytosis and actin dynamics in human macrophages in two genome-wide knockout screens (Haney et al., 2018; Yeung et al., 2019). It is essential to embryonic development in mice, since loss of NHLRC2 leads to embryonic lethality (Hiltunen et al., 2022).

Certain bi-allelic variants of *NHLRC2* are associated with a multiorgan disease named FINCA disease (OMIM #618278) based on histologic findings (fibrosis, neurodegeneration and cerebral angiomatosis) (Uusimaa et al., 2018). The FINCA disease was first identified in three Finnish infants (Uusimaa et al., 2018); after that, it has also been diagnosed in several cases in other countries (Badura-Stronka et al., 2022; Boschann et al., 2022; Brodsky et al., 2020; Rapp et al., 2021). The histopathological findings in the lung tissues of the Finnish patients resembled those of fibrotic non-specific interstitial pneumonia (NSIP) (Uusimaa et al., 2018), and the others resembled desquamative interstitial pneumonia, pulmonary alveolar proteinosis, or cholesterol pneumonia (Rapp et al., 2021).

NHLRC2 has previously been listed as a differentially expressed gene between relatively stable and rapidly progressing IPF patients in one genome-wide expression study (Boon et al., 2009). High *NHLRC2* gene expression combined with the expression of two other protein-coding genes and one long non-coding RNA in lung tumor samples has been associated with survival time in lung adenocarcinoma patients in one study utilizing publicly available gene expression

datasets (Ye et al., 2019). However, the physiological function of NHLRC2 is still unclear.

2.3 Interstitial lung diseases

ILDs are a heterogeneous group of distinct diseases affecting the interstitium of the lung by inflammation or fibrosis (Travis et al., 2013; Wijsenbeek et al., 2022). They can be classified into idiopathic ILDs, autoimmune-related ILDs (e.g., connective tissue disease-associated ILD), exposure-related ILDs (e.g., hypersensitivity pneumonitis), sarcoidosis, ILDs with cysts or airspace filling (e.g., pulmonary alveolar proteinosis), and other forms (Wijsenbeek et al., 2022). A major group of ILDs with unknown etiology are called idiopathic interstitial pneumonias (IIP) (Travis et al., 2013).

IIPs are classified into major IIPs, rare IIPs, and unclassifiable IIPs (Travis et al., 2013). Major IIPs include IPF, idiopathic NSIP, respiratory bronchiolitis-interstitial pneumonia, acute interstitial pneumonia, desquamative interstitial pneumonia, and cryptogenic organizing pneumonia (Travis et al., 2013). IPF is one the most common IIPs. Other ILDs can also display a progressive phenotype and a clinical course similar to IPF (Cottin et al., 2018).

2.4 Idiopathic pulmonary fibrosis

2.4.1 Definition and clinical presentation

The nomenclature used for IPF has varied in the past decades (Scadding & Hinson, 1967; Turner-Warwick et al., 1980). In the 1960s, the term “diffuse fibrosing alveolitis” was used since it was thought to represent an inflammatory parenchymal process primarily affecting the alveoli (Scadding, 1967). In 1969, Liebow & Carrington introduced a spectrum of histologic features, including UIP, in patients with IIPs (Liebow & Carrington, 1969). The terms UIP and cryptogenic fibrosing alveolitis were used for IPF and occasionally also for other IIPs until the end of the 20th century (Turner-Warwick et al., 1980). In their IIP classification published in 1998, Katzenstein and Myers suggested that the histologic UIP pattern should be the manifestation of IPF and no other IIPs (Katzenstein & Myers, 1998). In 2000 the first international consensus statement on IPF was published, and in 2002 the current use of terms for IIPs was established in a consensus classification by the

American Thoracic Society (ATS) and European Respiratory Society (ERS) (King et al., 2000; Travis et al., 2002).

IPF is a chronic lung disease in which connective tissue replaces the normal gas-exchanging tissue (Raghu et al., 2011, 2018, 2022). IPF is restricted to the lungs, and it typically presents in older individuals (Raghu et al., 2022). The symptoms of IPF are typically nonspecific: slowly progressing dyspnea, desaturation, cough, bibasilar inspiratory crackles, and clubbed fingers (Raghu et al., 2018, 2022). An unpredictable clinical course of disease is typical for IPF.

2.4.2 Epidemiology and risk factors

The estimated prevalence and incidence vary greatly between studies due to different methodologies, case definition, and classification systems (Hutchinson et al., 2015). The prevalence of IPF ranges between 7.8 and 24.3 cases per 100,000 persons (Hilberg et al., 2022). The prevalence of IPF in Finland was earlier reported to be 16–18 per 100,000 (Hodgson et al., 2002). Recently, the prevalence of IPF in specialized care in Finland was estimated to be 36.0 cases per 100,000 residents (Salonen et al., 2022).

The incidence of IPF in Europe and North America has been estimated to range from 3 to 9 cases per 100,000 persons per year between 1998 and 2012 in one meta-analysis (Hutchinson et al., 2015). The incidence of IPF has been estimated to vary between 2.1 and 6.3 cases per 100,000 persons in a study including six European countries (Hilberg et al., 2022).

Both genetic and environmental factors may contribute to the development of IPF, and it can occur sporadically and occasionally in families. Familial pulmonary fibrosis is defined as having at least two blood relatives with any fibrotic ILD among first- or second-degree family members (Borie et al., 2022). The familial cases have been reported to represent 25% of all IPF cases (Cutting et al., 2021). Genome-wide association and familial studies have associated IPF risk with several different common and rare risk variants. Common variants (minor allele frequency of > 5%) linked with IPF include single nucleotide polymorphism (rs35705950) of mucin 5B gene (*MUC5B*) (Dhindsa et al., 2021; Gong et al., 2020). MUC5B is a glycoprotein required for airway clearance and innate immune responses to bacteria (Roy et al., 2014). Additional variants include genes related to innate immune function, e.g., *TOLLIP*, epithelial barrier function, e.g., *DSP*, telomere maintenance, e.g., *TERT* and cell cycle regulation, e.g., *KIF15* (Allen et al., 2020). Rare variants (with minor allele frequency of < 0.1%) have been reported in telomere-related

genes such as *TERT, RTEL1*, *PARN*, *TERC* (Zhang et al., 2022), and surfactant biology related genes including *SFTPC* and *SFTPA* (Sutton et al., 2022; Zhang et al., 2022).

Non-genetic factors identified as risk factors for IPF include older age, male sex, and smoking (Ekström et al., 2014; Park et al., 2021). IPF is more common in males and in subjects with smoking history, and the incidence of IPF increases with age (Raghu et al., 2006). Environmental exposure, such as pollutants, occupational exposures, gastro-esophageal reflux, and viral infections may also increase the risk of IPF (Baumgartner et al., 2000).

2.4.3 Pathogenesis

IPF was initially thought to represent an inflammatory parenchymal process primarily affecting the alveoli (Scadding & Hinson, 1967). Fibrosis was considered to result from the activation of macrophages that produce growth factors and stimulate fibroblasts to produce ECM. Later, IPF has been considered to result from a failure of alveolar epithelial cell repair following repetitive injury leading to increased expression of profibrotic factors, such as TGF-β1, and activation of fibroblasts and aberrant wound healing responses (Figure 2) (Fernandez & Eickelberg, 2012a; Moss et al., 2022; Selman et al., 2001).

Type II pneumocytes function as epithelial stem cells in the distal lung and have an important role in the regeneration and repair of injured alveoli (Barkauskas et al., 2013). Recent single-cell RNA sequencing studies have identified diverse aberrant epithelial cell populations lining FF and honeycombs in IPF lung tissue (Adams et al., 2020; Carraro et al., 2020; Habermann et al., 2020). Apoptosis, senescence, abnormal differentiation, and impaired renewal capacity of type II pneumocytes have been observed in IPF lungs (Adams et al., 2020; Barbas-Filho et al., 2001). Cuboidal epithelial cells overlying FF termed “aberrant basaloid cells” express basal epithelial, mesenchymal, senescence, and developmental markers (Adams et al., 2020). Several factors that may underlie dysfunction of type II pneumocytes and lead to secretion of profibrotic mediators in IPF have been proposed; they include telomere shortening, oxidative stress, mitochondrial dysfunction, and increased endoplasmic reticulum stress (imbalances in protein homeostasis that lead to the accumulation of unfolded proteins and activation of unfolded protein response) (Estornut et al., 2022; Parimon et al., 2020).

Inability of type II pneumocytes to resolve endoplasmic reticulum stress may lead to apoptosis but also to recruitment of profibrotic macrophages and cytokines

(Moss et al., 2022). A pro-fibrotic macrophage subpopulation highly expressing secreted phosphoprotein 1 has been identified surrounding and within FF (Morse et al., 2019). It has been speculated that dysregulated crosstalk between aberrant basaloid cells and pro-fibrotic macrophages could represent a pathway of progressive lung fibrogenesis (Spagnolo et al., 2021). Signaling of both alveolar epithelial cells and macrophages may contribute to differentiation of fibroblasts to myofibroblasts (Moss et al., 2022; J. Wang et al., 2022).

Following epithelial cell injury, fibroblasts are recruited, and they differentiate into myofibroblasts that produce dense, poorly organized ECM and have the ability to generate contractile force for wound closure due to α-SMA stress fibers (Kuhn & McDonald, 1991). Unlike after normal wound healing, myofibroblasts are not removed in IPF. Instead, they accumulate, forming FF (Kuhn & McDonald, 1991). The factors mediating differentiation of myofibroblasts and their resistance to apoptosis include TGF-β, integrin αVβ6, connective tissue growth factor, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), overexpression of Wnt, and imbalance of oxidant-antioxidants (Moss et al., 2022). TGF-β pathway is one of the most upregulated pathways in the aberrant epithelial cell population (Adams et al., 2020). TGF-β signaling also promotes recruitment of inflammatory mediators that stimulate the production of ECM (Moss et al., 2022). The ECM proteins expressed within FF include collagens I, III, IV, V, VI, fibronectin, hyaluronan, fibrinogen, tenascin-C and versican (Herrera et al., 2019; Herrera et al., 2022; Kaarteenaho-Wiik et al., 1996).

Resident fibroblasts, pulmonary fibrocytes and fibrocytes derived from bone marrow and blood circulation are suggested to be possible progenitors of myofibroblasts (Andersson-Sjöland et al., 2008). It has also been suggested that alveolar epithelial cells undergo epithelial-to-mesenchymal transition and are one source of fibroblasts (Harada et al., 2010). However, single-cell RNA-sequencing data have not supported an epithelial origin of fibroblasts (Adams et al., 2020; Habermann et al., 2020). Instead of transforming into mesenchymal cells, it has been suggested that epithelial cells more likely acquire mesenchymal markers that promote fibrosis (Moss et al., 2022).

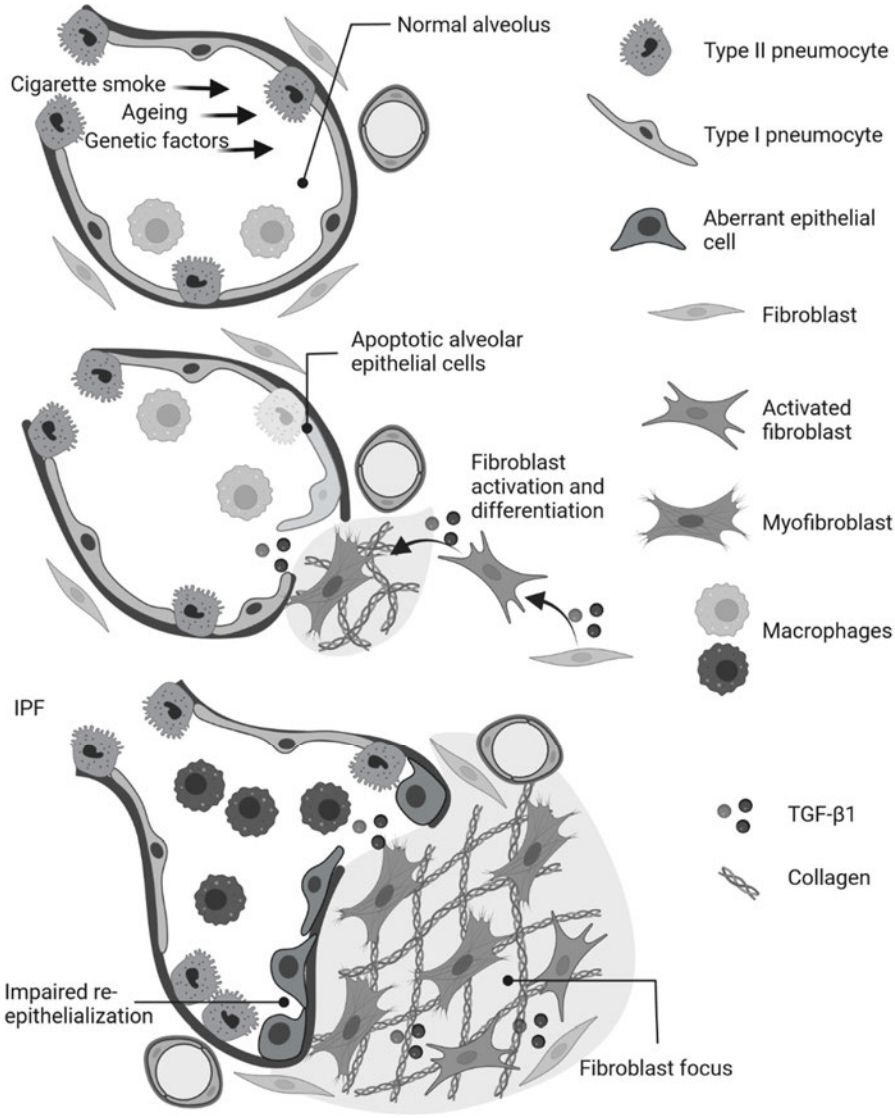

Fig. 2. Pathogenesis of idiopathic pulmonary fibrosis. Pulmonary fibrosis is characterized by the dysfunction of alveolar epithelial cells and accumulation of fibroblast, myofibroblasts and extracellular matrix. Following injury, epithelial cells secrete growth factors such as TGF-β1 and induce fibroblast proliferation and differentiation into myofibroblasts (Fernandez & Eickelberg, 2012a; Moss et al., 2022; Selman et al., 2001). Myofibroblasts produce extracellular matrix proteins such as

collagens and form structures called fibroblast foci. TGF-β1, transforming growth factor β1; IPF, idiopathic pulmonary fibrosis. Created with BioRender.com.

2.4.4 Diagnosis

Diagnostic criteria for IPF have changed several times in the past. In 2000, the first uniform international diagnostic criteria for IPF were established (King et al., 2000). In 2002, a consensus classification of IIPs was published to clarify the terminology used for IPF and other IIPs (Travis et al., 2002). The first guideline for diagnosing and managing of IPF made in collaboration with ATS, ERS, the Japanese Respiratory Society, and the Latin American Thoracic Association was published in 2011 (Raghu et al., 2011); it stated that high-resolution computed tomography (HRCT) is an essential diagnostic method for IPF. The diagnostic guideline was updated in 2018 and 2022 (Raghu et al., 2018, 2022)

According to the current international guidelines, the diagnosis of IPF requires evaluation of clinical data, the exclusion of other known causes of ILD, the presence of a UIP pattern on HRCT of the chest, or specific combinations of HRCT and histologic pattern in patients subjected to surgical lung biopsy (SLB) (Raghu et al., 2022). The diagnosis should be made after multidisciplinary discussion among radiologists, pathologists, and pulmonologists (Raghu et al., 2022).

The UIP pattern in HRCT includes honeycombing with or without traction bronchiectasis or bronchiolectasis, the presence of irregular thickening of interlobular septa, a reticular pattern with mild ground glass opacity, and occasionally, pulmonary ossification (Raghu et al., 2018, 2022). Honeycombing structure in computed tomography (CT) imaging is clustered cystic air spaces (between 3–10 mm in diameter, but occasionally up to 2.5 cm) that are usually subpleural, peripheral and basal in distribution. They develop after collapse of fibrotic alveolar septa and dilatation of terminal airways (Raghu et al., 2022). Traction bronchiectasis refers to dilated bronchi on CT due to peripheral retraction resulting from lung fibrosis.

The indications for SLB have changed during the past decades. In the 2000 guideline, SLB was recommended to be taken from most patients, especially those with suspected IPF who had any atypical clinical, physiological, or radiological features of IPF (King et al., 2000). In the following 2011 guideline, SLB was suggested to be taken if the HRCT findings were classified as possible UIP or as inconsistent with UIP pattern (Raghu et al., 2011). In the guideline updated in 2018, conditional recommendation of SLB was made if HRCT suggested probable UIP,

indeterminate UIP or an alternative diagnosis (Raghu et al., 2018). In the present guideline, histologic confirmation is recommended to be considered if HRCT findings suggest indeterminate UIP or an alternative diagnosis, but not for those with HRCT patterns of UIP and probable UIP (Raghu et al., 2022). The decision on lung biopsy is suggested to be made in multidisciplinary meetings, and the patient's age, other illnesses, medication, lung functions and the risks of complications should be taken into consideration. According to the present guideline, transbronchial lung cryobiopsy is currently an acceptable alternative to SLB for making a histopathological diagnosis in IPF patients (Raghu et al., 2022).

Several proteins, such as surfactant proteins, Krebs von den Lungen-6 (also known as mucin 1, MUC1), and MMPs, have been studied as diagnostic markers for IPF in bronchoalveolar lavage fluid (BALF) and in blood. Some of the studied proteins have shown evidence of diagnostic process (Ishii et al., 2003; Morais et al., 2015; Stainer et al., 2021). However, there are currently no diagnostic biomarkers recommended by guidelines for the diagnosis of IPF (Raghu et al., 2022).

Histopathological features

The histological UIP pattern in the absence of other known causes for ILD is strongly suggestive of IPF. According to the current classification, the histopathological patterns for UIP are divided into the following groups (Raghu et al., 2022):

1. UIP
    - Patchy dense fibrosis with architectural distortion, i.e., scarring or honeycombing
    - A predilection for subpleural and paraseptal lung parenchyma
    - FF
    - The absence of features that suggest an alternative diagnosis
2. Probable UIP
    - Some of the findings of UIP are present
    - Absence of features that suggest an alternative diagnosis
3. Intermediate for UIP

- Fibrosis with or without architectural distortion, with features favoring either a pattern other than UIP or features favoring UIP as secondary to another cause
- Some histologic features of UIP, but with other features suggesting an alternative diagnosis

4. Alternative diagnosis
    - Features of other histologic patterns of IIPs, e.g., absence of FF or loose fibrosis in all biopsies
    - Histologic findings indicative of other diseases

FF are regions of highly proliferative myofibroblast accumulations within a myxoid-appearing matrix. They are located immediately adjacent to regions of alveolar epithelial cells that are hyperplastic and/or apoptotic (Fukuda et al., 1995). The basement membrane beneath the epithelial cells has been observed to be absent or fragmented (Fukuda et al., 1995; Kuhn & McDonald, 1991). FF have been identified to be heterogenous structures with varying shape and volume in a three-dimensional model (Jones et al., 2016).

Honeycomb changes is characterized by expanded airspaces lined by bronchiolar epithelium or hyperplastic alveolar epithelial cells and are separated by thick walls containing collagen and varying amounts of chronic inflammation (Katzenstein & Myers, 1998). The airspaces often contain mucin, histiocytes, neutrophils, and other inflammatory cells, but may also be empty. Honeycombing is a manifestation of scarring and architectural remodeling that follows lung injury due to different causes, and it is not specific to UIP. They develop after collapse of fibrotic alveolar septa and dilatation of terminal airways (Leslie, 2012; Raghu et al., 2022).

2.4.5 Treatment

Previously, the target in treating IPF was to suppress the inflammation. A combination of corticosteroid and either azathioprine or cyclophosphamide was used for treatment of IPF (King et al., 2000). A combination corticosteroid, azathioprine, and N-acetylcysteine (NAC) as triple-therapy seemed to decrease the decline of FVC and DLCO more efficiently than prednisone and azathioprine alone (Demedts et al., 2005), and in the 2011 guideline, triple therapy or NAC monotherapy was suggested to be a reasonable choice for some patients (Raghu et al., 2011). NAC is an antioxidant (acting as scavenger and restoring glutathione),

anti-inflammatory, and mucolytic drug. However, it is not currently recommended for IPF therapy alone or combined with antifibrotic drugs due to the lack of beneficial effects in pulmonary functional tests parameters and in the mortality rate (Martinez et al., 2014; Raghu et al., 2015b).

The current pharmacological treatment of IPF includes antifibrotic drugs, namely pirfenidone and nintedanib, which have been shown to reduce disease progression in patients with IPF (Cameli et al., 2020; Petnak et al., 2021). The Social Insurance Institution of Finland (Kela) gave pirfenidone reimbursement status for IPF patients in 2013, and nintedanib in 2015. Pirfenidone is a synthetic compound that exerts anti-inflammatory, anti-fibrotic, and antioxidant properties by inhibiting the production and release of inflammatory cytokines (e.g., tumor necrosis factor-α), and through down-regulation of key pro-fibrotic growth factors including TGF-β, and reduction of lipid peroxidation and oxidative stress (Myllärniemi & Kaarteenaho, 2015). Nintedanib is an intracellular inhibitor of VEGF receptors 1–3, fibroblast growth factor (FGF) receptors 1–3, and platelet-derived growth factor receptor a and b (Wollin et al., 2015). Nintedanib inhibits these tyrosine kinase receptors and interferes with several processes that have been implicated in the pathogenesis of IPF, including proliferation and migration of lung fibroblasts and differentiation of fibroblasts to myofibroblasts (Lehtonen et al., 2016).

However, pirfenidone and nintedanib can only slow down disease progression and may prevent AEs, while no curative pharmacological treatment exists for IPF at the moment (Collard et al., 2017; Petnak et al., 2021; Spagnolo et al., 2021). Lung transplantation is the only potentially curative treatment option for IPF but due to age and comorbidities, it represents a therapeutic option for only a minority of patients (Laporta Hernandez et al., 2018). Nowadays, IPF and other ILDs are, however, the most common diseases leading to lung transplantation (Kapnadak & Raghu, 2021). Management of IPF also includes the use of supplemental oxygen, pulmonary rehabilitation, and the management of comorbidities (Raghu et al., 2011; Raghu et al., 2015b).

2.4.6 Prognosis

IPF is usually a lethal disease; patient survival after diagnosis varies around 3 years without lung transplant, although some patients might live longer (Bjoraker et al., 1998; Raghu et al., 2011). Studies performed after the new classification system and antifibrotic medication suggest longer survival time (Kaunisto et al., 2019).

The prediction of disease progress of IPF patients is difficult since the information on prognostic markers is limited.

Clinical and radiological prognostic factors

Older age at diagnosis has been associated with poorer survival (Fell et al., 2010; Kaunisto et al., 2019; Raghu et al., 2006). In one study, median survival for patients younger than 50 years was 116.4 months compared with 62.8 months for subjects aged 50–60 years, 27.2 months for patients aged 60 to 70 years, and 14.6 months for patients older than 70 (King et al., 2001). Another study suggested that age, combined with findings on HRCT, is a useful diagnostic tool that identifies patients with IPF (Fell et al., 2010).

In some studies, males have been reported to have poorer survival than females (Han et al., 2008; Kärkkäinen et al., 2018). Ex-smokers have been shown to have a shorter survival time compared to current smokers and non-smokers in unadjusted univariate analysis (Kärkkäinen et al., 2017). Low baseline values of pulmonary function tests, i.e., forced vital capacity (FVC) and diffusing capacity of the lung for carbon monoxide (DLCO), have also been suggested to indicate poor prognosis of IPF patients (Reichmann et al., 2015). The Gender, Age, Physiology (GAP) index and Composite Physiologic Index (CPI) are the most common risk-prediction models used for IPF and other ILDs. The formula for calculation of CPI is as follows: 91.0 – (0.65x DLco % predicted) – (0.53x FVC % predicted) + (0.34 x FEV1 % predicted) (Wells et al., 2003). Although GAP staging may be useful for predicting mortality, it has been reported to be unable to identify all of the patients with rapid disease progression (Kärkkäinen et al., 2019).

Comorbidities such as cardiovascular diseases, diabetes, asthma, lung cancer and chronic obstructive pulmonary disease (COPD) are common in IPF patients (Kärkkäinen et al., 2017; Raghu et al., 2015a). Some of the comorbidities, e.g., cardiovascular diseases, COPD, and lung cancer, are related to poorer survival (Kärkkäinen et al., 2017; Raghu et al., 2015a).

In radiology, a definite UIP pattern, honeycombing, and traction bronchiectasis have been shown to predict poor prognosis in some (Mononen et al., 2021; Salisbury et al., 2017), but not all studies (Yamauchi et al., 2016). Patients with IPF and other ILDs without honeycombing with a high extent of reticulation on HRCT have been shown to have shorter survival time than patients with low extent of reticulation, and the high reticulation pattern can predict disease progression (Mononen et al., 2022).

Histopathological prognostic markers

Among different histological subgroups of pulmonary fibrosis, the UIP pattern has shown the worst survival (Bjoraker et al., 1998). A later study revealed that either a histological or radiological UIP pattern predicted shorter survival than NSIP pattern (Flaherty et al., 2003). Among specific histologic features of UIP, a high amount of FF has been shown to be a marker for poor prognosis in patients with IPF (Kaarteenaho, 2013; King et al., 2001; Lee et al., 2011; Mäkelä et al., 2021; Nicholson et al., 2002; Tiitto et al., 2006), although the association has not been shown in all studies (Collard et al., 2007; Flaherty et al., 2012; Hanak et al., 2008). Recently, the high density of FF containing p16 (cyclin-dependent kinase inhibitor 2A)-positive fibroblasts with an overlying p16-positive epithelium was associated with reduced lung transplant-free survival in patients with fibrosing ILD and IPF patients (Keow et al., 2022). High numbers of intra-alveolar macrophages and interstitial mononuclear inflammatory cells were associated with long survival time in patients with IPF in one study utilizing an artificial intelligence model (Mäkelä et al., 2021).

There are also some promising biomarkers for IPF, especially related to alveolar epithelial dysfunction, ECM remodeling, fibroproliferation, fibrogenesis, and immune dysregulation (Stainer et al., 2021). A small amount of immunohistochemical expression of surfactant protein A in alveolar epithelial cells in diseased areas and its high serum levels have been shown to predict a poor prognosis for patients with IPF (Greene et al., 2002; Nagata et al., 2011). In one study, high α-SMA and interleukin 4 expression within fibrotic areas in SLB samples was linked with poor survival (Waisberg et al., 2012). The high expression of ECM protein tenascin-C in lung tissue samples is associated with a poor prognosis in IPF (Kaarteenaho-Wiik et al., 1996). Another ECM-protein, periostin (also known as osteoblast specific factor 2), is highly expressed in fibrotic areas of lung tissues of IPF patients, and the serum levels of periostin may predict disease progression and survival in IPF patients (Naik et al., 2012; Okamoto et al., 2011). Serum levels of MMP-7 (a marker of fibrogenesis and ECM remodeling) has also been shown to predict prognosis and transplant-free survival in IPF patients (Tzouvelekis et al., 2017). However, there is currently no validated biomarker for disease progression of IPF.

Acute exacerbations of IPF

AE of IPF is defined as an acute, clinically significant respiratory deterioration and new, ground-glass opacification or consolidation on HRCT (Collard et al., 2016). AEs are the leading cause of hospitalization and death in patients with IPF (Daniels et al., 2008). AE can occur at any time of the disease course, and it can even be the initial presentation of the disease (Raghu et al., 2018). AE-IPF is thought to be triggered by an acute event, such as microaspiration, infection, or mechanical stretch (Collard et al., 2016).

The most common histopathological finding of AE-IPF is diffuse alveolar damage (DAD) superimposed on underlying UIP (Kaarteenaho & Kinnula, 2011; Oda et al., 2014). Severe damage of the alveolar epithelium and alveolar capillary endothelium triggers the acute, exudative phase of DAD, which is characterized by alveolar and interstitial edema and hyaline membrane formation (Collard et al., 2016; Katzenstein et al., 1976). The acute phase is followed by an organizing, fibroproliferative phase where hyaline membranes are replaced by accumulation of fibroblasts and myofibroblasts to alveolar spaces and proliferation of type II pneumocytes (Katzenstein et al., 1976).

2.5 Lung cancer

Lung cancer is a heterogeneous group of tumors. The most common histologic types of lung cancer are ADC and SCC, which are further divided into several subtypes according to the WHO classification (WHO Classification of Tumours Editorial Board, 2021). ADC and SCC originate from different cells and have several differences in biological patterns, molecular characteristics, and therapeutic strategies (Herbst et al., 2018).

2.5.1 Epidemiology and risk factors

Epidemiology

Lung cancer was reported to be the second most common cancer in the world with an estimated 2.2 million new cases (Sung et al., 2021) and the third most common cancer in Europe with 480,000 new cases in 2020 (Dyba et al., 2021). Lung cancer incidence rates are reported to be the highest in Eastern Asia, Northern America, Europe, and while the lowest rates are found in Sub-Saharan Africa (Zhang et al.,

2021). Men are more frequently affected than women (Lortet-Tieulent et al., 2014). In Finland, the incidence of lung cancer was 33.7 in women and 60.3 in men per 100,000 residents in 2020 (Finnish Cancer Registry, n.d.). Lung cancer has been reported to be the most common cause of cancer death with an estimated 380,000 deaths in Europe in 2020 (Dyba et al., 2021).

Risk factors

Smoking is the most important risk factor for developing lung cancer (Bade & Dela Cruz, 2020). Approximately 85 to 90% of lung cancer cases are caused by smoking. The risk is greater for squamous cell carcinoma (SCC) and small cell carcinoma (SCLC) than for adenocarcinoma and large cell carcinoma (LCC), and depends on the age when smoking was started, the years smoked, and the number of smoked cigarettes (Kenfield et al., 2008; Pesch et al., 2012). The risk decreases after quitting smoking (Pesch et al., 2012). The majority of non-smoking lung cancer patients are reported to be women (Pesch et al., 2012).

Ionizing radiation (radon), air pollution, exposure to occupational and environmental carcinogens, like arsenic, beryllium, cadmium, chromium, nickel, silica, diesel fumes, may increase the risk of having lung cancer (Dela Cruz et al., 2011). Asbestos is the most common occupational risk factor of lung cancer (Dela Cruz et al., 2011). Infections like human papilloma virus have been suggested to have a role in lung cancer (Bade & Dela Cruz, 2020).

Intrinsic factors, such as IPF, COPD, family history of cancer, and genetic and hormonal factors may increase the risk of lung cancer (Bade & Dela Cruz, 2020). One case-control study revealed that individuals with first-degree relatives affected by lung cancer had a 1.6-fold higher risk of lung cancer (Lissowska et al., 2010). The lung cancer risk also increases with age; this may be caused by mutations that accumulate in somatic cells throughout a person's lifetime (Alexandrov et al., 2013).

2.5.2 Pathogenesis

Lung cancer has one of the highest mutation frequencies compared to other cancers; this may be related to the exposure of lung epithelial cells to carcinogens in tobacco smoke (Alexandrov et al., 2013). Driver mutations are variants that give a growth advantage for the cells that carry them and promote cancer development. The remaining mutations that do not provide a growth advantage are called passenger

mutations (Stratton et al., 2009). Cancer genes are genes identified as drivers in at least one cancer type (Stratton et al., 2009). The most commonly altered genes in NSCLCs include *TP53*, *KRAS*, *EGF),* and *BRAF* (Mäki-Nevala et al., 2016; Suster & Mino-Kenudson, 2020; Talvitie et al., 2022; Wang et al., 2022). The frequency of genetic alterations varies depending on the histologic type of the tumor, patient's smoking history, and the population studied (Majeed et al., 2021; Mäki-Nevala et al., 2016; Talvitie et al., 2022; Wang et al., 2022). Somatic alterations leading to an oncogenic activation include point mutations and gene rearrangements, such as amplifications and fusions.

Studies utilizing mouse models have shown that lung ADCs may originate from type II pneumocytes; ADC tumors are most frequently diagnosed at the distal airways where type II pneumocytes are located (Sutherland et al., 2014). Single-cell RNA sequencing studies have shown that early cancer cells are similar to type II pneumocytes, supporting those findings (Wang et al., 2021). Type II pneumocytes have been suggested to undergo dedifferentiation into a stem-like state that can initiate tumor progression and give rise to the heterogeneous populations of cancer cells observed in ADC (Figure 3) (Wang et al., 2021). Instead, SCCs are believed to arise from basal cells in the large airways through a stepwise process where the epithelium changes from normal to hyperplasia, squamous metaplasia, dysplasia, and carcinoma *in situ,* progressing eventually to invasive SCC (Ishizumi et al., 2010; WHO Classification of Tumours Editorial Board, 2021).

In addition to cancer cells, tumors include a complex tumor microenvironment which consists of a tumor immune microenvironment with macrophages and T cells, vascular components with endothelial cells and pericytes, ECM, and stromal components with CAFs and mesenchymal stem cells (Hanahan, 2022; Sinjab et al., 2022; Wong et al., 2022; Wu et al., 2021). CAFs are a heterogeneous cell population originating from different sources such as resident fibroblasts, and they are detected in the early stages of tumor development (Wong et al., 2022). In normal circumstances, fibroblasts have antitumorigenic activities that can suppress tumor growth (Wong et al., 2022). However, during cancer development, continuous damaging stimuli and persistent emergence and accumulation of cancer cells initiates the activation of fibroblasts (Wong et al., 2022). Activated fibroblasts are transformed into CAFs, which are not removed by apoptosis (Wong et al., 2022). This results in a chronic repair response in tumors that is known as cancer fibrosis, stroma, or desmoplastic reaction.

Fibroblasts can be activated by various mechanisms. Epithelial cancer cells secrete growth factors, such as TGF-β, FGF, and platelet-derived growth factor,

into the microenvironment, which stimulates the recruitment and activation of fibroblasts (Wong et al., 2022). Cancer cells also produce various inflammatory modulators like the interleukin family, tumor necrosis factor factor-α, and VEGF-A, which promote tumor progression, invasion and angiogenesis, but may also be related to fibroblast activation (Wong et al., 2022). Additionally, exosomes derived from cancer cells, certain microRNAs, ROS, and hypoxia can activate CAFs (Wong et al., 2022).

CAFs themselves secrete cytokines (e.g., C-X-C motif chemokine ligands such as CXCL12), chemokines, growth factors (e.g., TGF-β) and ECM proteins (e.g., MMPs), which are involved in cancer cell proliferation, and invasion as well as tumor metastasis and chemoresistance (Nazareth et al., 2007; Wong et al., 2022). CAFs can stimulate tumor invasion and metastasis by ECM remodeling and by inducing EMT (Wong et al., 2022). CAFs produce factors that can initiate angiogenesis (VEGF receptors, FGF, type I collagen and fibronectin). They are also involved in shaping the immunosuppressive tumor microenvironment (Wong et al., 2022).

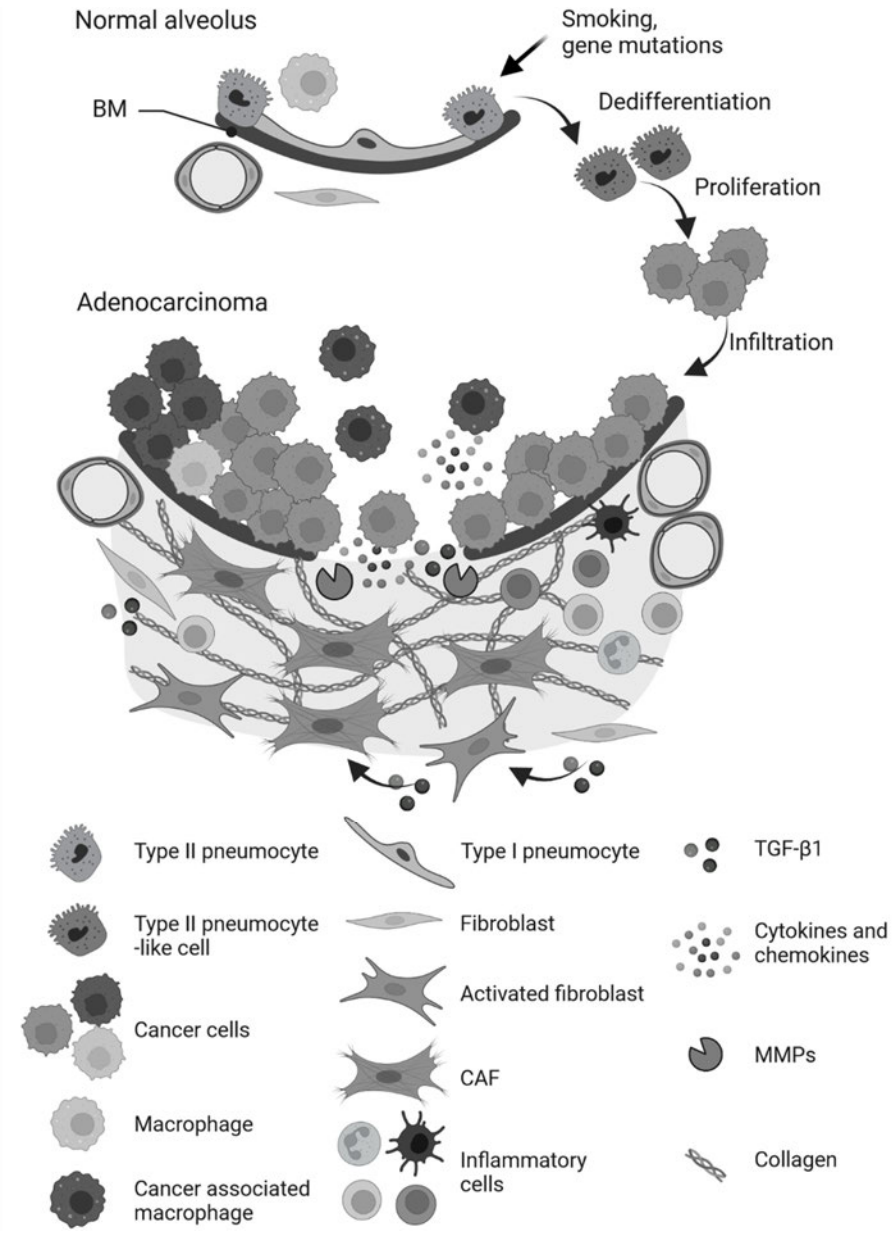

Fig. 3. Pathogenesis of lung adenocarcinoma. Early cancer cells are suggested to dedifferentiate into a stem-like state that resemble type II pneumocytes and give rise to cancer cells in lung adenocarcinoma (Wang et al., 2021). Cancer cells secrete various growth factors including TGF-β1, which results in the recruitment and activation of

fibroblast and their differentiation into CAFs (Wong et al., 2022). CAFs produce extracellular matrix proteins such as collagens and secrete different growth factors, cytokines, and chemokines to stimulate cancer cell proliferation, invasion and metastasis. Additionally, macrophages and other inflammatory cells may contribute tumor development (Sinjab et al., 2022). CAF, cancer-associated fibroblast; MMP, matrix metalloproteinase, TGF-β1, transforming growth factor β1. Created with BioRender.com.

2.5.3 Diagnosis

The symptoms of lung cancer are usually non-specific, being similar to other pulmonary diseases, and they often occur late in the disease course (Ruano-Raviña et al., 2020). The size and location of the primary tumor and metastasis affect the symptoms. The symptoms can also be paraneoplastic or general symptoms. Lung cancer is often detected at advanced stages and the majority of NSCLCs are metastatic at the time of diagnosis (Ruano-Raviña et al., 2020).

Abnormal findings in chest radiography may raise a suspicion of lung cancer, but it is less sensitive than CT, which is primarily used for determination of the size, location, shape, borders, density, and distribution of the tumor. Diagnostic methods comprise bronchoscopy including biopsies, brushings, or washings, endobronchial or esophageal ultrasound guided biopsies, and ultrasound or CT guided transthoracic biopsies. Tests should be selected in multidisciplinary collaboration (Rivera et al., 2013). Majority of lung cancers present in advanced stages and are, and the diagnosis for these patients is primarily based on small biopsy and cytology specimens rather than surgical resection samples (Travis et al., 2011).

Following the initial diagnosis of lung cancer, accurate staging is needed for determining appropriate treatment and for estimating the prognosis (Detterbeck et al., 2017). Lung cancer is staged based on the TNM (tumor, node, metastasis) classification system that defines the anatomical extent of the disease (Brierley et al., 2016).

Histologic classification of lung cancer

Advances in lung cancer diagnosis and treatment in the last decade have given new importance to classifying lung cancers further into specific subtypes (Nicholson et al., 2022). The latest World Health Organization (WHO) Classification of Thoracic Tumors was published in 2021 (WHO Classification of Tumours Editorial Board, 2021). Previous versions of the WHO classification were published in 1967, 1981,

1999, 2004 and 2015 (Travis et al., 2015a; Travis et al., 1999, 2004; World Health Organization, 1967, 1981).

Approximately 80% of all lung cancers are NSCLC type. NSCLCs are further divided into several histologic subtypes, including adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma. ADC is the most common subtype of NSCLCs while the second most common type is SCC (Cheng et al., 2016).

Adenocarcinoma

ADCs are malignant epithelial tumors with morphologic or immunohistochemical evidence of glandular differentiation, and with either mucin production or pneumocyte marker expression (WHO Classification of Tumours Editorial Board, 2021). The most common immunohistochemical pneumocyte markers are thyroid transcription factor 1 (TTF-1) and napsin A (Travis et al., 2015b). The subclassification of adenocarcinomas of the lung remained largely unchanged between the WHO 2015 and 2021 classifications (Nicholson et al., 2022; Travis et al., 2015a; WHO Classification of Tumours Editorial Board, 2021).

Invasive ADCs consist of a complex heterogeneous mixture of histologic subtypes, and ADCs with a pure single pattern are quite rare (Kerr, 2009). Lepidic ADC consists of neoplastic cells growing along pre-existing alveolar structures, without evidence of stromal or vascular invasion (WHO Classification of Tumours Editorial Board, 2021). Acinar predominant ADC, which is the most common ADC subtype, is characterized by the formation of round or oval-shaped glandular structures, with a central luminal space surrounded by cuboidal or columnar cells which may contain mucin (WHO Classification of Tumours Editorial Board, 2021). Papillary ADC consists of malignant cuboidal or columnar cells growing along central fibrovascular cores that form papillae with secondary and tertiary branching (WHO Classification of Tumours Editorial Board, 2021). Micropapillary adenocarcinoma is defined as papillary tufts forming florets without fibrovascular cores and solid pattern as sheets of polygonal cells with possible mucin formation, lacking acini, tubules, and papillae. The tumor cells of invasive mucinous ADC have goblet or columnar cell morphology, small basally located nuclei, and abundant intracytoplasmic mucin. The lepidic growth pattern is the most common, although any pattern except solid may be seen (WHO Classification of Tumours Editorial Board, 2021).

According to the 2021 WHO classification, all growth patterns should be recorded semiquantitatively in 5 to 10% increments to determine the predominant histologic pattern, i.e., subtype, and quantify other patterns to determine the tumor grade (WHO Classification of Tumours Editorial Board, 2021). The grading is based on a combination of the predominant histologic pattern and the worst pattern (solid, micropapillary, cribriform, and complex glandular pattern), if it accounts for at least 20% of the tumor (Nicholson et al., 2022).

Squamous cell carcinoma

Lung squamous cell carcinomas (SCC) are malignant epithelial tumors showing keratinization and/or intercellular bridges that arise from bronchial epithelium. According to the 2021 classification, SCCs are divided into keratinizing, nonkeratinizing, and basaloid subtypes (WHO Classification of Tumours Editorial Board, 2021). Additionally, lymphoepithelial carcinoma is currently classified as SCC (WHO Classification of Tumours Editorial Board, 2021).

SCC tumors are classified as keratinizing subtype if any amount of keratinization is present and as basaloid squamous cell carcinoma if this component is greater than 50% of the tumor, regardless of the presence of any keratinization (WHO Classification of Tumours Editorial Board, 2021). The non-keratinizing tumors require immunohistochemistry for squamous markers such as p40 or p63 for proof of squamous differentiation (WHO Classification of Tumours Editorial Board, 2021).

2.5.4 Treatment

The treatment of lung cancer depends on the histologic subtype, stage, and patient's general condition, such as lung function and the presence of comorbidities (Planchard et al., 2018; Postmus et al., 2017; Remon et al., 2021; Santos & Rodriguez, 2022). For early-stage NSCLCs, lobectomy is considered as the standard therapy, but anatomical segmentectomy is currently acceptable for small, non-invasive or minimally invasive lesions (Postmus et al., 2017; Remon et al., 2021). Adjuvant chemotherapy is recommended to patients with resected stage II and III NSCLC taking into account comorbidities, time from surgery, and postoperative recovery (Postmus et al., 2017; Remon et al., 2021).

In advanced disease, the treatment may be based on molecular pathology tests which include testing for possible activating mutations and predictive

immunohistochemical markers. Certain driver mutations, such as *EGFR* and *KRAS* gene mutations and *ALK* and *ROS1* rearrangements, are effective targets for therapies (Majeed et al., 2021). *EGFR* mutations and *ALK* or *ROS1* rearrangements are more common in ADCs than in SCCs although they can occur in SCCs, especially among young never-smoking patients; thus, molecular testing for these driver mutations is essential in these patients (Lindeman et al., 2018).

If the tumor does not contain any targetable driver mutations, cytostatic or immunological treatment or combination of the two can be used based on the expression of programmed cell death ligand 1 (PD-L1) (Planchard et al., 2018; Santos & Rodriguez, 2022). PD-L1 is a key immunoregulatory molecule which inhibits the cluster of differentiation 8 (CD8) cytotoxic immune response and the resultant antitumor immune response through its receptor PD-1. New immune checkpoint inhibitors and specific drugs targeting different genetic alterations in ADCs and SCCs are under development (Majeed et al., 2021; Santos & Rodriguez, 2022)

2.5.5 Prognosis

Clinical prognostic factors

In Finland, the relative 5-year survival rate of lung cancer is 23.6% in females and 15.9% in males (Hemminki et al., 2022). Surgically operated NSCLC patients have higher survival rates than patients without operation. Clinical prognostic factors include gender, age, smoking status, comorbidities, and nutritional status (Garinet et al., 2022). Women with resected lung cancer have been reported to have a better prognosis than men (Sachs et al., 2021). Older age has been shown to predict poor postoperative survival (Shewale et al., 2020). Underweight has been reported to be associated with worse prognosis and obesity with better prognosis than normal weight (Alifano et al., 2021). Non-smokers have been reported to have better prognosis than smokers (Garinet et al., 2022; Molinier et al., 2020).

Histopathological prognostic factors

Histopathological prognostic factors include TNM classification, histologic lung cancer type and subtype, pleural and lymphovascular invasion, and spread through airspaces (STAS) (Garinet et al., 2022). Previously, SCC has been reported to have

better prognosis than ADC (Kawaguchi et al., 2010). However, the histological classification has changed since those studies were performed, and a more recent publication reported patients with ADC having longer survival time than those with SCC (Shewale et al., 2020). Of histologic ADC subtypes, lepidic predominant ADC has the best prognosis while solid and micropapillary predominant ADCs have worse prognosis (Mäkinen et al., 2015; Travis et al., 2015a). Lepidic and micropapillary/solid patterns as minor components in acinar and papillary predominant subtypes may also predict prognosis of lung ADC patients (Hou et al., 2022; Mäkinen et al., 2015).

Lymphovascular invasion has been reported to predict poor prognosis (Mäkinen et al., 2017; Wang et al., 2011). STAS (invasive pattern of lung cancer with the presence of cancer cells within the air spaces outside the main tumor) has been shown to be associated with poor prognosis in some (Han et al., 2021) but not in all studies (Mäkinen et al., 2017).

2.6 Associations of idiopathic pulmonary fibrosis and lung cancer

IPF and lung cancer share similar risk factors, similar survival, and common genetic and epigenetic mechanisms (Ballester et al., 2019). The common risk factors of IPF and lung cancer include smoking, older age, and male sex, and IPF itself has been shown to increase the risk of developing lung carcer as compared to general population (le Jeune et al., 2007). The reported prevalence of lung cancer in IPF patients varies between studies (Raghu et al., 2015a). In a recent European study, the prevalence of lung cancer among IPF patients was estimated to be 10.2% (Karampitsakos et al., 2023). In that study, IPF preceded lung cancer in the majority of the cases having IPF and lung cancer (Karampitsakos et al., 2023). The incidence of lung cancer in patients with IPF has been reported to increase with the duration of follow-up (Yoo et al., 2019). The most common histologic lung cancer type in patients with IPF is SCC, followed by ADC, and it is often located peripherally in lower lobes adjacent to fibrotic and honeycomb areas (Guyard et al., 2017; Karampitsakos et al., 2023; Khan et al., 2015). Bronchiolar hyperplastic/dysplastic cells are suggested to be the driver of lung cancer in ILDs (Kewalramani et al., 2022).

Smoking has been found to increase the risk of IPF and lung cancer in several studies (Ekström et al., 2014; Park et al., 2021). Smoking has also been associated with lung cancer development in IPF patients (Yoo et al., 2019). Current smokers have been shown to develop IPF at a younger age than non- and ex-smokers

(Kärkkäinen et al., 2017). In unadjusted models, current smokers have been shown to have longer survival time than non- and ex-smokers (Antoniou et al., 2008; Kärkkäinen et al., 2017; King et al., 2001). However, in models adjusted with CPI, IPF patients with a smoking history had shorter survival time than never-smokers (Antoniou et al., 2008; Kärkkäinen et al., 2017). Among IPF patients, current smokers have been reported to be younger than non- and ex-smokers (Kärkkäinen et al., 2017; King et al., 2001). Non-smoking NSCLC patients have been reported to be older than patients with a smoking history, the majority are female and have tumors with ADC histology (Dias et al., 2017; Löfling et al., 2019). Additionally, some genetic alterations, e.g., *EGFR* mutations, are more common in never-smokers than in ever-smokers (Chapman et al., 2016). Cigarette smoke has been associated with many IPF and lung cancer related mechanisms, e.g., overexpression of genes associated with EMT and fibroblast-like phenotype (Checa et al., 2016), telomere shortening (Astuti et al., 2017), and endoplasmic reticulum stress (Jorgensen et al., 2008).

Several growth factors, including TGF-β1, are involved in the pathogenesis of IPF and lung cancer. TGF-β1 is a major profibrotic growth factor that can promote lung fibrosis and cancer (Ballester et al., 2019). In pulmonary fibrosis, the *TGFB1* gene has been shown to be expressed in cells within the FF, aberrant basaloid cell lining FF, and macrophages as well as adjacent alveoli (Herrera et al., 2022). TGF-β1 induces myofibroblast differentiation and ECM production. Additionally, it induces the production of MMPs, regulators of small GTPases, protease inhibitors, and integrins, which participate in ECM remodeling and cell-ECM interactions (Ballester et al., 2019). In lung cancer, TGF-β may act as a tumor suppressor in the early stage of tumorigenesis by suppressing epithelial cell proliferation (Saito et al., 2018). TGF-β can also facilitate cancer progression by inducing EMT and endothelial-to-mesenchymal transition and suppressing immune surveillance. TGF-β activates CAFs, which influence tumor development, progression, and metastasis by interacting with carcinoma cells and other factors of the cancer microenvironment (Wong et al., 2022).

Both IPF and lung cancer patients have poor prognosis (Hemminki et al., 2022; Khor et al., 2020). In Finland, the five-year survival rate for IPF has been reported to be 45% (Kaunisto et al., 2019) and for lung cancer, 23.6% in females and 15.9% in males (Hemminki et al., 2022). Furthermore, comorbid lung cancer in IPF patients affects the survival since patients with both IPF and lung cancer have been shown to have higher risk of all-cause mortality than IPF patients without lung

cancer (Karampitsakos et al., 2023) and lung cancer patients without IPF (Kanaji et al., 2016).

Lung cancer with IPF complicates the treatment of those patients, and the optimal treatment for lung cancer with IPF remains to be established. Patients with IPF and lung cancer treated with antifibrotic drugs have been shown to have decreased all-cause mortality compared to patients who were not treated with antifibrotics (Karampitsakos et al., 2023). Patients with IPF and otherwise operable lung cancer who did not undergo surgical resection have been shown to have higher risk of all-cause mortality than surgically treated patients (Karampitsakos et al., 2023). However, lung cancer surgery may increase the risk of AEs (Sato et al., 2014). Some patients with advanced NSCLC with IPF are treated with platinum-based cytotoxic chemotherapy, although such treatment can also induce AEs of IIPs (Minegishi et al., 2020). Nintedanib was initially discovered as an anticancer agent and after that, it was also approved for the therapy of IPF (Reck et al., 2014; Richeldi et al., 2015). Recently, nintedanib combined with chemotherapy was shown to be an effective treatment option for patients with advanced NSCLC with non-squamous histology and IPF (Otsubo et al., 2022).

Previously, the gene expressions of IPF and lung cancer have mostly been evaluated separately with microarray studies performed on RNA isolated from whole lung tissues. Few studies have compared publicly available NSCLC and IPF microarray datasets performed on lung tissues (Leng et al., 2020; Ulke et al., 2019). IPF and tumor tissues from patients with both IPF and lung cancer have been investigated using microarray analysis (Takenaka et al., 2009) and next-generation sequencing (Otsubo et al., 2020). A few studies have used cultured stromal cells derived from IPF and normal lung tissues (Table 1) (Lee et al., 2017; Lindahl et al., 2013; Peng et al., 2013; Rodriguez et al., 2018; Vuga et al., 2009). Fibroblasts cultured from lung cancer were investigated by microarray analysis in one study (Navab et al., 2011). However, cultured stromal cells from IPF and lung cancer have not been compared in the same study. The comparison of IPF and LC may help to better understand the molecular mechanisms involved in both diseases and may contribute to development of new therapeutic strategies.

Table 1. Microarray studies using cultured stromal cells derived from idiopathic pulmonary fibrosis or lung adenocarcinoma.

Study	Patient samples	Control samples	Smoking	Microarray platform
Vuga et al., 2009	4 UIP histology (explants)	3 normal lungs (transplant)	Not available	CodeLink UniSet Human I Bioarray
Hsu et al., 2011	9 SSc-PF, 9 SSc-PAH, 10 IPF, 6 IPAH (explants)	9 normal lungs (transplant)	3 SSc-PF, 4 SSc-PAH, 3 IPF, 4 IPAH and 3 controls smokers, 6 SSc-PF, 5 SSc-PAH, 7 IPF, 4 IPAH and 3 controls non-smokers	Illumina HumanRef-8 v3.0
Navab et al., 2011	15 ADC (cancer resection surgery)	15 tumor-free lungs (cancer resection surgery)		Affymetrix Human Exon 1.0 ST Array
Lindahl et al., 2013	3 IPF, 8 SSc-ILD (SLBs)	10 tumor-free lungs (cancer resection surgery)	4 SSc-ILDs and 2 IPFs ex-smokers, not available for all control cases	Affymetrix human U133Av2
Peng et al., 2013	4 rapidly progressing IPF, 6 stable IPF	4 tumor-free lungs (cancer resection surgery)	Controls non-smokers	Affymetrix Human Genome U133 Plus 2.0
Lee et al., 2017	8 IPF (SLBs)	4 tumor-free lungs (cancer resection surgery)	Controls and 4 IPFs non-smokers, 4 IPFs ex-smokers	Illumina HumanHT-12 V4.0
Rodriguez et al., 2018	8 IPF (explants)	4 normal lungs (transplant)	6 IPFs ex-smokers, controls non-smokers	Duke Operon Human 36K oligonucleotide array V4.0

 ADC, lung adenocarcinoma; IPAH, idiopathic pulmonary arterial hypertension; IPF, idiopathic pulmonary fibrosis; UIP, usual interstitial pneumonia; SLB, surgical lung biopsy; SSc-ILD, scleroderma-associated interstitial lung disease; SSc-PF, scleroderma-related pulmonary fibrosis; SSc-PAH, scleroderma-related pulmonary arterial hypertension.

3 Aims of the study

The overall aim of this study was to compare the mRNA expressions, especially the expression of ECM-related genes, in stromal cell lines derived from IPF, ADC and normal lung and investigate the NHLRC2 protein and mRNA expression patterns in lung tissues from patients with IPF and lung cancer.

The specific aims of this study were:

1. to quantify the gene expressions of fibroblastic cell lines isolated from patients with lung ADC from tumor and corresponding normal lung as well as from lung tissues of patients with IPF by microarray analysis, and to determine the ECM genes and factors related to their regulation whose gene expression is higher or lower in IPF compared to lung adenocarcinoma or normal lung. The expression of certain ECM proteins i.e., collagen α1(IV) chain, periostin, MMP-1 and MMP-3 in stromal cells and lung tissues was studied further with quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR) and immunohistochemistry (IHC), respectively (I).
2. to investigate the NHLRC2 expression pattern in SLB samples derived from IPF patients and examine whether immunohistochemical NHLRC2 expression correlates with the occurrence of AE-IPF and clinical features of the patients (survival, age, sex, smoking status, and pulmonary function test results). The effect of TGF-β1 on NHLRC2 mRNA and protein expression in primary lung stromal or epithelial cell lines was investigated *in vitro* (II).
3. to study the expression pattern of NHLRC2 in lung tissue samples derived from lung ADC and SCC patients and examine whether the immunohistochemical expression of NHLRC2 correlates with histologic (subtype, nuclear atypia, mitotic activity, tumor necrosis, desmoplasia, and lymphovascular invasion) and clinical (survival, age, sex, smoking status, pulmonary function test results, COPD) data of the lung cancer patients (III).

4 Material and methods

4.1 Patients and lung cell lines (I, II, and III)

The study material consists of primary cell lines and lung tissue samples taken from IPF or lung cancer patients who underwent a SLB or a complete surgical resection, respectively, at the Oulu University Hospital (Table 2). Lung tissue samples were retrieved from the files of the Biobank Borealis and the Department of Pathology, Oulu University Hospital. Stromal cells were cultured from tumor and corresponding tumor-free peripheral lung tissue of the ADC patients, and from the patients with IPF. In addition, one IPF cell line was derived from peripheral lung outside the tumor from a patient operated for lung cancer. To eliminate the changes caused by smoking, control samples were derived from histologically normal-appearing lung tissues from non-smoking lung adenocarcinoma patients who had undergone lung cancer surgery.

The IPF patients had undergone SLB for diagnostic purposes between 1991 and 2019 (I, II). Lung tissue specimens taken at autopsy were also studied (II). IPF was diagnosed according to the international guidelines (Raghu et al., 2011, 2018). The patients experiencing AE-IPF during follow-up were identified either based on the SLB and autopsy material showing DAD in parallel with UIP or by applying the current criteria for AE-IPF (Collard et al., 2016). Sex, pharmacological treatment, age, pulmonary function test results and smoking status at the time of biopsy were collected from medical records. IPF patients with a smoking history of less than five pack-years were considered as non-smokers. Death dates were collected from death certificates obtained from the national registry of Statistics Finland. Survival time was calculated from the date of biopsy to death, transplantation, or last follow-up date (11/05/2021, II).

The lung cancer patients had undergone a surgical resection between 1998 and 2007 (III). Clinical information, including age, sex, smoking history, pulmonary function test results, and follow-up data were gathered systematically from medical records. Patients whose lifetime tobacco consumption was fewer than 100 cigarettes were considered non-smokers. Overall survival (OS) was defined as the time from the date of diagnosis to the date of last follow-up or death from any cause. Disease-specific survival (DSS) was defined as the time from the date of diagnosis to the date of death from lung cancer.

Table 2. Study material.

Material	Study I	Study II	Study III
Cultured stromal cells, n			
IPF, SLB	4[1]	5	-
Lung cancer, tumor	4 ADC	-	2 ADC, 2 SCC
Lung cancer, tumor-free peripheral lung	4 ADC	4 ADC	2 ADC, 2 SCC
Paraffin-embedded tissue samples, n			
IPF, SLB	14	50	-
IPF, autopsy		8	-
Lung cancer, resection, tumor	14 ADC	-	102 ADC, 111 SCC
Lung cancer, resection, tumor-free peripheral lung	13 ADC	10 ADC	10 ADC
Frozen lung tissue samples, n			
Lung cancer, tumor	-	-	3 ADC, 2 SCC
Lung cancer, tumor-free peripheral lung	-	-	3 ADC, 2 SCC

[1] One cell line was cultured from peripheral lung outside the tumor from a patient who had undergone cancer resection surgery; ADC, adenocarcinoma; IPF, idiopathic pulmonary fibrosis; SLB, surgical lung biopsy; SCC, squamous cell carcinoma.

4.2 Cell culture (I, II, and III)

Stromal cells were cultured from SLB samples of the patients with IPF and lung cancer resections from tumor and corresponding tumor-free peripheral lung tissue samples. Cells were cultured in Minimum essential medium Eagle (α modification) (Sigma-Aldrich) supplemented with 13% heat-inactivated fetal bovine serum (FBS-Good, Pan Biotech, Aidenbach, Germany), 2 mM L-glutamine, 10 mM HEPES, 100 U/mL penicillin, and 0.1 g/L streptomycin, and 2.5 mg/L amphotericin B (all from Sigma-Aldrich) at 37°C in humidified atmosphere containing 5% CO_2. According to the electron microscopic analyses published previously, these cell lines are composed of both fibroblasts and myofibroblasts (Karvonen et al., 2012, 2014). Stromal cells were used for experiments in passages three, four, five or six.

Normal human primary small airway epithelial cells (SAEC, American type culture collection, ATCC, Virginia, USA PCS-301-010) and normal human primary bronchial/tracheal epithelial cells (PBTE, ATCC PCS-300-010) were cultured in airway cell basal medium supplemented with bronchial epithelial growth kit (ATCC). SAEC were used for experiments in passages six and seven and PBTE in passage five.

Epithelial lung cancer cell lines SK-MES-1 (ATCC HTB-58, RRID:CVCL_0630, SCC), H1650 (ATCC CRL-5883, Research Resource Identifier (RRID):CVCL_1483, minimally invasive lung ADC), and SK-LU-1 (ATCC HTB-57, RRID:CVCL_0629, ADC) were cultured in Minimum essential medium Eagle (α modification) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 0.1 g/L streptomycin, 2 mM L-glutamine, 10 mM HEPES, and 2.5 mg/L amphotericin B.

4.3 RNA extraction (I, and II)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA concentrations in samples were measured using the NanoDrop spectrophotometry system (Thermo Fisher Scientific, Vilnius, Lithuania).

4.4 Microarray analysis (I)

Microarray analysis was performed in Biocenter Oulu Sequencing Center core facility. The quality of RNA was analyzed with a Qiaxcel electrophoresis system (Qiagen). By using a 3'IVT Express Kit (Affymetrix Inc., Santa Clara, CA), biotinylated cRNA were prepared from the total RNA according to the manufacturer's instructions. Following labeling, cRNA was hybridized to Affymetrix human hgu133Aplus2 chips. The microarray chip was washed and stained on an Affymetrix GeneChip Fluidics Station 450 according to the manufacturer's instructions. Affymetrix GeneChip Scanner 3000 7G was used for scanning the chips.

The microarray expression data were analyzed using the R/Bioconductor through a graphical user interface, Chipster (3.12.5, CSC, Finland, http://chipster.csc.fi/) (Kallio et al., 2011). Normalization was done using a custom chip description file for hgu133Aplus2. Intensity data were log2-transformed, and quantile normalized using robust multi-array average (RMA). Statistical analysis was performed using empirical Bayes and Benjamini-Hochberg was used as multiple correction for false discovery rate (Smyth, 2004). Lists of differentially expressed genes between IPF, ADC and control were generated using a $\log_2$ fold change lower than -1 or higher than 1. Microarray data were deposited in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo) with accession number GSE144338.

Matrisome annotator (Naba et al., 2017) was used for annotation of differentially expressed genes as being or not being part of the matrisome. With that tool, each entry is tagged with matrisome division (core matrisome or matrisome-associated) and category (ECM glycoproteins, proteoglycans, collagens, ECM regulators, ECM-affiliated proteins, or secreted factors) (Naba et al., 2017).

4.5 Quantitative real-time reverse transcriptase polymerase chain reaction (I, and II)

Five hundred-ng to one-µg aliquots of RNA were reverse-transcribed by using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Reactions were performed by using iQTM SYBR Green Supermix (Bio-Rad Laboratories, Inc., USA). Samples were processed for qRT-PCR using CFX ConnectTM Real-Time PCR Detection System (Bio-Rad Laboratories). After an initial denaturation at 95˚C for three minutes, the samples were subjected to 40 cycles of amplification, consisting of denaturation at 95˚C for ten seconds, annealing at appropriate temperature for each primer pair (see Table 3) for 10 seconds, and extension at 72˚C for 15 seconds. Following a final extension phase of 72°C for two minutes, a melt curve analysis was performed, in which the temperature was increased from 55 to 95˚C at a linear rate of 0.5˚C every five seconds. PCR amplification was performed in triplicate and the threshold cycle values were averaged. Non-template controls were included for each gene. Primers were designed with NCBI Primer-BLAST (Ye et al., 2012).

The Livak method (Livak & Schmittgen, 2001) was used for quantification of relative gene expressions. Gene expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) or to hydroxymethylbilane synthase (*HMBS*) to confirm that the data are reproducible. To calculate the fold changes, the normalized values were compared to the average ΔCT value of the control stromal cell lines when different cell lines were studied (I, II) or non-treated samples in TGF-β1 experiments (II).

Table 3. Sequences, annealing temperatures, and amplicon sizes of quantitative RT-PCR primers used in this study.

Target	Primer	Sequence 5'-3'	Ta (°C)	Amplicon size (bp)	Study
COL4A1	Forward	CCCCAAAGGTGTTGACGGCT	61.4	126	I, II
	Reverse	AGACCAACTCCAGGCTCTCC			
GAPDH	Forward	GAGTCAACGGATTTGGTCGT	63	185	I, II
	Reverse	GACAAGCTTCCCGTTCTCAG			
HMBS	Forward	TGCAACGGCGGAAGAAAA	59	66	I
	Reverse	AGCTGGCTCTTGCGGGTAC			
MMP1	Forward	TCACAGCTTCCCAGCGACTC	60	128	I
	Reverse	CTGGGCCACTATTTCTCCGCT			
MMP3	Forward	TGAGGACACCAGCATGAACC	63	99	I
	Reverse	GGACCACTGTCCTTTCTCCTAAC			
NHLRC2	Forward	AGCTGAAGGCAATGAATGGCTACT	59	173	II
	Reverse	TACAAGCACTGCTGTCTGCACTA			
POSTN	Forward	GGAGACAAAGTGGCTTCCGA	62.2	104	I
	Reverse	CCTTCCAGCGTCTCAAAGACT			

4.6 Histopathology (III)

Tumors were re-evaluated according to the 2015 WHO classification based on hematoxylin and eosin (HE) and alcian blue mucin stains (Mäkinen et al., 2015). The predominant growth pattern analysis of ADC has been presented in detail previously (Mäkinen et al., 2015, 2017). In addition, the following histological parameters were analyzed: mitotic activity, nuclear atypia, desmoplasia, tumor necrosis, and lymphovascular invasion as described previously (Mäkinen et al., 2017). The pathological stage was determined according to the TNM classification of malignant tumors as described previously (Mäkinen et al., 2015, 2017; Rusch et al., 2010).

4.7 Immunohistochemistry (I, II, and III)

Immunohistochemical stainings were performed in formalin-fixed and paraffin-embedded lung specimens that were cut into 3.5 or 4-µm sections, de-paraffinized in xylene, and rehydrated in a descending ethanol series. Following antigen retrieval (see Table 4), endogenous peroxidase was blocked with aqueous 0.3% hydrogen peroxide (Peroxidase-Blocking Solution, Dako, Glostrup, Denmark) for 10 minutes. Stainings were performed by using Dako REAL EnVision Detection

System (Dako) (Study I and II) or by using EnVision Flex Kit with DAB+ chromogen (3,3' diaminobenzidine) (Dako) (Study III). Primary antibodies listed in Table 4 were diluted in Dako antibody diluent (Agilent). Replacement of primary antibody with phosphate-buffered saline, mouse isotype control or rabbit isotype control (Invitrogen, Carlsbad, USA) was used as negative control. The sections were counterstained with Mayer's hematoxylin (Sigma-Aldrich).

Table 4. Antibodies used for immunohistochemistry.

Antibody	Supplier, catalogue number and RRID	Epitope retrieval	Dilution	Incubation	Study
Monoclonal mouse anti-human CD31 antibody, clone JC70A	Agilent Cat# M0823, RRID:AB_2114471	Tris-EDTA buffer (pH 9)[1]	1:100	30 min at RT	I, II
Monoclonal mouse anti-human CD68, clone PG-M1	Agilent, Cat# M0876, RRID:AB_2074844	Tris-EDTA (pH 9.0)[1]	1:300	20 min at RT[2] or 30 min at RT[3]	I, II, III
Monoclonal mouse anti-thyroid transcription factor antibody, clone 8G7G3/1	Agilent, Cat# M3575, RRID:AB_2877699	Tris-EDTA (pH 9)[1]	1:200	30 min at RT	I, II
Monoclonal mouse smooth muscle actin (clone 1A4)	Agilent, Cat# M0851, RRID:AB_2223500	Tris-EDTA (pH 9.0)[1]	1:1,000	20 min at RT[2] or 30 min at RT[3]	I, II, III
Polyclonal rabbit anti-collagen IV alpha 1	Novus Biologicals, Cat# NB120-6586, RRID:AB_789360	Pepsin treatment for 30 min at 37°C	1:75	1 hour at RT	I, II, III
Monoclonal mouse anti-MMP-1 antibody (clone Ab-1)	Oncogene Research Products, IM35L	Citrate buffer (pH 6)[1]	1:75	1 hour at RT	I
Polyclonal rabbit anti-MMP-3 antibody	Abcam, Cat# ab137659	Citrate buffer (pH 6)[1]	1:100	1 hour at RT	I
Polyclonal rabbit anti-NHLRC2	Sigma-Aldrich Cat# HPA038493, RRID:AB_10672519	Tris-EDTA (pH 9.0)[1]	1:500	o/n at +4°C	II, III

Antibody	Supplier, catalogue number and RRID	Epitope retrieval	Dilution	Incubation	Study
Polyclonal rabbit anti-periostin antibody	Abcam Cat# ab14041, RRID:AB_2299859	Citrate buffer (pH 6)[1]	1:3000	o/n at +4°C	I

[1] Microwave heat treatment for 15 minutes; [2] Dako REAL EnVision Detection System (Dako); [3] Envision Flex Kit (Dako); IHC, immunohistochemistry; o/n, overnight; RRID, Research Resource Identifier; RT, room temperature.

In order to identify the phenotype of the cells expressing collagen α1(IV), periostin, MMP-1, MMP-3, and NHLRC2, some cases were also studied for markers of macrophages and monocyte lineage cells (cluster of differentiation 68, CD68), type II pneumocytes (thyroid transcription factor 1, TTF-1), endothelial cells (CD31) and myofibroblasts (α-SMA) (Table 4).

4.8 Analysis of the immunoreactivity (I, II and III)

4.8.1 Expression of extracellular matrix proteins (I)

In study I, the slides were studied under a light microscope. The extent of the immunoreactivity for collagen α1(IV), periostin, MMP-1 and MMP-3 was scored as negative or positive in stromal cells within widened alveolar tips, FF, and tumor stroma of ADC. Widened alveolar tips were defined as widened endings of free interalveolar septa as described previously (Karvonen et al., 2013). Additionally, immunoreactivity was evaluated in alveolar epithelium, alveolar macrophages, endothelial cells, smooth muscle cells, bronchiolar epithelial cells, and cancer cells.

4.8.2 Scanning of slides (II and III)

Whole slide images were acquired by using a NanoZoom S60 scanner (Hamamatsu, Hamamatsu City, Japan) in Transgenic and Tissue Phenotyping core facility, Biocenter Oulu, University of Oulu or by using a Leica-Aperio AT2 (Leica Biosystems, Nussloch, Germany) in Biobank Borealis of Northern Finland, Oulu University Hospital at 40x magnification.

4.8.3 Digital image analysis (II and III)

Image analysis was performed using Visiopharm digital pathology image analysis software (Visiopharm Integrator System, Hoersholm, Denmark) provided by Transgenic and Tissue Phenotyping core facility, Biocenter Oulu, University of Oulu. The area of NHLRC2-positive staining in all types of lung cells considering all intensities (weak, moderate, strong) in relation to total area of the tissue section (IPF and control lung, II) or to total area of the lung cancer tissue (III) was determined. Necrotic areas within cancer tissue were excluded from the analysis (III).

4.8.4 Calculation of NHLRC2-positive fibroblast foci (II)

Digitized slides were examined using Aperio Image Scope (Version 12.4.3.5008, Leica Biosystems). The total number of NHLRC2-positive FF was calculated from 47 samples with UIP histology. NHLRC2-positive FF was determined as consisting of more than 50% of positive spindle-shaped cells considering all intensities. Additionally, the total number of FF was calculated from each section. The number of FF was presented in relation to the area of the tissue section.

4.8.5 NHLRC2 in cancer cells (III)

To study the NHLRC2 expression specifically in cancer cells, digitized lung cancer tissue specimens were examined by using NDP.view2 (Hamamatsu). The extent of NHLRC2 positivity in cancer cells was divided into five groups: negative, less than 25% of cells positive, 25–49% of cells positive, 50–75% of cells positive, and over 75% of cells positive. The extent of NHLRC2 expression in tumor cells in ADCs was compared to that in SCCs.

4.9 mRNA in situ hybridization (II, and III)

NHLRC2 mRNA *in situ* hybridization was performed using RNAscope 2.5 HD assay - RED and probe Hs-NHLRC2 (555721) according to the manufacturer's instructions (Advanced Cell Diagnostics, ACD, Newark, CA, USA). Formalin-fixed and paraffin-embedded specimens were cut into 4-µm thick sections. Target retrieval was performed by boiling the sections at 98°C for 15 minutes in RNAscope target retrieval reagent using a KOS Microwave HistoSTATION

(Milestone, Sorisole, Italy). The nuclei were stained with Gill's Hematoxylin (Sigma-Aldrich, St. Louis, MO, USA), and coverslips were mounted with EcoMount (Biocare Medical, Pacheco, CA, USA). For control background noise and qualify samples, positive and negative control probes (Hs-UBC 310041 and DapB 310043, ACD) were used. Tissue specimens were digitized as described in section 3.8.2 Scanning of slides and examined by using Aperio Image Scope or NDP.view2. Specific signal was identified as red dots.

4.10 TGF-β1 exposure (II)

One control and one IPF stromal cell line and SAEC were plated at a density of 9,000 cells/cm^2 and grown for 24 hours. The culture medium of stromal cells was replaced with serum-free growth medium supplemented with 5 ng/mL TGF-β1 (Sigma-Aldrich) and the culture medium of SAECs was replaced with complete growth medium supplemented with 5 ng/mL TGF-β1. Cells were incubated for 24 to 72 hours. For controls, cells were cultured in similar conditions without TGF-β1.

4.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting (II, III)

Dodecyl maltoside (DDM, 1.5% in phosphate buffered saline) (II, III) or radio-immunoprecipitation assay (RIPA) lysis and extraction buffer (Thermo Fisher Scientific, Vilnius, Lithuania) (II) supplemented with a protease inhibitor cocktail tablet (Roche, Mannheim, Germany) were used for preparing cell lysates. After 45-minute incubation on ice, the samples were centrifuged at 20,000 g for 20 min. Frozen lung tissue samples were homogenized in 1.5% dodecyl maltoside (Thermo Fisher Scientific, in phosphate buffered saline) supplemented with a protease inhibitor cocktail tablet (Roche) by sonication and centrifuged at 20,000 g for 20 minutes. The protein concentrations of cell lysates and tissue homogenates were determined by DC Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions.

Twenty-µg aliquots of samples with Bolt LDS sample buffer (Thermo Fisher Scientific) were run on 8% polyacrylamide gel (Invitrogen Bolt Bis-Tris Mini Protein Gels, Thermo Fisher Scientific). The proteins were transferred onto nitrocellulose membrane (0.45 µm, Optitran reinforced NC, Whatman Schleicher and Schuell, Dassel, Germany). The membranes were stained with TotalStain Q (NC, Azure Biosystems, Dublin, CA, USA) (III). After blocking with 5% skim milk

in tris-buffered saline, the membranes were incubated with primary antibodies followed by appropriate labeled secondary antibody incubation (Table 5). The detection of protein bands was carried out using an Odyssey infrared imager (LI-COR Biosciences, Lincoln, NE, USA) (II) or an Azure 600 gel & blot imager (Azure Biosystems) (III). The band intensities were analyzed by using Image Studio Lite (LI-COR Biosciences). The expression levels of the target proteins were normalized to that of GAPDH (II) or total protein stain (III).

Table 5. Antibodies used for Western blotting.

Antibody	**Manufacturer, Catalogue number, RRID**	Dilution	Incubation	Study
Primary antibodies				
Monoclonal mouse smooth muscle actin, clone 1A4	Agilent, Cat# M0851, RRID:AB_2223500	1:1,000	1 hour at RT or o/n at +4°C	II
Monoclonal mouse anti-GAPDH, clone C65	Abcam Cat# ab8245, RRID:AB_2107448	1:5000	o/n at +4°C	II, III
Polyclonal rabbit anti-GAPDH	Abcam, Cat# ab9485, RRID:AB_307275	1:2,500	o/n at +4°C	II
Polyclonal rabbit anti-NHLRC2	Novus Biologicals, Cat# NB120-6586, RRID:AB_789360	1:500	o/n at +4°C	II, III
Secondary antibodies				
IRDye 680RD Donkey anti-Mouse IgG	LI-COR Biosciences Cat# 925-68072, RRID:AB_2814912	1:10,000	1 hour at RT	II, III
IRDye700DX Donkey anti-Rabbit IgG (H&L)	Rockland Cat# 611-730-127, RRID:AB_220156	1:5,000	1 hour at RT	II
IRDye 800CW Donkey anti-Mouse IgG	LI-COR Biosciences Cat# 926-32212, RRID:AB_621847	1:10,000	1 hour at RT	II
IRDye 800CW Donkey anti-Rabbit IgG	LI-COR Biosciences Cat# 925-32213, RRID:AB_2715510	1:10,000	1 hour at RT	II, III

o/n, overnight; RRID, Research Resource Identifier; RT, room temperature

4.12 Statistical Analyses (I, II, and III)

IBM SPSS Statistics for Windows, Version 25.0 (I, II) or 28.0 (III) (IBM Corp, Armonk, NY) was used for statistical analysis. OriginPro, Version 2019b (I, II) or version 2022 (III) (OriginLab Corporation, Northampton, MA, USA) was used for preparing graphs. The data were presented as median values with 25 and 75% quartiles for skewed variables (interquartile range, IQR), or as means with standard

deviation (SD) for those with a normal distribution. Independent samples t-test (III) was used for normally distributed variables and Mann-Whitney U test for skewed variables (I, II, III), Wilcoxon signed ranks test for comparison of paired control and tumor tissues (I, III), and Fisher-Freeman-Halton test for comparison of categorial variables (III). Comparisons of parameters that were not normally distributed between more than two groups were performed using the Kruskal-Wallis test and *post hoc* analysis (Dunn's test with Bonferroni correction). IPF patients were divided into groups based on the median values of FVC% and DLCO% (75% and 53%, respectively) (II). Survival was evaluated using the Kaplan-Meier method and differences in survival curves were evaluated using the log-rank test. The associations of relative immunohistochemical NHLRC2 expression and the number of NHLRC2-positive FF/cm^2 with survival or future AE were analyzed using univariate Cox regression analysis. Median values of relative NHLRC2 expression in IPF and lung cancer and median values of NHLRC2-positive FF/cm^2 in IPF patients were used as cut-off values for Kaplan-Meyer and Cox regression analyses. Values of $p < 0.05$ were considered statistically significant.

4.13 Ethical aspects

The Ethical Committee of Northern Ostrobothnia Hospital District in Oulu gave a favorable statement on the study protocol (64/2001, amendment 68/2005, 2/2008, amendments 12/2014, 2/2015, 2/2018 and 6/2022). National Supervisory Authority for Welfare and Health approved the research use of paraffin-embedded tissue samples (Dnro: V/25054/2019 and V/25090/2019). For collection of cell culture materials, all subjects provided a written informed consent.

5 Results

5.1 Patients' characteristics (II, and III)

The characteristics of the IPF and lung cancer patients are shown in Table 6 and Table 7. Of the 50 IPF patients, 47 (94%) had a surgical lung biopsy sample taken at the stable phase of the disease and 3 (6%) during AE. Thirty-seven (76%) of the IPF patients, 97 (87%) of the 111 SCC patients, and 67 (66%) of the 102 ADC patients were males. Twenty-eight (62%) of the 45 IPF patients with known smoking history were ever-smokers, including 20 ex- and eight current smokers. Eighty-one (83%) of the 98 ADC patients and 107 (99%) of the 108 SCC patients with known smoking history were ex- or current smokers. The median follow-up time was 46.0 months (range 1–178 months) in SCC patients and 32.5 months (range 0–172 months) in ADC patients.

Table 6. Characteristic of idiopathic pulmonary fibrosis patients.

Parameters	IPF (n = 50)
Tissue sample histology, n (%)	
UIP	47 (94)
UIP and DAD	3 (6)
Two separate lung tissue samples	
UIP in biopsy and UIP with DAD in autopsy	6 (12)
UIP in biopsy and autopsy	2 (4)
Age years, mean (SD)	62.3 (7.9)
Gender, n (%)	
Male	37 (74)
Female	13 (26)
Smoking status[1]	
Non-smoker	17 (38)
Ex-smoker	20 (44)
Current smoker	8 (18)
Pack-years of ever-smokers, median (IQR)[2]	25.0 (19.5–37.0)
FVC%, mean (SD)[3]	73.8 (15.5)
FEV1%, mean (SD)[3]	78.4 (16.7)
DLCO%, median (IQR)[4]	53.0 (45.0–62.1)
Follow-up time, months, median (IQR)[5]	41.3 (15.1–73.8)
Episode of AE during follow-up, n (%)	22 (44)
Diseased or transplanted, n (%)	39 (78)
Transplanted, n (%)	4 (8.0)

Parameters	IPF (n = 50)

Reprinted (adapted) under CC BY 4.0 license from Paper II © 2022 Kreus, M., Lehtonen, S., Salonen, J., Porvari, K., Kaarteenaho, R. The values were from the time of surgical lung biopsy. For follow-up time, death or lung transplantation was used as an endpoint event. Follow-up time for patients having no endpoints was defined as the time between biopsy date and May 11, 2021. [1] Information missing from five patients; [2] Information missing from 25 patients; [3] Information missing from eight patients; [4] Information missing from nine patients; [5] Information missing from one patient; AE, acute exacerbation; DAD, diffuse alveolar damage; DLCO%, percent predicted diffuse capacity for carbon monoxide; FEV1%, percent predicted forced expiratory volume at one second; FVC%, percent predicted forced vital capacity; IQR, interquartile range; n, number; SD, standard deviation; UIP, usual interstitial pneumonia.

Table 7. Characteristics of lung cancer patients.

Parameters	Adenocarcinoma n = 102	Squamous cell carcinoma n = 111
Age (years), mean (SD)	65 (8.70)	67.86 (7.10)
Gender		
Male	67 (65.69)	97 (87.39)
Female	35 (34.31)	14 (12.61)
Smoking status, n (%)[1]		
Non-smoker	17 (16.67)	1 (0.90)
Ex-smoker	20 (19.61)	56 (50.45)
Current smoker	61 (59.80)	51 (45.95)
Pack-years of ever-smokers, median (IQR)[2]	30.00 (16.50–40.00)	40.00 (26.00-50.00)
Stage, n (%)[3]		
IA	30 (29.4)	29 (26.1)
IB	25 (24.5)	17 (15.3)
IIA	18 (17.6)	30 (27.0)
IIB	12 (11.8)	7 (6.3)
IIIA	14 (13.7)	8 (7.2)
IV	3 (2.9)	2 (1.8)

Reprinted (adapted) with permission from Paper III © 2023 AME Publishing Company. [1] Information missing from four adenocarcinoma and three squamous cell carcinoma patients; [2] Information missing from 39 adenocarcinoma and 26 squamous cell carcinoma patients; [3] Information missing from 18 squamous cell carcinoma patients; IQR, interquartile range; n, number; SD, standard deviation.

5.2 The expression of extracellular matrix proteins in stromal cells of idiopathic pulmonary fibrosis, adenocarcinoma, and control lung (I)

5.2.1 The mRNA expression of several extracellular matrix genes in stromal cells

Gene expression levels in stromal cells cultured from lung tissue of four patients with IPF, four patients with ADC, as well as in four control samples consisting of histologically normal-looking lung collected outside of the tumor were determined using an Affymetrix platform (U133Aplus2). Although modest differences were observed between the groups, statistical significances were not achieved due to the small sample size. With our selection criteria (log_2 fold change lower than -1 or higher than 1), 36 genes between primary fibroblasts derived from ADC and normal lung, 157 genes between IPF and normal lung, and 152 genes between IPF and ADC were differentially expressed. Forty matrisome genes annotated by using Matrisome Annotator (Naba et al., 2017) were differentially expressed in IPF and 15 in ADC as compared to control. Of the matrisome genes, *COL4A1* expression was higher in IPF than in ADC and control, *POSTN* expression was higher in IPF and ADC than in control, and the expression of *MMP1* and *MMP3* was higher in IPF than in ADC and control (Table 8).

Table 8. Selected differentially expressed genes in idiopathic pulmonary fibrosis, lung adenocarcinoma, and control.

Gene	Log_2FC IPF/Control	Log_2FC ADC/Control	Log_2FC IPF/ADC
COL4A1	-1.03	ND	-1.18
POSTN	1.03	1.70	ND
MMP1	1.50	ND	2.44
MMP3	3.10	ND	2.40

List of differentially expressed genes between IPF and ADC was generated using log_2FC lower than -1 or higher than 1 and the differentially expressed genes were annotated using Matrisome annotator (Naba et al., 2017). ADC, adenocarcinoma; IPF, idiopathic pulmonary fibrosis; log_2FC, log_2 fold change; ND, no difference.

Because of their differential expression profiles and suggested role in fibrosis (Okamoto et al., 2011; Urushiyama et al., 2015; Yamashita et al., 2011), *COL4A1* (encoding collagen α1(IV), *POSTN* (encoding periostin), *MMP1* (encoding MMP-1, also known as interstitial collagenase or fibroblast collagenase), and *MMP3*

(encoding MMP-3, also known as stromelysin-1 or transin-1) were chosen for further analysis. The mRNA levels of the above-mentioned genes measured by RT-qPCR showed a trend in the direction of a change observed in the microarray results. However, due to the small sample size, statistical significance was not achieved.

5.2.2 Localization of collagen α1(IV), periostin, MMP-1 and MMP-3 in idiopathic pulmonary fibrosis and lung adenocarcinoma

The expression of collagen α1(IV), periostin, MMP-1 and MMP-3 at the protein level in stromal cells within lung tissues was studied by immunohistochemistry. Strong collagen α1(IV) immunoreactivity was observed extracellularly within stromal cells of the widened alveolar tips in control lung, FF in IPF, and stroma of ADC. Collagen α1(IV) was also detected in the basement membranes of alveolar epithelium and endothelium and smooth muscle cells. Occasionally, a weak expression was observed in bronchiolar epithelial cells and alveolar macrophages, hyperplastic alveolar epithelium lining FF in IPF, and in the cancer cells in a few ADC cases.

Periostin was observed extracellularly in some widened alveolar tips of control lungs, within alveolar epithelium, and within the area of bronchiolar basement membrane. In IPF, periostin was mainly observed in stromal cells within FF. In ADC, periostin immunoreactivity was observed in tumor stroma, but not in the cancer cells.

Very weak MMP-1 immunoreactivity was observed occasionally within widened alveolar tips and smooth muscle cells in controls while strong MMP-1 expression was detected in the alveolar macrophages, bronchiolar epithelium, some alveolar epithelial cells, and endothelial cells. In IPF, some stromal cells within FF were positive for MMP-1, and strong MMP-1 expression was observed in the hyperplastic alveolar epithelial cells lining FF. MMP-1 expression was observed in both stromal cells and cancer cells in ADC.

Faint MMP-3 immunoreactivity was detected in some of the widened alveolar tips in normal control lung. MMP-3 was mainly expressed in bronchiolar epithelial cells, alveolar macrophages and monocyte lineage cells. Weak MMP-3 expression was detected in some alveolar epithelial cells and smooth muscle cells. In IPF, hyperplastic alveolar epithelial cells and some stromal cells of FF were positive for MMP-3. In ADC, cancer cells and spindle-shaped stromal cells were mainly positive for MMP-3.

5.3 NHLRC2 expression pattern in lung tissue

5.3.1 Idiopathic pulmonary fibrosis and normal lung (II)

The cell-type specific NHLRC2 protein and mRNA expressions in lung tissues were studied by immunohistochemistry and *in situ* hybridization. In control lung, alveolar type II pneumocytes, small airway epithelial cells and alveolar macrophages showed strong cytoplasmic NHLRC2 expression. Mainly weak NHLRC2 immunoreactivity was occasionally observed in type I pneumocytes and smooth muscle cells. NHLRC2 expression in endothelial cells varied from negative to quite strong.

In SLB samples of IPF patients, obtained at the stable phase or during AE, mainly moderate to strong cytoplasmic NHLRC2 expression was detected in hyperplastic alveolar and bronchiolar type epithelial cells lining FF and honeycombs (Figure 4A). Some α-SMA-positive stromal cells within FF were also positive for NHLRC2 in IPF. Despite varying amounts of autolysis, mainly moderate or strong NHLRC2 expression was observed in bronchiolar and hyperplastic alveolar epithelial cells in autopsy lung tissue samples, similarly to the SLB samples.

NHLRC2 mRNA expression was in line with the protein expression, being observed mainly in alveolar and bronchial epithelial cells in control lung and hyperplastic alveolar epithelial cells in IPF.

5.3.2 Lung cancer (III)

Negative to moderate cytoplasmic immunohistochemical NHLRC2 expression was observed in cancer cells in ADC and SCC (Figure 4). Moderate to strong cytoplasmic NHLRC2 expression was observed in inflammatory cells within cancer stroma. Macrophages within the tumor were mainly weakly positive. Spindle-shaped stromal cells and endothelial cells showed occasionally very weak immunoreactivity for NHLRC2. Normal and metaplastic epithelial cells of bronchi outside the tumor were mainly positive for NHLRC2.

NHLRC2 mRNA expression studied by mRNA *in situ* hybridization in cancer cells in ADC and SCC tumors was in line with the immunohistochemical results.

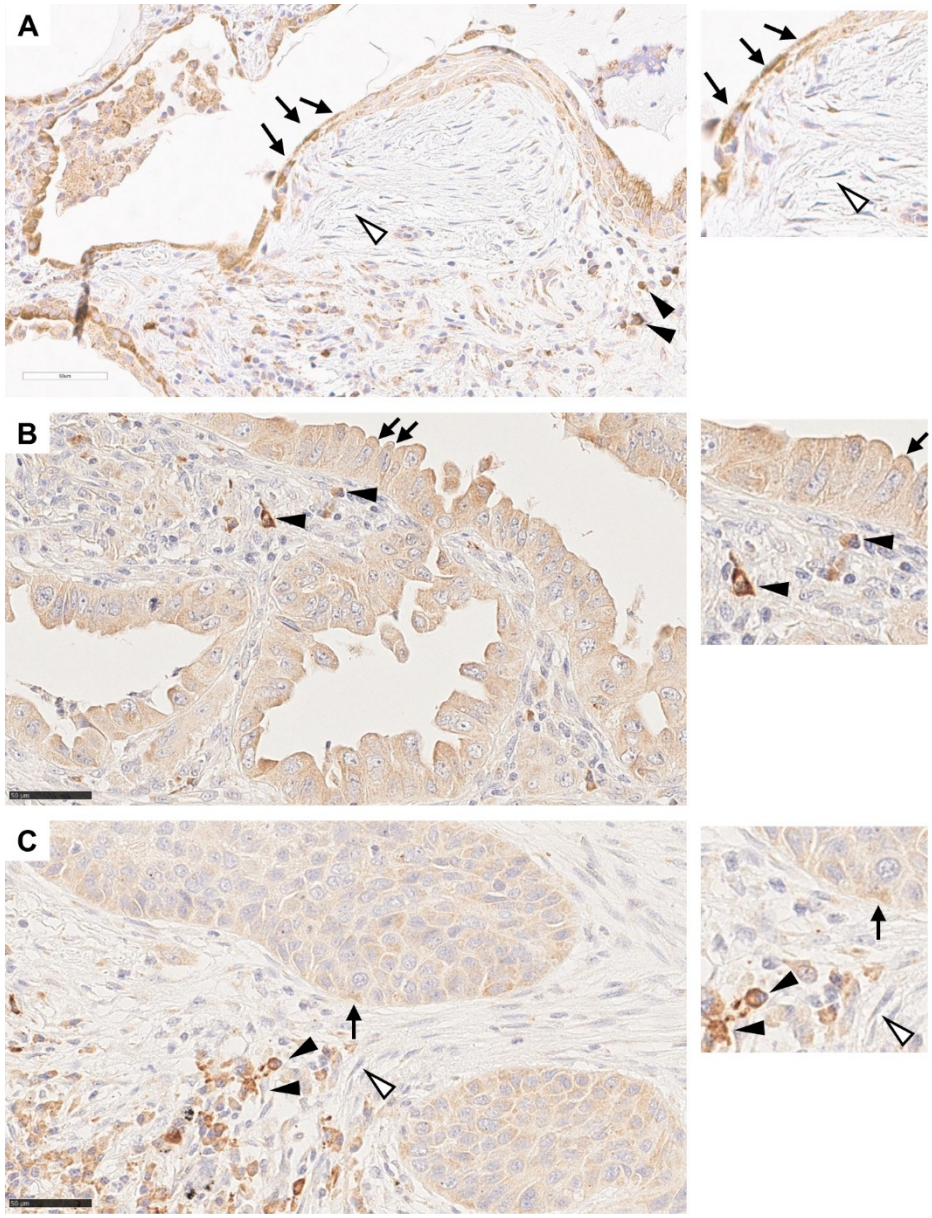

Fig. 4. Immunohistochemical NHLRC2 expression in idiopathic pulmonary fibrosis and lung cancer. A) In idiopathic pulmonary fibrosis, cytoplasmic NHLRC2 expression (brown color) was detected mainly in alveolar epithelial cells lining fibroblast foci (arrows). Some inflammatory cells were also positive (black arrowheads). Spindle

shaped stromal cells within fibroblast foci (white arrowheads) were occasionally positive. B) and C) In lung adenocarcinoma and squamous cell carcinoma, mainly tumor cells (arrows) and inflammatory cells (black arrowheads) within tumor stroma were positive for NHLRC2 whereas spindle-shaped stromal cells (white arrowheads) were mainly negative. Scale bar 60 µm (A) or 50 µm (B, C). Figure by Mervi Kreus.

5.4 Association of immunohistochemical NHLRC2 expression with clinical data

5.4.1 Idiopathic pulmonary fibrosis (II)

The relative NHLRC2-positive area determined by digital image analysis was higher in IPF at a stable phase of the disease (median = 6.56, IQR = 5.37–9.31) than in control (median = 4.29, IQR = 3.49–5.47) ($p < 0.001$). AE-IPF patients (n = 3, median = 9.35%, IQR = 9.10–15.32) seemed to have higher NHLRC2 expression than patients at the stable phase of the disease (n = 47, median = 6.56%, IQR = 5.37–9.31) ($p = 0.080$), although the difference did not reach statistical significance. There was no difference in NHLRC2 expression in IPF patients experiencing AE during follow-up and IPF patients who did not.

There was a trend of ever-smokers having a higher relative NHLRC2 expression than non-smokers, although the difference was not statistically significant ($p = 0.060$). However, when the two AE-IPF patients with known smoking history were included in the analysis, ever-smokers (median = 8.01%, IQR = 5.66–9.37) had significantly higher NHLRC2 expression than non-smokers (median = 5.82%, IQR = 4.72–6.56) ($p = 0.037$). The relative NHLRC2 expression did not differ between females and males, high and low FVC%, or high and low DLCO%.

5.4.2 Lung cancer (III)

ADC patients having tumors with high relative NHLRC2 expression (over 16% of tumor area) determined using image analysis software had lower DSS and OS rates than patients having tumors with low NHLRC2 expression (less than 16% of tumor area, $p = 0.002$ and $p = 0.001$, respectively). NHLRC2 expression did not correlate with survival in SCC. NHLRC2 expression did not correlate with the clinical characteristics (age, gender, smoking status, COPD, or pulmonary function test results, or stage) of ADC or SCC patients.

5.5 Association of immunohistochemical NHLRC2 expression with histopathological features (II, and III)

5.5.1 Idiopathic pulmonary fibrosis (II)

To study the expression of NHLRC2, especially in fibroblasts and myofibroblasts, the numbers of NHLRC2-positive FF (more than 50% positive spindle-shaped cells within FF) were calculated. There were no differences in the numbers of NHLRC2-positive FF/cm^2 between IPF patients experiencing AE during follow-up time and patients who did not, ever-smokers and non-smokers, females and males, high and low FVC%, or high and low DLCO%. The number of NHLRC2-positive FF did not correlate with survival or predict a future episode of AE-IPF.

In addition to the NHLRC2-positive FF, the total number of FF was counted. Ever-smokers had less FF (median = 29.9, IQR = 19.5–45.0 FF/cm^2) than non-smokers (median = 69.4, IQR = 52.6–81.2 FF/cm^2) ($p < 0.001$). Current smokers seemed to have less FF/cm^2 than ex-smokers, although the result did not reach statistical significance (p = 0.068). Additionally, patients with low FVC% had more FF/cm^2 (median = 61.7, IQR = 38.7–79.0 FF/cm^2) than patients with higher FVC% (median = 32.2, IQR = 19.6–54.9 FF/cm^2) (p = 0.020).

5.5.2 Lung cancer (III)

Immunohistochemical NHLRC2 expression determined by image analysis software was higher in ADC (mean = 17.72, SD = 8.99) than in SCC (mean = 12.04, SD = 7.37, $p < 0.001$). NHLRC2 expression was higher in ADC and SCC than in controls (mean = 4.23, SD = 1.14, $p < 0.001$ and $p < 0.001$, respectively).

High NHLRC2 expression was associated with high mitotic activity in ADC (p = 0.042), but the expression of NHLRC2 did not correlate with other histopathological parameters (nuclear atypia, tumor necrosis, desmoplasia, lymphovascular invasion) in ADC or SCC. All SCC tumors included in this study had moderate/high mitotic activity, thus NHLRC2 could not be compared between tumors with low and moderate/high mitotic activity in SCC.

The most frequent histologic ADC subtype was acinar (n = 52, 51.0%), including seven tumors with cribriform pattern. Twenty-three (22.5%) out of 102 ADCs were solid, eight (7.8%) were papillary, and seven (6.9%) were micropapillary predominant ADCs. One tumor (1.0%) was lepidic predominant ADC. Eleven (10.8%) out of 102 ADCs were invasive mucinous variants. Due to

the low number of each ADC subtype group, they were divided into two groups based on a previous publication showing that solid and micropapillary ADCs have worse DSS than other histologic subtypes (Mäkinen et al., 2015). The relative NHLRC2 expression did not differ between the histologic subtypes of ADC. Ninety-nine (89.2%) out of the 111 SCC tumors were non-keratinizing type, while eight (7.2%) were keratinizing and four (3.6%) were basaloid type. There were no differences in NHLRC2 expression among SCC subtypes.

5.6 Semi-quantitative immunohistochemical NHLRC2 expression in cancer cells (III)

To study further the NHLRC2 expression, especially in cancer cells, the percentage of positive cancer cells was evaluated semi-quantitatively. Fifty (49%) out of 102 ADCs had over 75% positive cancer cells while 18 (16.2%) out of 111 SCCs had over 75% of positive cells; thus, ADC tumors contained more NHLRC2-positive cancer cells than SCC tumors ($p < 0.001$).

5.7 NHLRC2 protein levels in lung cancer tissue samples (III)

To confirm the image analysis results of NHLRC2 expression in lung cancer, frozen ADC ($n = 3$) and SCC ($n = 2$) tissue samples were subjected to immunoblotting. In line with the results of semiquantitative analysis and digital image analysis, the NHLRC2 expression levels were variable in different ADC and SCC tumor samples. In three out of the five tumor tissue samples, NHLRC2 expression level was higher than in the corresponding tumor-free lung, but due to the limited number of samples the result was not statistically significant.

5.8 NHLRC2 mRNA and protein levels in cultured lung cell lines (II, III)

NHLRC2 mRNA and protein levels were measured in different types of cultured cells by RT-qPCR and Western blot analysis, respectively, to evaluate whether the expression differs between stromal cells and epithelial cells. NHLRC2 mRNA and protein levels in SAEC, PBTE, and stromal cells derived from IPF and control lung and NHLRC2 protein levels in epithelial cancer cell lines (H1650, SK-LU-1 and SK-MES-1) and stromal cells from tumor and areas outside tumor were equal.

5.9 The effect of TGF-β1 in NHLRC2 expression in vitro (II)

To study whether TGF-β1 regulates NHLRC2 mRNA or protein expression *in vitro*, cultured stromal cells (IPF and control) and SAEC were exposed to 5 ng/mL TGF-β1 for 24 to 72 hours. TGF-β1 exposure did not have an effect on NHLRC2 mRNA or protein levels in stromal cells or SAEC in cell culture conditions. To confirm the fibroblast to myofibroblast activation by TGF-β1, *COL4A1* and α-SMA mRNA and protein levels were measured. *COL4A1* and *ACTA2* levels were higher in TGF-β1-treated samples than in non-treated samples in stromal cells and SAEC. Additionally, α-SMA protein levels were higher in samples exposed to TGF-β1 than in non-treated samples.

6 Discussion

In this study, mRNA expressions in stromal cell lines derived from IPF, ADC and control lung were studied by microarray analysis. We were especially interested in the ECM-associated genes and selected *COL4A1*, *POSTN*, *MMP1* and *MMP3* due to their previously shown association with pulmonary fibrosis. Additionally, we described for the first time the expression pattern of NHLRC2 in lung tissue samples from patients with IPF, ADC and SCC and investigated the effect TGF-β1 on NHLRC2 mRNA and protein expression in primary lung stromal or epithelial cell lines *in vitro*.

6.1 Gene expression studies on IPF and lung cancer

IPF and NSCLC have been shown to share similarly dysregulated genes in tissue samples in studies comparing publicly available NSCLC and IPF gene expression datasets (Leng et al., 2020; Spek & Duitman, 2019; Ulke et al., 2019). Spek and Duitman found 512 common differentially expressed genes in IPF and NSCLC lung tissues as compared to control, although some of them were differentially up- and down-regulated in IPF and lung cancer (Spek & Duitman, 2019). Ulke (2019) performed a gene set enrichment analysis on three publicly available NSCLC datasets and one IPF microarray dataset, revealing 92 genes upregulated in NSCLC datasets and enriched in IPF. These genes were associated with a fibrotic type II pneumocyte cell phenotype (Ulke et al., 2019). Another study identified 79 common signature genes in IPF and NSCLC, including *MMP1* (Leng et al., 2020). Yao (2022) identified differentially expressed genes in IPF and control lung overlapping in four different IPF microarray datasets from gene expression omnibus and found 31 differentially expressed genes. Next, they identified five hub genes (*COL1A1*, *COL3A1*, *POSTN1*, *MMP1*, and *TIMP3*) by analyzing protein-protein interaction networks (Yao et al., 2022). They found that *COL1A1*, *COL3A1*, *POSTN1*, and *MMP1* expression levels were up-regulated while *TIMP3* was down-regulated in IPF patients, as well as in ADC and SCC datasets from the Cancer Genome Atlas (Yao et al., 2022). A microarray-based study comparing gene expression in lung tissues from five patients with both IPF and lung cancer revealed five genes, i.e., *MT1A*, *MMP7, P21*, *SMAD4*, and *TIMP1,* that were down-regulated in cancer tissue in comparison to IPF in at least two of the patients (Takenaka et al., 2009). Some of the genes identified in the above-mentioned studies were similarly up- or down-regulated in IPF and lung cancer but differentially expressed in the

current study (e.g., *MMP1*). However, those studies looked at the gene expression in whole lung tissue consisting of multiple cell types while in this study, the gene expression was studied in cultured stromal cells.

CAFs have been previously compared to paired normal lung fibroblasts in a study where 46 differentially expressed genes were identified (Navab et al., 2011). Some of these genes, e.g., *CHI3L1*, *COL11A1*, *MFAP5*, *ST6GALNAC5*, and *TNFSF4,* were also identified in this study as differentially expressed genes between ADC and control or between IPF and control. A previous study on stromal cells derived from IPF and normal lung identified 178 differentially expressed genes in IPF (Peng et al., 2013). Fourteen of those genes were also differentially expressed between IPF and control in this study, and the expression of two of these genes (*POSTN* and *S100A4*) was also different between ADC and controls. Another study identified 547 differentially expressed genes in IPF and controls (Lindahl et al., 2013). Thirty-nine of these genes were also differentially expressed in our microarray analysis, although not all in the same direction, since only 14 genes were similarly up- or down-regulated in IPF in our study. The similarities between our study and the others (Lindahl et al., 2013; Navab et al., 2011; Peng et al., 2013) suggest that at least some disease-related changes in the transcriptome are maintained during *in vitro* culture. Rodriguez (2018) could not identify statistically significant gene expression differences between cultured fibroblasts derived from IPF and normal lung. However, they showed that the gene expression of cultured fibroblasts differs from that of freshly isolated fibroblasts (Rodriguez et al., 2018).

Single-cell RNA-sequencing studies have revealed that human lung fibroblasts are a very heterogeneous population (Tsukui et al., 2020). The heterogeneity of the original cell populations before culture may at least partly explain the differences between microarray studies. Additionally, in some previous microarray studies the IPF cells were derived from biopsies (Lee et al., 2017; Lindahl et al., 2013), and in others, from lung explants (Hsu et al., 2011; Rodriguez et al., 2018; Vuga et al., 2009), and the gene expression between biopsy and explant tissues has been shown to differ (Meltzer et al., 2011), which may in part explain the variation between the studies. Although we observed some differences in gene expression between the cells from tumor and corresponding controls, it is possible that the histologically normal lung outside the tumor is also affected. Normal lung tissue from healthy donors is rarely, if ever, obtained from lung surgery, and tumor-free lung from lung cancer resection surgery has often used as a control, including some of the other microarray studies performed on lung stromal cells (Lee et al., 2017; Lindahl et al., 2013; Navab et al., 2011; Peng et al., 2013). Others have used tissues from lung

transplant donors instead (Hsu et al., 2011; Rodriguez et al., 2018; Vuga et al., 2009).

We identified 20 similarly up- or down-regulated genes in IPF and ADC as compared to control, while the majority of the altered genes in IPF and ADC were different. In line with our study, Spek and Duitman (2019) reported that the majority of the genes differentially expressed between NSCLC and control lung tissues were not altered in IPF in their study utilizing publicly available datasets. A few previous studies have compared the expression of certain genes (e.g., *TRL2*, *TRL3*, *TRL4*, *TRL7*, *TRL8*, *TRL9*, *hTERT*, *hTERC*) in BAL fluid cells and/or lung tissue samples derived from IPF and lung cancer (Antoniou et al., 2013; Samara et al., 2012). These studies have revealed distinct expression profiles of those genes between IPF and lung cancer.

ADC and SCC differ from each other in several ways, including cellular origin, since ADC is thought to arise from alveolar type II epithelial cells and SCC from basal cells of the larger proximal airway (Cheung & Nguyen, 2015). Microarray-based gene expression analyses performed on lung tumor samples have revealed differences in the gene expression profiles of ADC and SCC (Charkiewicz et al., 2017; Girard et al., 2016; Hou et al., 2010). Some of the studies comparing the gene expression of IPF and lung cancer (Leng et al., 2020; Spek & Duitman, 2019; Ulke et al., 2019) have combined data of ADC and SCC. However, due to the differences between ADC and SCC, it could be useful to compare IPF to ADC and SCC separately due to the differences between them.

6.2 Extracellular matrix proteins in idiopathic pulmonary fibrosis and lung cancer

In this study, *COL4A1* was found to be down-regulated in IPF-derived stromal cells in comparison to control cells and ADC. In contrast, in previous microarray-based and RNA sequencing studies, *COL4A1* has been listed as an up-regulated gene in cultured fibroblasts and tissues derived from IPF patients as compared to control (Lindahl et al., 2013; Luzina et al., 2018). A single-cell analysis has shown *COL4A1* expression in fibroblasts, smooth muscle cells, endothelial cells, and alveolar type I cells within non-diseased lung (Karlsson et al., 2021; The Human Protein Atlas, n.d.-b). We detected extracellular collagen α1(IV) immunoreactivity in the surrounding spindle-shaped cells within FF, which supports the previous studies showing collagen IV expression in the FF and early fibrotic lesions of IPF (Herrera et al., 2019; Urushiyama et al., 2015). In lung cancer, *COL4A1* expression has been

reported in stromal fibroblasts surrounding tumor cells by mRNA *in situ* hybridization (Soini et al., 1993), and discontinuous collagen α1(IV) protein expression has been observed around well-differentiated clusters in ADCs (Polette et al., 1997); these results are in line with our results showing strong collagen α1(IV) expression in the stroma of ADC. Based on the results of microarray analysis it could be speculated that the fibroblastic cells in IPF and ADC might have differences in their ability to produce collagen α1(IV).

Periostin has previously been studied in both IPF and lung cancer. It has been reported that *POSTN* is up-regulated in both cultured and non-cultured fibroblasts derived from IPF as compared to normal lung fibroblasts (Emblom-Callahan et al., 2010; J. U. Lee et al., 2017). By showing that periostin mRNA and protein were up-regulated in both IPF and ADC derived stromal cells, we confirmed the previous findings. *POSTN* gene expression level has previously been shown to be higher in IPF lung tissues as compared to normal lungs (Cecchini et al., 2018; DePianto et al., 2015; Yang et al., 2013). Recently, *POSTN* up-regulation in tissue samples of both IPF and lung cancer was reported in a study comparing publicly available IPF and lung cancer gene expression datasets (Yao et al., 2022). Similarly to our results, periostin immunoreactivity has been observed in areas of FF in IPF (Naik et al., 2012; Okamoto et al., 2011). Additionally, *POSTN* expression has been reported to occur within FF (Guillotin et al., 2021). Periostin gene and protein expression has been shown to be up-regulated in NSCLC tissues compared to normal lung tissue (Edlund et al., 2012; Ratajczak-Wielgomas et al., 2022), and it has been detected in lung cancer stroma, but not in cancer cells, by mRNA *in situ* hybridization and immunohistochemistry (Hong et al., 2013; Inoue et al., 2019; Ratajczak-Wielgomas et al., 2022; Sasaki et al., 2001), which supports our findings. Although periostin is highly expressed in the stroma of both ADC and SCC, the expression has been shown to be higher in SCC than in ADC (Ratajczak-Wielgomas et al., 2022).

We observed that *MMP1* was up-regulated in stromal cells cultured from IPF lung compared to ADC, which is in line with the previous study showing *MMP1* up-regulation in non-cultured fibroblasts derived from IPF lung (Emblom-Callahan et al., 2010). Several microarray-based studies have shown higher *MMP1* gene expression in IPF than in normal lung tissues (Cecchini et al., 2018; Estany et al., 2014; Konishi et al., 2009; Yang et al., 2013; Zuo et al., 2002). *MMP1* expression has also been shown to be higher in ADC and SCC tissues than in normal lung (Nakamura et al., 2003). In some studies, MMP-1 has been reported to be mainly expressed in epithelial cells and alveolar macrophages in IPF (Selman et al., 2006;

Zuo et al., 2002), although other studies have also reported MMP-1 immunoreactivity in stromal cells in IPF and ADC, in line with our observations (An et al., 2016; Thomas et al., 2000).

We identified *MMP3* as an up-regulated gene in fibroblasts derived from IPF in comparison to control and ADC. Several microarray-based studies have also reported up-regulated *MMP3* gene expression in lung tissues derived from IPF patients (DePianto et al., 2015; Estany et al., 2014). In normal lung, immunohistochemical MMP-3 expression has been mainly reported in alveolar macrophages while in IPF, it has been shown to be expressed in epithelial cells, macrophages, fibroblasts and intravascular leukocytes (DePianto et al., 2015; Yamashita et al., 2011). A previous study reported increased *MMP3* gene expression in NSCLC lung tissues compared to paired normal lung tissues (Nakamura et al., 2003) whereas our microarray analysis did not identify differences in *MMP3* gene expression between stromal cells derived from ADC and normal lung. We detected MMP-3 expression in both tumor cells and stromal cells while previous studies have shown stronger MMP-3 immunoreactivity in tumor cells than in stromal cells in lung ADC (Mehner et al., 2015; Thomas et al., 2000).

6.3 NHLRC2 in IPF and lung cancer

The expression pattern of NHLRC2 in IPF and lung cancer was observed to be similar since the expression in both lung diseases was mainly detected in epithelial cells and macrophages while stromal cells were weakly positive or negative. Additionally, according to the microarray data from the study I, *NHLRC2* was expressed at equal levels in fibroblasts derived from control, IPF and ADC. Single-cell RNA sequencing data show that NHLRC2 is widely expressed in different cell types within normal lung (Karlsson et al., 2021; The Human Protein Atlas, n.d.-a). The NHLRC2 expression pattern in normal lung, ADC and SCC shown in our study is in line with the pattern shown in the Human Protein Atlas with the same antibody (The Human Protein Atlas, n.d.-a; Uhlén et al., 2017). In normal lung, NHLRC2 was shown to be expressed in epithelial cells of bronchi as well as in type II pneumocytes and macrophages (The Human Protein Atlas, n.d.-a; Uhlén et al., 2015). According to the data in Human Protein Atlas, NHLRC2 expression in SCC cancer cells is negative (n = 2) or weak (n = 2) (The Human Protein Atlas, n.d.-a; Uhlén et al., 2017). In ADCs, NHLRC2 expression was negative (n = 1), weak or moderate in under 25% of the cells (n = 2), or moderate in over 75% of the cells (n = 3) (The Human Protein Atlas, n.d.-a; Uhlén et al., 2017).

NHLRC2 contains a thioredoxin (Trx)-like domain, although it has not been shown to have thioredoxin activity so far (Uusimaa et al., 2018; Yeung et al., 2019). NHLRC2 expression in the lung resembles that of Trx, since in the previous studies Trx1 was expressed in the hyperplastic alveolar epithelium, bronchiolar epithelial cells, and alveolar macrophages in IPF (Iwata et al., 2010; Tiitto et al., 2003) and in cancer cells to varying extent (Azuma et al., 2007; Fernandes et al., 2009; Soini et al., 2001). Additionally, similarly to NHLRC2, Trx expression has been reported in bronchial and alveolar epithelial cells and macrophages in normal lung outside tumor (Kakolyris et al., 2001; Soini et al., 2001). In addition, many of the antioxidative enzymes (e.g., superoxide dismutases, catalase, peroxiredoxins, and glutaredoxin) are expressed in airway epithelial cells, type II pneumocytes, and alveolar macrophages in human lung (Coursin et al., 1996; Kakolyris et al., 2001; Lakari et al., 2000; Lehtonen et al., 2004; Peltoniemi et al., 2004), and these cells are resistant to oxidative stress. ROS have a role in the initiation and progression of IPF and lung cancer (Valavanidis et al., 2013; Veith et al., 2019). Tumor cells are typically sensitive to oxidative stress since they have higher levels of ROS than normal cells due to the imbalance of oxidant and antioxidant levels (Nakamura & Takada, 2021).

NHLRC2 has been suggested to have a role in the regulation of ROS-induced apoptosis since it has been shown to be cleaved by caspase-8 at Asp580 in ROS-induced apoptosis in a human colon cancer cell line (HCT116) (Nishi et al., 2017). Loss of NHLRC2 increased the susceptibility of these cells to apoptosis (Nishi et al., 2017). Different expression patterns of caspase-8 have been detected in lung SCC and ADCC since strong cytoplasmic immunoreactivity in single cells was observed in SCC while ADC showed mainly diffuse cytoplasmic expression (Törmänen-Näpänkangas et al., 2001). Additionally, the single-cell staining pattern detected mainly in SCC was associated with high apoptotic activity (Törmänen-Näpänkangas et al., 2001).

We observed that immunohistochemical NHLRC2 expression determined by image analysis method was higher in IPF, ADC and SCC than in control lung. Similar to NHLRC2 expression, Trx has been shown to be higher in tumor than in normal lung tissue by immunohistochemistry and Western blot analysis (Deng et al., 2011; Fernandes et al., 2009; Kim et al., 2003; Park, Joo Kim et al., 2006). Trx1-system is also elevated in IPF (Iwata et al., 2010; Tiitto et al., 2003) , and Trx levels in serum have been shown to be higher in IPF than in control and in patients who later experience AE compared to patients not having AE (Iwata et al., 2010).

NHLRC2 gene expression has been reported to be lower in lung tissues of IPF patients whose FVC% and DLCO% values declined significantly up to 12 months following lung biopsy compared to slowly progressing disease (Boon et al., 2009). However, the protocol used in that study was different from ours since we analyzed the pulmonary function test results at the time of biopsy and information of the patients experiencing AE-IPF during the follow-up time. We did not find associations in NHLRC2 protein expression and occurrence of AE-IPF, FVC%, or DLCO%. However, three patients with AE-IPF had slightly higher NHLRC2 expression than patients at stable phase of the disease.

We observed that high NHLRC2 protein expression was associated with poor prognosis of ADC patients. NHLRC2 expression did not associate with survival in SCC or IPF patients. The gene expression data presented in the Human Protein Atlas shows that *NHLRC2* mRNA expression is not associated with survival in ADC or SCC (The Human Protein Atlas, n.d.-a; Uhlén et al., 2017). In contrast, a low gene expression of NHLRC2 combined with the expression of one long non-coding RNA and two other protein coding genes in tumor tissues has been reported to predict poor prognosis of lung ADC patients in a study generating a transcriptomic prognostic model for ADC utilizing publicly available gene expression datasets (Ye et al., 2019). The contradictory results between the study of Ye (2019) and our study may be due to different methods used. Furthermore, mRNA and protein levels do not always correlate even in samples from the same subjects, as shown previously in a study comparing data of microarray and proteomic analysis performed on lung ADC tissues (Chen et al., 2002). That phenomenon may also explain the differences in the results between our study and those reported in the Human Protein Atlas (The Human Protein Atlas, n.d.-a; Uhlén et al., 2017) and by Ye (2019).

TGF-β1 has a role in the pathogenesis of both pulmonary fibrosis and lung cancer. It has been reported to induce epithelial-to-mesenchymal transition in lung epithelial cells, differentiation of fibroblasts to myofibroblasts, and production of ECM proteins (Evans et al., 2003; Ji et al., 2014; Kasai et al., 2005). Immortalized skin fibroblasts derived from FINCA patients associated with *NHLRC2* variants have been reported to show enhanced differentiation into myofibroblasts (Paakkola et al., 2018). However, the effect of TGF-β1 on NHLRC2 expression has not been studied before. In our study, TGF-β1 exposure *in vitro* did not show any effect on NHLRC2 mRNA or protein levels in SAEC or primary lung stromal cells. Similarly, it has been reported that TGF-β1 exposure does not affect the protein levels of

peroxiredoxins in two human epithelial lung cell lines (A549 and BEAS-2B) (Lehtonen et al., 2005).

According to data presented in the Human Protein Atlas, NHLRC2 is widely expressed in different cell types in all tissues, and its mRNA expression is reported to be enhanced only in cardiomyocytes (Karlsson et al., 2021; The Human Protein Atlas, n.d.-a). Most normal tissues show moderate to strong cytoplasmic immunohistochemical staining for NHLRC2 (The Human Protein Atlas, n.d.-a; Uhlén et al., 2015). Similar to NHLRC2 expression in lung cancer, variable expression in cancer cells of different cancers including head and neck cancer was shown in the Human Protein Atlas (The Human Protein Atlas, n.d.-a; Uhlén et al., 2017). Thus, NHLRC2 may have a role in the pathogenesis of cancers in different organs.

6.4 Smoking, IPF and lung cancer

Smoking has been found to increase the risk of having IPF and lung cancer in several studies (Ekström et al., 2014; Park et al., 2021). Additionally, among IPF patients, current smokers have been shown to have longer unadjusted survival time compared to non- and ex-smokers (Antoniou et al., 2008; Kärkkäinen et al., 2017; T. E. King et al., 2001). However, smoking status has rarely been associated with immunohistochemical observations. We observed that the immunohistochemical NHLRC2 expression was higher in ever-smokers than in non-smokers. Futhermore, ever-smokers had less FF than non-smokers. In contrast, current smokers were shown to have less mast cells than non- or ex-smokers in a previous study with the same the IPF cases as used in this study (Salonen et al., 2021) and thus, it could be useful to compare smoking history and clinical parameters to immunohistochemical data since it might reveal novel information of disease pathogenesis. The NHLRC2 expression in non-smoking IPF patients was higher than that in controls and thus, smoking may not alone explain the higher NHLRC2 expression in IPF as compared to controls. NHLRC2 expression was not associated with smoking history in ADC. SCC has been observed more frequently among smokers than ADC (Pesch et al., 2012) and only one non-smoking SCC case was included in this study. Thus, NHLRC2 expression could not be compared between non-smokers and smokers among SCCs. The NHLRC2 expression in SCC did not differ between ex- and current smokers.

Smoking status has been shown to affect the phenotype of cultured primary fibroblasts (Karvonen et al., 2014; S. Lehtonen et al., 2021) and gene expression in

lung ADC tissues (Bossé et al., 2012; Landi et al., 2008). One of the IPF cases included in our microarray analysis was a non-smoker, two were ex-smokers, and one was a current smoker, while the control samples of our study were from non-smoking ADC patients. Thus, some of the differences observed between IPF and ADC or control may be caused by smoking. Smoking data of control cases has not been available for all previous microarray studies (Lindahl et al., 2013; Vuga et al., 2009), or some of the controls have been smokers (Hsu et al., 2011).

6.5 Strengths and limitations

The small number of cell lines examined by the microarray analysis was the most serious limitation in study I. The number of primary stromal cell samples of IPF in particular was restricted since only a few patients with IPF undergo a surgical lung biopsy operation, which limits access to material. However, the numbers of cell lines in some of the previously published studies have also been relatively small (Hsu et al., 2011; Lee et al., 2017; Lindahl et al., 2013; Peng et al., 2013; Rodriguez et al., 2018; Vuga et al., 2009). As also previously speculated, the phenotypic changes in the fibroblastic cells during their passage in culture as well as the differences in the cell collection and culture methods might have affected the results (Rodriguez et al., 2018). Some disease-specific alterations in gene expressions are not maintained during cell culture and the disappearance of these differences might be enhanced if cell culture conditions including cell confluency are not controlled. Therefore, stringently controlled conditions from the beginning of the cell collection and culture were adopted in this study.

The study design was retrospective, resulting in some missing patient data and possible exclusion of potential cases. The number of IPF and lung cancer cases was limited, especially when the patients were divided into groups based on clinical and histologic information. The patients with IPF were treated with various pharmacological therapies, which may have affected the occurrence of AE-IPF and survival. However, the collection of the patients' clinical information was conducted in a very detailed manner, we were able to gather rather comprehensive data, and all available data was thoroughly evaluated.

Image analysis has many differences compared to the more traditional semiquantitative evaluation of immunohistochemical staining in different cell types. By digital image analysis, total staining in all cell types in the sample is counted while in semiquantitative analyses, staining in different cell types can be scored separately. Based on the experience obtained during this study, anthracosis,

necrosis, background staining, and variable sample quality may be difficult to deal with when using digital image analysis. On the other hand, different staining intensities can be distinguished more reliably by the software than human eye (Lykkegaard Andersen et al., 2018). Altogether, these two analysis methods have their own strengths and limitations, and thus it may be worthwhile to use them both in certain situations.

7 Conclusions

1. Some similarities were found in the gene expressions of IPF and lung ADC derived stromal cells, although there were also several differences, suggesting that the molecular changes occurring in these two lung diseases are mainly different. Further studies are needed on the factors similarly dysregulated in IPF and lung cancer.
2. NHLRC2 was mainly expressed in alveolar and bronchiolar epithelium and macrophages in normal lung and hyperplastic alveolar epithelial cells in IPF. Additionally, the immunohistochemical expression of NHLRC2 determined by digital image analysis method was higher in IPF than in control lung and it was associated with smoking. TGF-β1 did not have an effect on NHLRC2 expression in primary stromal cells or small airway epithelial cells *in vitro*.
3. NHLRC2 was mainly expressed in cancer cells and inflammatory cells in lung ADC and SCC tumors. The immunohistochemical NHLRC2 expression was higher in lung ADC than in SCC and its high expression was associated with mitotic activity and poor survival in ADC patients. Further studies are needed to elucidate the pathogenetic role of NHLRC2 in both IPF and lung cancer.

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