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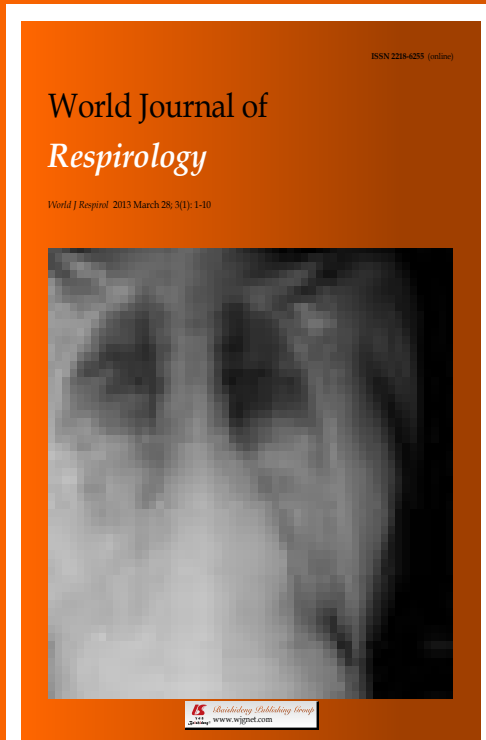

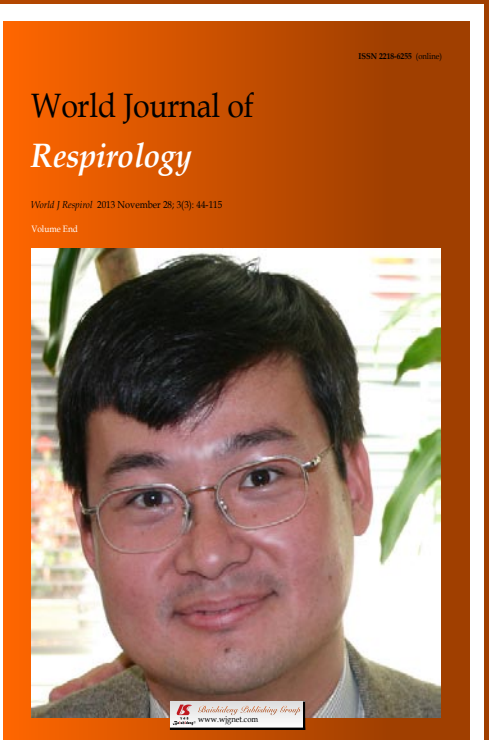

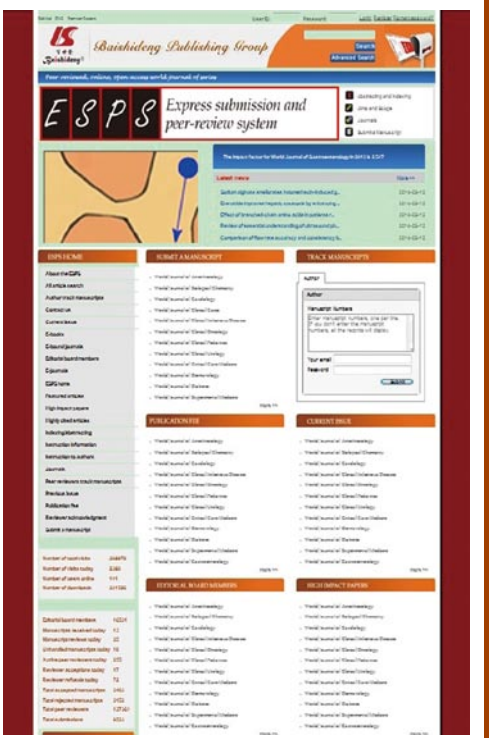

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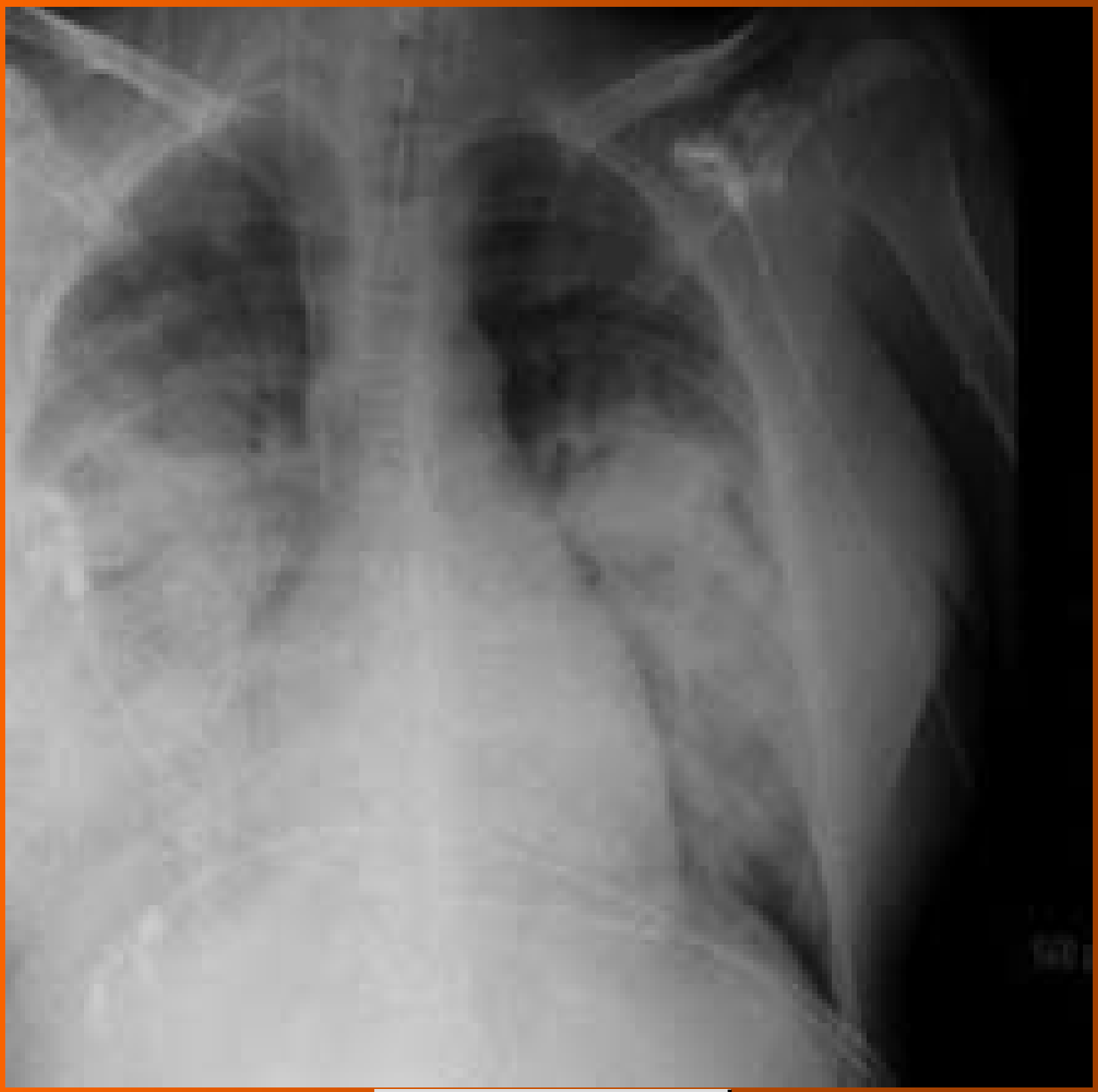

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BRIEF ARTICLE

Cytokine gene polymorphisms in idiopathic pulmonary fibrosis

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Abstract

AIM: To characterize cytokine gene polymorphisms in patients with idiopathic pulmonary fibrosis (IPF) compared to healthy controls.

METHODS: Fifty-six IPF patients were involved in the study. The control population consisted of 144 healthy volunteers without history of lung disease. All of the patients were diagnosed with IPF according to the American Thoracic Society/European Respiratory Society consensus statement. Polymorphisms in the interleukin (*IL*)-*1*, *IL-1*, *IL-1R*, *IL-1RA*, *IL-2*, *IL-4*, *IL-6*, *IL-10*, *IL-12*, tumour necrosis factor, interferon, transforming growth factor, *IL-1*, *IL-2*, *IL-4* and *IL-4RA* genes were characterized by polymerase chain reaction with sequence-specific primers. Statistical analysis was performed using the MedCalc statistical software. A Bonferroni correction of significance at an alpha of 0.05 was used for multiple analyses. A corrected *P* value less than 0.0023 (0.05/22) was considered significant.

RESULTS: We found significant differences in the *IL-4* promoter region polymorphisms between IPF patients and controls. Namely, polymorphisms of *IL-4* (*-590*) [computed tomography (CT) in 32 of 56 patients *vs* 27 of 144 controls; $P < 0.0001$] and *IL-4* (*-33*) (CT in 25 of 56 patients *vs* 27 of 144 controls; $P = 0.0006$) differed between both groups. With regard to haplotypes, we found differences in the frequencies for haplotype 1 of *IL-4* (*-1098*) (*-590*) (*-33*) between IPF and controls (TCC in 23 of 56, TTC in 10 of 56, and TTT in 21 of 56 patients *vs* TCC in 112 of 144, TTC in 0 of 144, and TTT in 32 of 144 controls; $P < 0.0001$). We did not find significant differences in gene polymorphism frequencies of other cytokines in the IPF group *vs* the controls.

CONCLUSION: We hypothesize that *IL-4* promoter polymorphisms could be involved in the pathogenesis of IPF, likely *via* enhancement of the T_H2 cytokine milieu with exaggerated fibroproliferative healing.

Key words: Cytokine genes; Idiopathic pulmonary fibrosis; T_H2-type immune response

Core tip: Enhanced fibroproliferation resulting in terminal fibrosis is the main feature of idiopathic pulmonary

fibrosis (IPF). Various mechanisms of alveolar damage and its healing are involved in IPF development. One of the potential contributing pathogenic factors is the genetically encoded imbalance of cytokine production. We found differences between the frequencies of interleukin (*IL*)*-4* gene promoter polymorphisms in IPF patients *vs* controls. Based on these results and on the observation that *IL-4* promoter polymorphisms can influence IL-4 production, we hypothesize that *IL-4* promoter polymorphisms could be involved in the pathogenesis of IPF, likely by enhancing the T_H2 cytokine milieu with subsequent fibroproliferative healing.

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most severe interstitial lung disease (ILD) and can be characterized by uncontrolled fibroproliferation as a sequelae of the pathological pattern of the healing of multiple alveolar lesions[1,2]. The role of the regulatory, profibrotic and antifibrotic cytokines and chemokines in the pathogenesis of IPF has been the subject of much conjecture[3-6]. One of the possible mechanisms of fibroproliferative healing in IPF could be the alternative activation of alveolar macrophages (AM) under the influence of predominantly T_H2-type cytokines. These alternatively activated AMs induce an increase of collagen production by fibroblasts. This process appears to be mediated by C-C chemokine 18, which is overexpressed in the presence of interleukin (IL)-10, IL-4 and IL-13[7,8]. One of the possible mechanisms of overexpression of T_H2 cytokines is the expression level of redox-sensitive transcription factors. Kikuchi et al. observed that the expression of T_H2 cytokines, such as IL-4 and IL-13, was significantly elevated in the lungs of Nrf2-deficient mice, with an increase in the number of T_H2 cells that expressed the GATA-binding protein 3[9]. However, the pathogenesis of IPF is a complex, multi-event process in which the T_H2 cytokines might be only one of multiple pathogenic factors. Nevertheless, it seems that cytokine imbalance likely plays an important role in coordinating the healing of alveolar lesions resulting in fibrosis.

One of the mechanisms resulting in increased production of cytokines is a polymorphism in a region of a cytokine gene that causes a change in its expression. Cytokines and their receptors are highly conserved within their coding regions. Although polymorphisms outside of the coding region do not influence the amino acid sequence, they may affect protein expression by influencing alternative mRNA splicing, mRNA stability, or transcription levels. Polymorphisms within the 5'- and 3'-regulatory regions of cytokines are particularly notable given that they can determine the transcription factor binding sites within the cytokine gene promoters and the structure of enhancers and silencers. In part, individual diversity in immune responses might be explained by such polymorphisms because they result in interindividual differences in the capacity to produce cytokines, which results in a variety of biological consequences. Hence, the investigation of IPF immunogenetics has been focused on identifying the cytokine polymorphisms that contribute to IRF or modify its severity.

Previous studies have described cytokine gene polymorphisms that could possibly be involved in IPF pathogenesis: *IL-10* (Whittington HA)[10], *IL-6* and tumour necrosis factor-receptor (*TNF-R*) II (Pantelidis P)[11], *IL-1-receptor antagonist* (*RA*) (Whyte M, Korthagen NM, Hutyrova B)[12-14], transforming growth factor-beta1 (*TGF-β1*) (Xaubet A, Son JY, Awad MR)[15-17], tumour necrosis factor-alpha (*TNF-α*) (Whyte M, Freeburn RW)[12,18] and *IL-8* (Renzoni E, Ahn MH)[19,20].

In our previous studies, we have suggested the involvement of *IL-1*, *IL-4*, *IL-12* and interferon (*IFN*) *-gamma* gene polymorphisms in the pathogenesis and clinical presentation (functional parameters, bronchoalveolar lavage fluid characteristics, high resolution computed tomography score) of sporadic IPF. Polymorphisms of the promoter region of *IL-4*, which were not previously described in any of the cited studies, were the most significant results[21-23]. Previous studies have shown that *IL-4* promoter variants, namely, *IL-4* (*-589*) *T* and *IL-4* (*-33*) *T*, could result in up to three-fold higher transcriptional activity of IL-4 *in vitro*, which was subsequently confirmed *in vivo*[24,25].

In our most recent study, we aimed to reinforce our hypothesis regarding the potential role of cytokine gene polymorphisms, namely, the high-producer *IL-4* promoter gene polymorphisms, for IPF pathogenesis in a larger number of patients.

MATERIALS AND METHODS

Ethics

The study design and the informed consent form were approved by the Central Ethical Committee of the University Thomayer Hospital and the Institute for Clinical and Experimental Medicine. All patients signed the informed consent form before submitting a blood sample for genotyping.

Study subjects

Fifty-six IPF patients were involved to our study. All patients with IPF were Caucasians [age: mean ± SD (67.29 ± 11.85), range (36-87 years), male/female (37/19)] Patients were diagnosed according to the American Thoracic Society/European Respiratory Society consensus statement[26]. We used the following criteria: insidious onset of dyspnea, bilateral basal crackles and digital clubbing, restrictive ventilatory pattern and lowered diffusion

Table 1 Demographic and basic clinical parameters of idiopathic pulmonary fibrosis patients, mean ± SD (range)

Parameters	Value
Men:women ratio	37:19
Mean age (yr)	67.29 ± 11.85
Mean FVC (%)	70.96 ± 20.60 (21-127)
TL_{CO} (%)	39.78 ± 16.63 (13-81)
BALF LY (%)	17.86 ± 18.93 (0-72)
BALF PMN (%)	15.84 ± 17.58 (1-70)
BALF EOS (%)	3.21 ± 6.95 (0-28)

BALF: Bronchoalveolar lavage fluid; FVC: Forced vital capacity; TL_{CO}: Transfer factor for CO; LY: Lymphocytes; PMN: Polymorphonuclear leukocytes; EOS: Eosinophilic leukocytes.

capacity for carbon monoxide, and typical radiological changes on high resolution tomography of the lungs (HRCT) with prevailing fibrotic changes and granulocytic bronchoalveolar lavage (BAL). A videothoracoscopic lung biopsy was performed in 16 patients who did not meet all above-mentioned criteria, and in all cases, the histopathological investigation revealed changes characteristic for usual interstitial pneumonia (UIP). We excluded other potential causes of ILD that can present with a UIP HRCT pattern, namely, hypersensitivity pneumonitis and connective tissue diseases (Table 1).

The control population of 144 unrelated individuals (24 males, 120 females) was made up of Caucasians from the Czech Republic. All control subjects were potential bone marrow donors in generally good health status, without any known diseases, at the time of the study. They did not state asthma, pulmonary fibrosis and systemic inflammatory disease in their histories. The normal controls had a mean age of 43.1 (SD = 16.17) years (range 19-80 years).

Methods

Polymorphisms in the promoter regions of *IL-1α*, *IL-1β*, *IL-1R*, *IL-1RA*, *IL-2*, *IL-4*, *IL-6*, *IL-10*, *IL-12*, *TNF-α* and *IFN-γ* and the polymorphisms in the translated regions of the *TGF-β*, *IL-1β*, *IL-2*, *IL-4* and *IL-4RA* genes were characterized (Table 2).

DNA extraction

Two milliliter of peripheral venous blood were collected into ethylen-diamino-tetraacetic acid (EDTA) tubes. Isolation of genomic DNA was performed from 350 μL of blood using a DNA Blood Kit Cartridge B350 (Qiagen, Germany) on an automated BioRobot EZ1 instrument. The quality of DNA samples was checked on a spectrophotometer and DNA samples were archived at -20 ℃.

Cytokine genotyping: We evaluated the polymorphisms of thirteen different cytokine genes utilizing the CYTOKINE GENOTYPING KIT (Dynal, Biotech, Norway). The test is designed as a polymerase chain reaction (PCR) with sequence-specific primers. In detail, each well of a 48-well tray contains a specific primer pair for amplifying the desired unique sequence. The entire procedure was performed according to the manufacture's manual. The obtained pattern of positive and negative PCR was documented and interpreted according to the manufacture's worksheet.

Table 2 List of investigated cytokine gene polymorphisms

Polymorphism	Genotype
IL-1α (-889)	C/C C/T T/T
IL-1β (-511)	C/C C/T T/T
IL-1β (+3962)	C/C C/T T/T
IL-1R pst 1970	C/C C/T T/T
IL-1 RA mspa 11100	C/C C/T T/T
IL-1 RA (+1902)	A/A A/G G/G
IL-12 (-1188)	A/A A/C C/C
INF-β UTR 5644	A/A A/T T/T
TGF-β1 codon 10	C/C C/T T/T
TGF-β1 codon 25	C/C C/G G/G
TNF-α (-308)	A/A A/G G/G
TNF-α (-238)	A/A A/G G/G
IL-2 (-330)	G/G G/T T/T
IL-2 (+166)	G/G G/T T/T
IL-4 (-1098)	G/G G/T T/T
IL-4 (-590)	C/C C/T T/T
IL-4 (-33)	C/C C/T T/T
IL-6 (-174)	C/C C/G G/G
IL-6 (+565)	A/A A/G G/G
IL-10 (-1082)	A/A A/G G/G
IL-10 (-819)	C/C C/T T/T
IL-10 (-592)	A/A A/C C/C

IL: Interleukin; TNF: Tumor necrosis factor; INF: Interferon; UTR: Untranslated region.

Statistical analysis

The basic statistical characteristics, *i.e.*, the mean values and SD, were calculated for continuous variables (demographic and basic clinical parameters). The genotype frequencies and allele carriage frequencies in IPF were determined by direct counting and were compared with those in the control population using a Fisher's exact or χ^2 test. A Bonferroni correction of significance level at an alpha of 0.05 was used for multiple analyses. Statistical analysis was performed using the MedCalc statistical software. A P value less than 0.0023 (0.5/22) was considered significant.

RESULTS

Demographic and clinical data of the IPF group

The statistical analysis of the basic demographic data, functional parameters, and BAL fluid cell counts of the group of IPF patients is presented in Table 1.

Frequencies of cytokine gene polymorphisms in the IPF vs control group

When comparing the frequencies of gene polymorphisms between IPF individuals and controls, we found significant differences for the investigated *IL-4* promoter region polymorphisms (-590) and (-33) ($P < 0.0001$; $P = 0.0006$, respectively). In the IPF group, we more fre-

Table 3 *IL-4* (*-590*), *IL-4* (*-33*) and *IL-4 haplotype 1* polymorphisms in idiopathic pulmonary fibrosis and healthy controls *n* (%)

Locus	Genotype	Controls	IPF group	*P* value
IL-4 (*-590*)	CC	112 (77.8)	24 (42.9)	< 0.0001
	CT	27 (18.8)	31 (55.4)	
	TT	5 (3.5)	1 (1.8)	
IL-4 (*-33*)	CC	112 (77.8)	29 (52.7)	0.0006
	CT	27 (18.8)	25 (45.5)	
	TT	5 (3.5)	1 (1.8)	
IL-4 haplotype 1	GCC	0 (0.0)	1 (1.8)	< 0.0001
	TCC	112 (77.8)	23 (41.1)	
	TCT	0 (0.0)	1 (1.8)	
	TTC	0 (0.0)	10 (17.9)	
	TTT	32 (22.2)	21 (37.5)	

IPF: Idiopathic pulmonary fibrosis; IL: Interleukin.

quently observed the T allele at these positions (Table 3).

With regard to haplotypes, we observed differences in the frequency of haplotype 1 in the promoter region of *IL-4* (*-1098*) (*-590*) (*-33*) between the IPF and control groups (haplotype 1: TCC in 23 of 56 (41.1%), TTC in 10 of 56 (17.9%), and TTT in 21 of 56 (37.5%) patients *vs* TCC in 112 of 144 (77.8%), TTC in 0 of 144 (0%), and TTT in 32 of 144 (22.2%) controls; $P < 0.0001$) (Table 3).

The differences in the frequency of haplotype 2 in the promoter region of *IL-4* between IPF and controls were not significant after Bonferroni correction (Haplotype 2: GCC in 16 of 56 (28.6%), GCT in 2 of 56 (2.6%) and TCC in 35 of 56 (62.4%) patients *vs* GCC in 22 of 144 (15.3%), GCT in 0 of 144 (0%) and TCC in 117 of 144 (81.2%) controls; $P = 0.0061$). We also observed differences between IPF and controls for the frequencies of haplotype 1 polymorphisms at *IL-6* (*-174*) and (*nt565*), but these differences could not be declared as significant after Bonferroni correction [CA in 4 of 56 (7.1%) patients *vs* in 25 of 144 (17.4%) controls; $P = 0.0438$].

We did not find significant differences in gene polymorphisms frequencies for any other cytokines in the IPF group compared to the healthy population.

Hardy-Weinberg equilibrium

We tested for Hardy-Weinberg (HW) equilibrium for all examined SNPs as well. With the exception of *IL-4* (*-590*) and (*-33*), all SNPs were in equilibrium. We had to reject HW equilibrium in the control group for both *IL-4* (*-590*) and *IL-4* (*-33*) at $P = 0.05$ and in the IPF group for *IL-4* (*-590*) at $P = 0.01$ and for *IL-4* (*-33*) at $P = 0.07$. The less significant *P* value in the IPF group for *IL-4* (*-33*) was because of the smaller number of samples in that group compared to controls.

DISCUSSION

In our study, we have reinforced our hypothesis that *IL-4* gene promoter polymorphisms might be involved in IPF pathogenesis. The involvement of *IL-4* promoter gene polymorphisms has previously been shown to be involved in the pathogenesis of other diseases and their presentations, including: asthma severity, common variable immune deficiency, autoimmune thyroid disease, atopy, allergic rhinitis, eczema, Crohn's disease and renal transplantation outcome[27-29]. However, *IL-4* promoter gene polymorphisms have not yet been investigated, to our knowledge, in IPF Individuals who carry the T allele at the (-590) position of the *IL-4* gene, as more than half of our IPF patients did, were previously found to have a higher proportion of IL-4-producing T-helper cells (Nakashima H)[25]. IL-4 is required for the subsequent appearance of IL-4-producing cells, and thus for T_H2 lineage commitment. Regarding the biological effect of IL-4, RNA hybridization studies have demonstrated that IL-4 suppresses the expression of T_H1 and inflammatory cytokines (IL-1, IL-1 and TNF). These results suggest that IL-4 modulates monocyte production of TNF and IL-1 by down-regulating gene expression. This unique property of IL-4 may be important in regulating the immune response[30]. Regarding the direct role of IL-4 in the pathogenesis of fibrosis, *in vitro* studies have shown that this cytokine can regulate fibroblast function, including chemotaxis, proliferation, collagen synthesis, and myofibroblast differentiation[31,32]. In accordance with these observations, other reports have observed increased IL-4 expression in bleomycin-, silica- and radiation-induced lung injuries and suggested that activated macrophages represent the major source of IL-4 during the establishment of active lung fibrosis. In human studies, the progression of IPF was also shown to be associated with sustained IL-4 production[33-35]. These results strongly suggest that *IL-4* gene polymorphisms with consequent IL-4 overproduction are involved in the pathogenesis of many diseases in which the role of T_H1/T_H2 equilibrium is crucial. IPF is one such disease, and our study supports the hypothesis of an encoded genetic predisposition for T_H2 immune response to environmental stimuli, which could result in alternative activation of AMs and the triggering of pathologic healing of alveolar lesions[8]. *IL-4* promoter variants, namely, *IL-4* (*-589*) *T* and *IL-4* (*-33*) *T*, result in up to three-fold higher transcriptional activity *in vitro*, which likely results in increased IL-4 production and thus T_H2 dominant cytokine milieu[25]. We have found in our study that this high-producer *IL-4* genotype is significantly more present in IPF patients than controls. Aside from the potential direct role of *IL-4* gene polymorphisms in IPF aetiology, these polymorphisms could also have disease-modifying effects. This notion was hypothesized in our previous studies on the correlation of clinical parameters of IPF with *IL-4* gene polymorphisms, in which we put forth the suggestion of the protective role of *IL-4* (*-33*) *CC* homozygosity (the low-producer genotype) against the progression of HRCT interstitial changes[22].

The *IL-6* (*-174*) *G→C* polymorphisms were previously mentioned in relationship with IPF development in the two studies by Pantelidis *et al*[11] and Riha *et al*[36]. In our

study, the statistical significance for the difference of frequencies of this polymorphism between IPF and control individuals was not reached after Bonferroni correction. This is likely because of our investigation of a greater number of polymorphisms in one kit and because the correction for multiple comparisons substantially influenced the result. The *IL-6* (*-174*) *G→C* polymorphism appears to be functionally relevant and codes for higher IL-6 production (allele G), which was shown in neonates and later in patients with liver cirrhosis and hepatocellular carcinoma[28,37]. The role of IL-6 in the pathogenesis of fibrosing interstitial lung diseases has been hypothesized in recent studies, which supports the potential involvement of *IL-6* polymorphisms in the pathogenesis of IPF[38-40].

Cytokine gene polymorphisms and cytokine milieu are only a part of the net of potential mechanisms and factors leading from alveolar epithelial injuries to pathological fibroproliferative healing and eventual fibrosis. Chemokines, reactive oxygen species, antioxidants, transcription factors, growth factors and enzymes (particularly metalloproteinases and their inhibitors) are also known to be involved.

We are aware that our group of patients is not large compared to other genetic studies, but IPF is a rare disease and our study was performed at one center. We also recognize that the control and IPF patient group differ in sex and age and that this variation could influence our results. This is because IPF is more prevalent in men, not because of the sampling. Nevertheless, there are not any data in the literature on the influence of age and sex on genetic polymorphisms of cytokines, and we posit that the occurrence of cytokine polymorphisms might not be influenced by these variables.

When considering the influence of HW equilibrium, we found disequilibrium for IL-4 (-590) and for IL-4 (-33) in both the control and the IPF groups. The deviation from HW equilibrium is likely because we cannot expect Mendelian inheritance in the Czech population as in other European populations. We suggest that the influence of migration, random mutations, selection pressure and non-panmixia is similar for both populations, *i.e.*, our IPF and control group. Therefore, we consider our results as significant despite the suboptimal size of our cohorts from the genetic perspective[41]. Optimally, multi-centre studies of the effect of cytokine genetic polymorphisms in IPF will be required to support our findings.

COMMENTS

Background

Idiopathic pulmonary fibrosis (IPF) is a serious primary fibrosing interstitial lung disease with an aetiology that is not known to date. Multiple pathogenic pathways and factors contribute to the pathological pattern of healing of alveolar damage in the lungs of individuals with IPF. One of the predisposing factors that are responsible for IPF development is likely the genetically encoded type of immune response, *i.e.*, cytokine gene polymorphisms. Polymorphisms of the cytokine genes, namely, that of T_h2-type cytokines, can cause an imbalance in the cytokine milieu with T_h2 prevalence, which can then contribute to triggering fibroproliferative healing in the lungs.

Research frontiers

Knowledge of the processes contributing to the development of lung fibrosis could help find a way to influence the pathways involved in IPF pathogenesis. Thus, research of the mechanisms and factors of fibroproliferative healing in the lungs helps to find potential new drugs for treatment of this serious disease.

Innovations and breakthroughs

Previous studies of the immunogenetics of IPF revealed some cytokine gene polymorphisms that could potentially be involved in fibroproliferative healing. Namely, genetic polymorphisms of transforming growth factor, tumor necrosis factor, interleukin (*IL*)-*1RN*, *IL-6* and *IL-8* might be involved in IPF pathogenesis. The authors believe that T_h2 prevalent cytokine milieu could be caused by increased production of IL-4 through its polymorphisms. The authors' previous study on 30 patients with IPF suggested involvement of *IL-4* genetic polymorphisms in IPF development. The authors' studies have shown that *IL-4* polymorphisms, namely, at loci causing higher expression of the cytokines, could be related to more severe fibrotic changes in IPF and also influence chemokine milieu in the lungs. In this study, they have tested the frequencies of cytokine gene polymorphisms in an extended group of IPF patients to further support their previous findings.

Applications

The results support a reason to investigate genetic polymorphisms in patients with IPF to find patients whose enhanced fibroproliferation in the lung could be driven by increased expression of T_h2 cytokines, namely, *IL-4*. This subgroup of IPF patients might profit from treatment to block the IL-4 pathogenetic pathway.

Terminology

The polymorphisms of the genes indicate that some loci are variable, and different individuals and populations can bear different alleles. These different alleles can cause changes in expression of the gene, *i.e.*, influence the production of the cytokine and cause changes in cytokine milieu.

Peer review

This study reported on significant differences in polymorphisms in the *IL-4* and *IL-6* promoter regions between IPF patients and controls. This study is an extension of prior work in a larger cohort of IPF patients and presents novel and important results. The article covers a topic of clinical relevance and novelty. Their findings could have future implications in understanding the pathogenesis of IPF and its treatments. The article is well structured and presents no ethical problems.

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CASE REPORT

Successful management of life-threatening respiratory failure from H1N1 influenza

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Author contributions: All authors contributed equally to this work; Al-Attar N and Altaani H performed the ECMO procedure, collected and reviewed clinical information about the patient and wrote the report; Bouadma L and Wolff M were involved with daily care at the intensive care unit and assisted with report writing; Nataf P was consulted on management of the patient and assisted with report writing.
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Abstract

We report the outcome of a pregnant woman with a life-threatening acute respiratory distress syndrome from a novel influenza A (H1N1) virus infection 3 d postpartum successfully managed by veno-arterial extracorporeal membrane oxygenation. The patient was successfully weaned from extracorporeal membrane oxygenation (ECMO) on day 10. Novel H1N1 virus infection was identified by real-time reverse transcription-polymerase chain reaction. Veno-arterial ECMO in this patient carried a number of specific advantages namely maintaining haemodynamic stability obviating the need for inotrope support and improving oxygenation compared to alternative approaches such as veno-venous ECMO and pumpless devices. Femoral arterial and venous cannulae were inserted in a pure percutaneous method allowing rapid establishment of extracorporeal circulation. Given the high mortality of acute respiratory distress syndrome following novel H1N1 virus infection, we advocate the use of ECMO when conventional mechanical ventilation fails.

Key words: Respiratory failure; Influenza A; Extracorporeal membrane oxygenation; Pregnancy; Outcome

Core tip: Novel influenza A virus infection can result in acute respiratory distress syndrome extremely difficult to manage with conventional mechanical ventilation and non-invasive therapy. Veno-arterial extracorporeal membrane oxygenation can provide respiratory and circulatory support in these patients. We successfully weaned a postpartum patient after 10 d of extracorporeal membrane oxygenation. Furthermore, by employing the pure percutaneous method of cannulae insertion, we minimized implantation time and trauma.

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INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a life-threatening complication of influenza. It has been reported after novel influenza A (H1N1) virus infection and is a major cause of death in these patients[1]. The virus causes lung damage from direct injury to the respiratory epithelium and can be associated with secondary cytokine storm[1]. The management comprises aggressive supportive care, with positive pressure ventilation and high oxygen concentrations. However, mechanical ventilation carries the risk of barotrauma and oxygen toxicity,

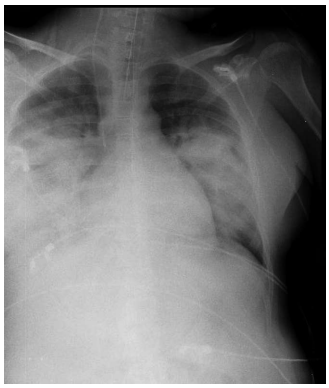

Figure 1 Chest-X ray showing bilateral infiltrates.

further aggravating lung injury[2]. In a series of ARDS in patients with H1N1 virus infection, mortality among the patients requiring mechanical ventilation was 58%[1] compared to 30% for other causes of ARDS. A number of adjuvant strategies have been employed for treatment of ARDS, and the use of extracorporeal membrane oxygenation (ECMO) has shown to reduce the ventilator-induced lung injury and mortality in certain patients[3,4]. The outcome of ECMO has not been specifically reported in patients with ARDS secondary to novel H1N1 virus infection but can improve survival by 50% regardless of the etiology[5,6].

CASE REPORT

A 41-year-old woman (para 5, gravida 2) was transferred from-the-department of obstetrics for acute respiratory distress 3 d following a normal vaginal delivery of a 37 wk term baby boy. The previous gynaecological, medical and surgical history were unremarkable. A few days before delivery she had fever (39 ℃) and a flu-like illness. On the third post-partum day, the patient developed increasing dyspnoea. This was followed by severe hypoxemia, acute respiratory distress syndrome, and an inability to achieve adequate oxygenation with conventional ventilation modalities (SaO_2 < 90%, arterial pO_2 = 49 mmHg and pCO_2 = 29 mmHg). Her chest X-ray showed bilateral widespread alveolar infiltrates (Figure 1). She had a white cell count of 2.98 × 10^9/L with predominant neutrophilia (88.6%) and lymphocytosis (10.1%), and C-reactive protein of 179 mg/L. She was transferred to the intensive care unit where orotracheal intubation and advanced mechanical ventilation (fraction of inspired oxygen of 1.0 and positive end-expiratory pressure of 15 cm H_2O including inhaled nitric oxide at 10 PPM) did not significantly improve the respiratory status (SpO_2 < 70%). Consequently, veno-arterial ECMO was established with an 18Fr cannula inserted into the right common femoral artery and a 22Fr venous cannula in the right femoral vein, associated with volume resuscitation. This lead to reversal of hypercapnia and improved oxygenation.

The patient was successfully weaned from ECMO on day 10. Bacterial cultures of blood, urine and tracheal aspirate were negative. Novel H1N1 virus infection was documented by testing of respiratory (bronchoalveolar lavage) specimens with real-time reverse transcription-polymerase chain reaction.

DISCUSSION

The patient was previous healthy and although pregnancy is known to increase the risk for influenza-associated morbidity and mortality during seasonal influenza epidemics[7,8] and pandemics[9,10], there seems to be a disproportionate increase in lethality for young, healthy individuals infected with HP strains of H1N1 viruses which correlates with a significant increase in the recruitment of inducible NOS-producing dendritic cells to the pneumonic lung, explaining the severity of the presentation[11]. The Centers for Disease Control and Prevention have reported a total of 20 cases of novel H1N1 virus infection among pregnant women in the United States, of which one patient developed ARDS and died[12]. In metropolitan France, 3 cases of infection in pregnant women were reported (out of a total of 921 confirmed or probable cases), none of which were associated with complications[13]. The use of veno-arterial ECMO in this patient maintained hemodynamic stability obviating the need for inotrope support and is less cumbersome than veno-venous ECMO. It was inserted in a pure percutaneous method using the Seldinger's technique allowing rapid establishment of extracorporeal circulation. After puncture of the vessel, a soft tipped guide-wire was passed through the needle. Dilatators were passed stepwise over the guide-wire to enlarge the access until the cannula size was achieved. The cannula was inserted and connected to the ECMO system. Thus percutaneous femoral cannulation carries the advantage of allowing an expeditious procedure in an emergency situation[14]. Because novel H1N1 virus infection complicated by ARDS is associated with a high mortality, ECMO should be considered when conventional mechanical ventilation fails.

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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Conference paper

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15 Morse SS. Factors in the emergence of infectious diseases.

Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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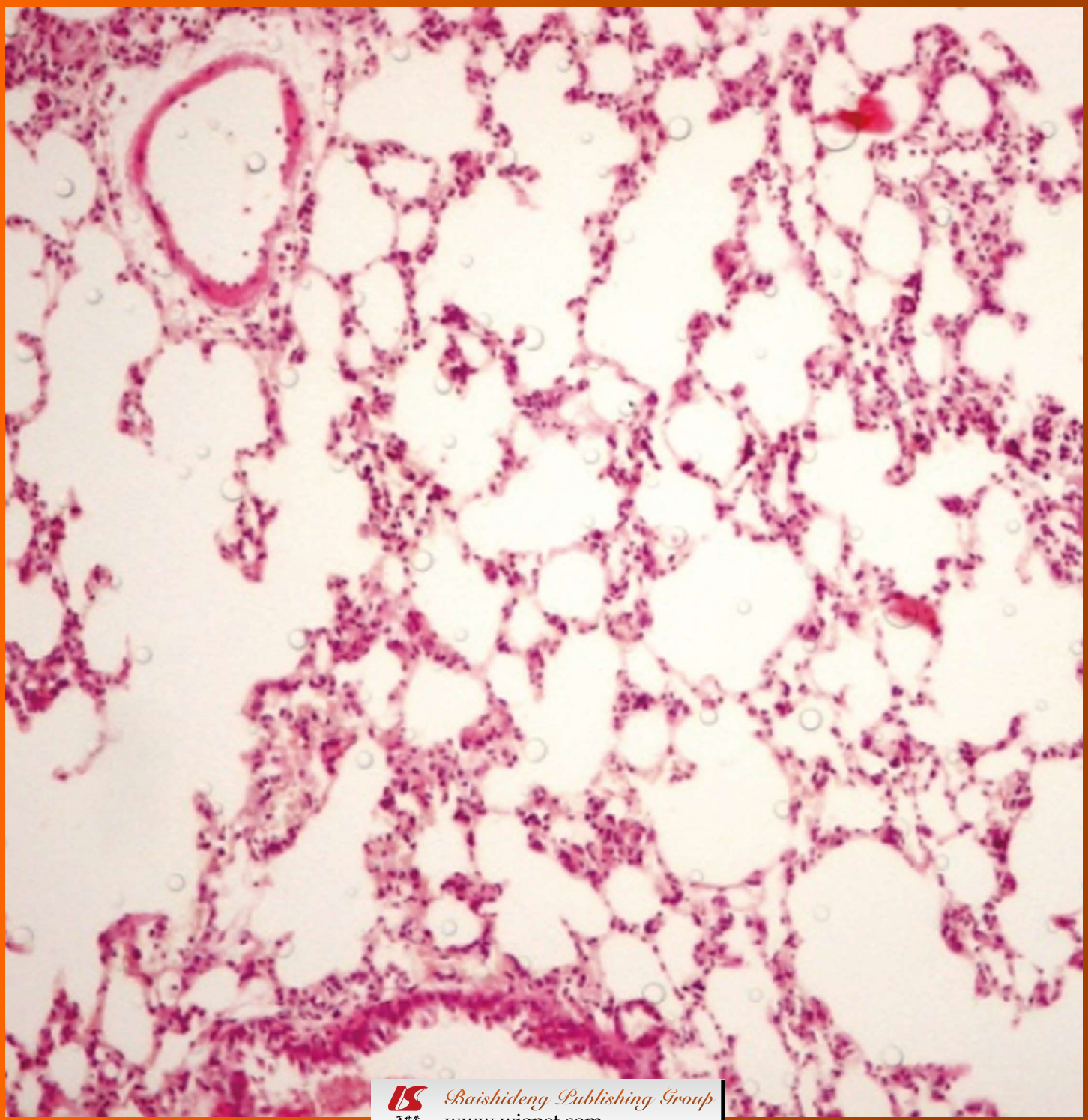

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ORIGINAL ARTICLE

High-frequency electrical stimulation of cervical vagi reduces airway response to methacholine

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Author contributions: Zhuang J and Xu F performed the majority of experiments, data analysis and statistics, manuscript preparation and revision; Bailet D, Curtis R, and Xu F involved in the study design and endeavored to obtain financial support.
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Abstract

AIM: To test whether high-frequency electrical stimulation (HES) of the bilateral cervical vagus nerves reduces the airway responses to methacholine (MCh).

METHODS: Guinea pigs were pretreated with saline (Sal, n = 9) or ovalbumin (Ova, n = 10) aerosol for two weeks (5 min/d, 5 d/wk) and subsequently anesthetized, paralyzed, tracheotomized and artificially ventilated. Both total lung resistance (R_L) and dynamic pulmonary compliance (C_{dyn}) were recorded. In addition, the effects of vagal low-frequency electrical stimulation (LES, monophasic, 50 Hz) and HES (monophasic and biphasic, 1 and 2.5 kHz) for about 10 s or 2 min on the responses of R_L and C_{dyn} to MCh aerosol-induced bronchoconstriction were compared in both groups of guinea pigs. In a few guinea pigs, the impact of bivagotomy on the R_L responses to MCh was assessed.

RESULTS: Before MCh challenge, LES, but not HES, significantly increased R_L by about 30% (P < 0.01) and decreased C_{dyn} by about 20% (P < 0.01) similarly in both groups. MCh aerosol for 2 min elevated R_L and diminished C_{dyn} more in Ova- than Sal-treated animals (R_L: 313% ± 52% *vs* 113% ± 17%, P < 0.01; C_{dyn}: -56% ± 7% *vs* -21% ± 3%, P < 0.01). During MCh-induced airway constriction, LES further enhanced, but HES decreased R_L and this decrease was greater in Ova- (about 45%) than Sal-treated animals (about 34%, P < 0.01) with little change in cardiovascular activity. On the other hand, LES further reduced whereas HES increased C_{dyn} more in Ova- (about 20%) than Sal-treated animals (about 13%, P < 0.01). In addition, bivagotomy almost eliminated the R_L and C_{dyn} responses to MCh.

CONCLUSION: We conclude that vagal HES is able to alleviate the bronchoconstriction induced by MCh in anesthetized guinea pigs, likely *via* reversible inhibition/blockade of vagal conduction.

Key words: Bronchodilation; Acetylcholine; Airway hyperreactivity; Ovalbumin; Asthma

Core tip: In summary, our study revealed that vagal high-frequency electrical stimulation (HES) significantly suppressed the airway response to methacholine (MCh) more greatly in the ovalbumin- than saline-treated animals, while vagal low-frequency electrical stimulation always increased airway resistance. Importantly, the HES-evoked bronchodilation during MCh challenge is concomitant with the on-and-off electrical stimulation and with no effect on cardiovascular activity. These, along with the greatly blunted airway resistance response to MCh after bivagotomy, suggest that vagal HES may be a potentially useful approach in alleviating asthmatic bronchoconstriction (likely *via* reversible inhibition or blockade of the vagal nerve conduction).

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INTRODUCTION

Airway hyperreactivity results predominantly from an increased acetylcholine release from parasympathetic nerves innervating the airways, leading to local smooth muscle contraction in asthmatic patients[1] and in animal models of asthma[2,3]. It was assumed that cholinergic agonists, such as methacholine (MCh), produced bronchoconstriction only *via* their direct effect on airway smooth muscle. However, this opinion has been challenged by several recent studies. Vagal activation by conventional electrical stimulation at low frequency (< 20 Hz) augments the airway responsiveness to MCh in rats[4]. Conversely, bivagotomy or vagal blockade by cooling profoundly decreases and even eliminates bronchoconstriction induced by application of cholinergic agonists into the respiratory tract in mice, rats, sheep, and dogs[5-8]. These results strongly demonstrate the critical role basic vagal tone per se plays in generating the airway smooth muscle responses to acetylcholine.

Accumulating evidence has shown a blocking effect of electrical stimulation at high frequencies [high-frequency electrical stimulation (HES) ⩾ 1 kHz] on mammal peripheral nerve fibers' conduction, thereby diminishing or abolishing the nerve's functions[9-11]. For example, HES applied to the sciatic nerve of anesthetized cats substantially decreases the alpha motor neural discharges as shown by recording of the antidromic potentials of single fibers of L7 ventral root[11]. Therefore, we hypothesized that vagal HES (similar to bivagotomy) would reduce the airway responses to MCh challenge in guinea pigs, especially those with ovalbumin (Ova)-sensitization as an established animal model of asthma[12,13].

MATERIALS AND METHODS

Animals

Pathogen-free Hartley Duncan guinea pigs were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and housed in the animal facility in filter top cages with ad libitum water and food access. The room was constantly ventilated and the temperature was kept at 23 ℃. The animals were quarantined for 2 wk before the experiments. The experimental protocols were approved by the Institutional Animal Care and Use Committee at Lovelace Respiratory Research Institute, Albuquerque, NM, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, United States.

Ova-exposure

Nineteen male guinea pigs were used and divided into two groups with matched-litter and body weight (initial weight about 250 g). Each animal was placed in a whole-body plethysmograph (SN 117829; Buxco Electronic Inc., Sharon, CT) for 1 h per day for 3 d. Following the habituation, the animals as previously reported[14,15] were either exposed to aerosolized Ova (n = 10) daily for 5 min, 5 d per week, for 2 wk (Ova-treatment) or to saline (Sal) aerosol (n = 9) in an identical manner to serve as a control. During the exposure, the individual guinea pig was placed in the plethysmograph under a negative-pressure exhaust hood. The plethysmograph was connected to an Aeroneb Pro nebulizer (Mountain View, CA) by which Ova (1% wt/vol) or its vehicle solution was introduced at 0.7 mL/min with a mass median aerodynamic diameter of 3.7 μm similar to those reported previously[14,15]. Diphenhydramine (8 mg) was injected intraperitoneally (*ip*) in both groups 1 h before each exposure in the second week to alleviate the bronchospasm caused by release of histamine during Ova-exposure[15]. Before the first and the last aerosol exposure, the animal's body weight was measured.

General animal preparation

One day after the last exposure with Ova or Sal, anesthesia was induced using a mixture of ketamine + xylazine (40 + 5 mg/kg; *im*). Supplementary anesthetics (4.0 + 0.5 mg/kg; *im*) were provided as needed to suppress corneal and withdrawal reflexes. The trachea was cannulated below the larynx and connected to a pneumotachograph (Frank's Mfg. Co., Albuquerque, NM) to measure the respiratory flow via a differential pressure transducer (ML141, AD Instruments, Castle Hill, Australia). The pneumotachograph had a linear flow-pressure relationship in the range of 0-20 mL/s with a flow resistance of 0.046 cmH_2O s/mL and a dead space of about 0.2 mL. The animals were exposed to a gas mixture of 30% oxygen in nitrogen throughout the experiment. The right femoral artery was cannulated for monitoring and recording arterial blood pressure (ABP) and heart rate (HR) through a blood pressure transducer (MLT0380, ADInstruments). The right femoral vein was cannulated for administration of agents. The cervical vagi were carefully isolated and placed on bipolar stimulating electrodes respectively. The guinea pigs were then paralyzed with pancuronium (0.1-0.3 mg/kg for induction and 0.1 mg/kg per hour for maintenance, *iv*) and ventilated at a constant frequency (f_R) of 70 breaths/min with tidal volume (V_T) adjusted to keep end tidal pressure of CO_2 ($P_{ET}CO_2$) at about 40 torr[4,16]. Sudden spontaneous increases in arterial blood pressure (> 10 mmHg) and/or any irregular rhythm or increase in heart rate (> 15%) were indicators for supplementary anesthesia after paralysis.

Measurement of airway resistance

As we reported previously[17], total lung resistance (R_L) and pulmonary compliance (C_{dyn}) were measured and recorded on a breath-by-breath basis. Briefly, a catheter was inserted into the intrapleural cavity between the 5th and 6th ribs and the incision subsequently sutured and sealed

Table 1 Baseline parameters in the Sal- and Ova-treated guinea pigs

Animals	R_L (cmH_2O s/mL)	C_{dyn} (mL/cmH_2O)	MABP (mmHg)	HR (bpm)
Sal (n = 9)	0.10 ± 0.02	0.35 ± 0.02	51.6 ± 1.6	327 ± 13
Ova (n = 8)	0.12 ± 0.02	0.34 ± 0.03	55.2 ± 3.7	311 ± 9

No significant differences of total lung resistance (R_L), dynamic pulmonary compliance (C_{dyn}), mean arterial blood pressure (MABP), or heart rate (HR) were found between the two groups.

airtight with silicone jelly in order to measure intrapleural pressure. The pneumothorax was corrected by briefly opening the intrapleural catheter to ambient air while holding hyperinflation (3 × V_T). Transpulmonary pressure was measured as the difference between the tracheal and intrapleural pressure.

Experimental procedures

In our pilot studies, the conventional stimulations (5, 10, 25, 50 and 100 Hz; 0.5 V; 0.2 ms; monophonic and biphasic square-waveform) were delivered from a biphasic stimulus isolator (BSI-950, Dagan Corporation Minneapolis, MN) and applied to stimulate the vagi. We found that among these stimulations, a stimulation at 50 Hz with monophonic square-waveform resulted in the reproducible and greatest R_L response, which was applied as the low-frequency stimulation (LES). In addition, HES at 1 or 2.5 kHz with monophasic waveform (HESm1 and HESm2) or with biphasic waveform (HESb1 and HESb2) were also applied. Both types of HES are reportedly able to block nerve conduction in previous studies[11,18-21]. These stimulations were randomly applied with the ratio of LES *vs* HES equal to 1:3 in each MCh exposure, and each stimulus lasted for a 10-s with an approximately 30 s interval or occasionally for 2-min period (only HES). Thirty minutes after completion of these electrical stimulations, the animals were subsequently exposed to MCh aerosol (100 μg/mL per minute for 2 min[4]) delivered by the nebulizer into the intake port of the ventilator by using an airflow 20 mL/s with a nebulizer output of 0.7 mL/min. The same patterns of electrical stimulations applied before MCh exposure were repeated with the first electrical stimulation applied at about 30 s of the plateau R_L response to MCh. After recovery from the MCh exposure, the animal received the same MCh exposure and electrical stimulations again once or twice to ensure the reproducibility of the R_L responses. The above procedures were performed in 9 Sal- and 8 Ova-treated animals. In the remaining two other Ova-treated guinea pigs, the R_L response to the same MCh challenge was measured before and after bivagotomy to determine whether bivagotomy would cause a similar inhibition of the R_L response to MCh.

Statistical analysis

Raw data of the airflow, tracheal and intrapleural pressures, $P_{ET}CO_2$, arterial blood pressure, and rectal temperature were digitized, monitored, and recorded by using a PowerLab/8sp (model ML 785; ADInstruments Inc., Colorado Springs, CO) connected to a computer employing the PowerLab Chart 5 software. Derived parameters, including the R_L, C_{dyn}, V_E, V_T, f_R, MABP, and HR, were calculated online using the functions of the software. After stabilization of these variables, the R_L baseline was determined by averaging R_L values for 30 s immediately before vagal electrical stimulation or MCh exposure. The electrical stimulation-evoked R_L responses before and after MCh aerosol were expressed as delta percent (Δ%) change from the baseline R_L values. The R_L responses to the 1st, 2nd, and 3rd MCh exposure were compared to determine the reproducibility. In our study, multiple trials of a given electrical stimulation in the individual animal were averaged to calculate our group data unless mentioned otherwise. All data were presented as means ± SE. Student *t*-test was applied to detect the significant differences in baseline values between the two groups (Ova- and Sal-treated). Two-way ANOVA was used to differentiate all responses evoked by the stimulations (chemical or electrical) between the two groups or among the R_L responses to different trials of MCh exposure. When an overall test was found to be significant, Fisher's post-hoc tests were followed for specific comparisons between individual groups. Differences were considered significant at a P value < 0.05.

RESULTS

Airway responses to vagal electrical stimulations before MCh challenge

After completion of the Sal- or Ova-pretreatment, animal body weights were not significantly different between the two groups (358 ± 12 g *vs* 363 ± 15 g). Following anesthesia and paralysis, animals' baseline airway and cardiovascular activities and their responses to the electrical stimulations were compared. As listed in Table 1, all of these baseline values were not significantly different between the two groups, which are consistent with the previously reported data from guinea pigs[5,14]. LES immediately increased the R_L by about 30% and decreased the C_{dyn} by approximately 18%; this is similarly associated with bradycardia in the Sal- and Ova-treated guinea pigs. The evoked responses disappeared rapidly after withdrawing the electrical stimulation. In sharp contrast, all HESs (HESm1, HESm2, HESb1, and HESb2) failed to significantly alter R_L, C_{dyn}, MABP, and HR. The typical experimental recordings and the corresponding group data are illustrated in Figures 1 and 2, respectively.

Airway responses to aerosol MCh

In both groups of guinea pigs, exposure to MCh aerosol for 2 min evoked significant bronchoconstrictive response as evidenced by an increase in R_L and decrease in C_{dyn} (Figure 3). The R_L response reached a plateau about 2 min after applying MCh, and the plateau lasted for 2.8 ± 0.2 min followed by a relatively rapid recov-

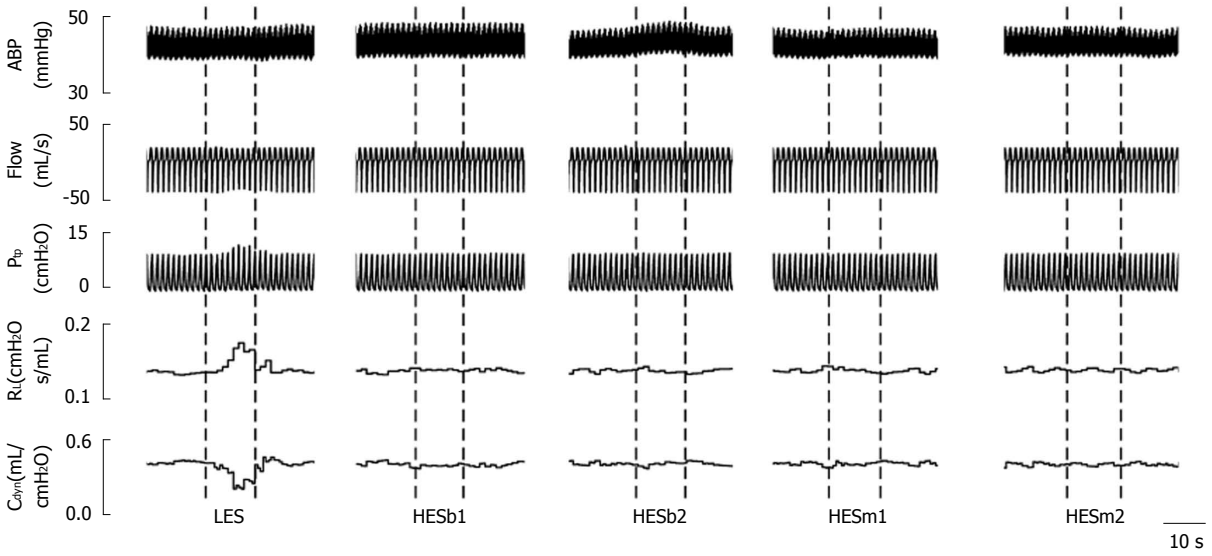

Figure 1 Typical total lung resistance and dynamic pulmonary compliance responses to electrical stimulations in an Ova-sensitized guinea pig. The traces from the top to bottom are arterial blood pressure (ABP), airflow (Flow), Trans-pulmonary pressure (P_{tp}), total lung resistance (R_L) and dynamic pulmonary compliance (C_{dyn}). The dashed lines in each column reflect the on- and off-set of a given electrical stimulation. Vagal electrical stimulation included low-frequency electrical stimulation (LES, monophasic stimulation at 50 Hz); HESb1 and HESb2 (biphasic waveform at 1 and 2.5 kHz); and HESm1 and HESm2 (monophasic waveform at 1 kHz and 2.5 kHz), which are the same for the following figures.

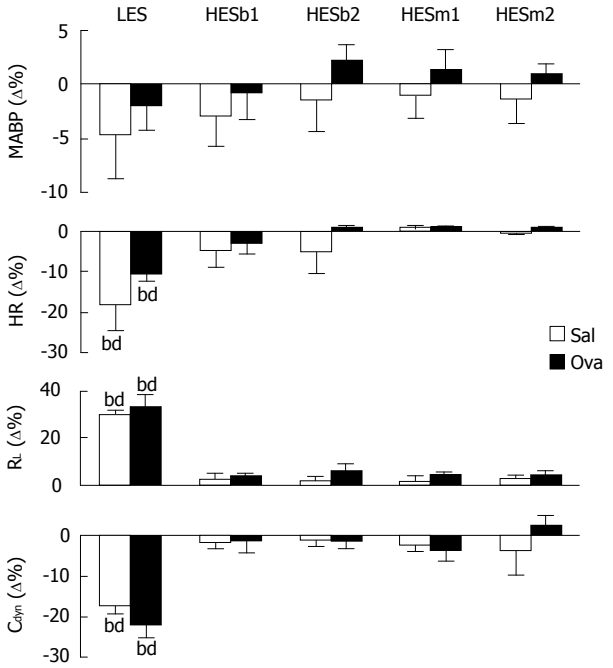

Figure 2 Group data showing the responses to vagal electrical stimulations. MABP: Mean arterial blood pressure; HR: Heart rate; R_L: Total lung resistance; C_{dyn}: Dynamic pulmonary compliance. n = 9 and 8 in the Sal- and Ova-pretreated guinea pigs; Mean ± SE. $^bP < 0.01$, vs the data immediately before electrical stimulation presented as "0"; $^dP < 0.01$, low-frequency electrical stimulation (LES) *vs* high-frequency electrical stimulations (HESs).

ery to their pre-exposure baselines. The amplitudes and durations of evoked R_L responses to the 1st, 2nd, and 3rd MCh exposure in Sal-treated and Ova-treated animals are listed in Table 2. The lack of significant difference among the R_L amplitude and duration in response to the three MCh exposures strongly indicates that these evoked

Table 2 The reproducibility of total lung resistance responses to methacholine exposure controls

Group	Amplitude (%)			Duration (min)		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Sal	127 ± 25	136 ± 28	128 ± 30	7.9 ± 0.4	8.0 ± 0.5	7.5 ± 0.5
Ova	355 ± 58	370 ± 68	351 ± 63	8.2 ± 0.5	8.4 ± 0.5	7.7 ± 0.6

n = 9 for Sal- and Ova-treated guinea pigs for trial 1 and 2, and n = 5 for trial 3 for both groups; mean ± SE; no significant difference was found among the R_L amplitude and duration in response to the three methacholine exposures in both groups of animals. Sal: Saline; Ova: Ovalbumin.

responses are reproducible. The MCh exposure caused a remarkable hypotension (phase Ⅰ) and a hypertension subsequently (phase Ⅱ) that gradually returned to its pre-exposure baseline level. In parallel to phase Ⅱ, there was a tachycardia evoked by MCh aerosol without HR change during phase Ⅰ. The group data (Figure 4) showed that the MCh-induced R_L increase and C_{dyn} decrease were significantly greater in the Ova- than Sal-treated animals (313% *vs* 113% and -57% *vs* -20% respectively; $P < 0.01$). The changes of MABP and HR, however, were not significantly different between the two animal groups. These data are consistent with previously reported airway and cardiovascular responses to MCh[22-25].

Airway responses to vagal electrical stimulations after exposure to aerosolized MCh

When the R_L response reached the plateau after MCh inhalation, the different electrical stimulations were randomly applied. Typical experimental recordings are exhibited in Figure 3, in which LES still enhanced R_L but HESs

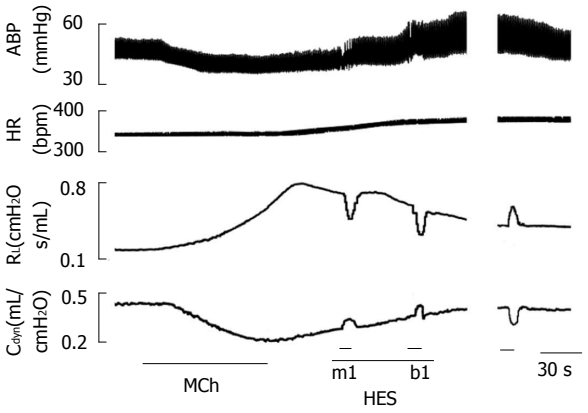

Figure 3 Typical examples exhibiting cardiovascular and airway responses to methacholine aerosol (100 μg/mL per minute for 2 min) and to vagal electrical stimulation in an anesthetized and paralyzed Ova-pretreated guinea pig. The traces from the top to bottom are arterial blood pressure (ABP), heart rate (HR), total lung resistance (R_L) and dynamic pulmonary compliance (C_{dyn}). The duration of the break is about 1 min. MCh: Methacholine. HES: High-frequency electrical stimulation.

suppressed the R_L response to MCh challenge. Statistically, the amplitude of the suppression was greater in the Ova- (49%) than the Sal-treated animals (35%). Similar results were also observed in C_{dyn}. The breakdown of the responses to individual HESs is detailed in Figure 5. The inhibitory effects on the R_L responses to MCh aerosol were not markedly different between the stimulations at 1 and 2.5 kHz and between the stimulations with different waveforms. In addition, the HES-induced alteration in R_L was not associated with statistical changes in ABP and HR, although ABP fluctuated a bit (Figures 3 and 5). In this study, 4 electrical stimulations were applied during each R_L plateau response to MCh aerosol. The bronchodilation responses (R_L) evoked by the first and last HES (usually with about 1.5 min apart) were not significantly different in amplitude (in Sal-treated GPs: -37% ± 5% *vs* -33% ± 6% and in Ova-treated GPs: -51% ± 6% *vs* -48% ± 7%; $P > 0.05$). In addition, no remarkable fatigue of the bronchodilation in response HES was observed during continuously stimulating vagi for 2 min (Figure 6). These data support the notion that detectable fatigue and/or nerve damage is minimal in the experimental timeframe of this study.

Airway responses to prolonged electrical stimulations after MCh challenge

We also tested whether prolongation of the vagal stimulation duration would produce similar bronchodilation after MCh exposure and whether the vagal nerve remained viable. As exhibited in Figure 6, the prolonged 2-min HES stimulation still produced a bronchodilation with a limited effect on both MABP and HR. Moreover, this response was reversible and repeatable.

Airway responses to MCh aerosol before and after bivagotomy

The objective of this study was to clarify whether, similar to vagal HES, bivagotomy would substantially diminish the airway responses to MCh aerosol in our experimental preparation. We found that as compared to the responses measured with intact vagi, bivagotomy did not significantly change the baseline R_L and C_{dyn} or the BP and HR in the Sal- and Ova-treated animals, which is similar to the previous reports in anesthetized rats[5,26]. Most imporatantly, the R_L and C_{dyn} responses to MCh aerosol were dramatically reduced by the bivagotomy (55% ± 11%) and the MCh-induced hypotension tended to be smaller (Figure 7).

DISCUSSION

Multiple mechanisms (parasympathetic nerve-dependent or -independent) are involved in generating airway hyperreactivity. In the present study, we addressed an essential issue as to whether HES reversibly impairs vagal conduction *via* blocking acetylcholine release from parasympathetic fibers, and thereby alleviates the MCh-produced bronchoconstriction. We found that, differing from an excitatory effect of LES, vagal HES greatly inhibited the airway constrictive responses to inhalation of MCh aerosol in anesthetized guinea pigs, especially in those sensitized by Ova. Actually, vagal LES-induced bronchoconstriction[22-24] and reduce C_{dyn}[27] have been observed in rats, rabbits, and guinea pigs. This excitatory response is achievable mainly *via* promoting release of acetylcholine from parasympathetic fibers innervating airway smooth muscle. Although vagal LES provoked a relatively greater R_L increase and lower C_{dyn} in Ova- than Sal-treated guinea pigs, these differences were not significant. There are four unique features of our HES-induced bronchodilation. (1) vagal HES during the bronchoconstrictive response to MCh challenge evokes a rapid bronchodilation that is consistent with the rapid tracheal relaxation by electrical stimulation *in vitro*[28]; (2) the electrical stimulation-evoked bronchodilation was reversible and reproducible, implying a minimal damage of vagal HES on the nerve in our study. This finding is consistent with the reported effects of HES characterized by its repeated and reversible blockade of nerve conduction regardless of the electrical parameters[20,29]; (3) the vagal HES-evoked bronchodilation was not associated with remarkable changes in HR and ABP; and (4) the HES-evoked bronchodilation after MCh challenge was significantly greater in Ova- than Sal-treated animals. Collectively, our data clearly suggest that vagal HES could substantially inhibit the bronchoconstrictive responses to cholinergic agonists. Asthma is a chronic disease; however, severe asthmatic attack is acute. In our study, the airway responses to MCh mimic the asthmatic attack, while HES-induced bronchodilation during the acute bronchoconstriction may provide a clue of alleviating the asthmatic attack.

A large body of evidence has shown multiple neural pathways responsible for regulating the airway smooth muscle tone[30]. Of them, two neural pathways are excitatory. Acetylcholine released from parasympathetic postganglionic nerves innervating airway smooth muscles produces airway contraction *via* acting on muscarinic 3

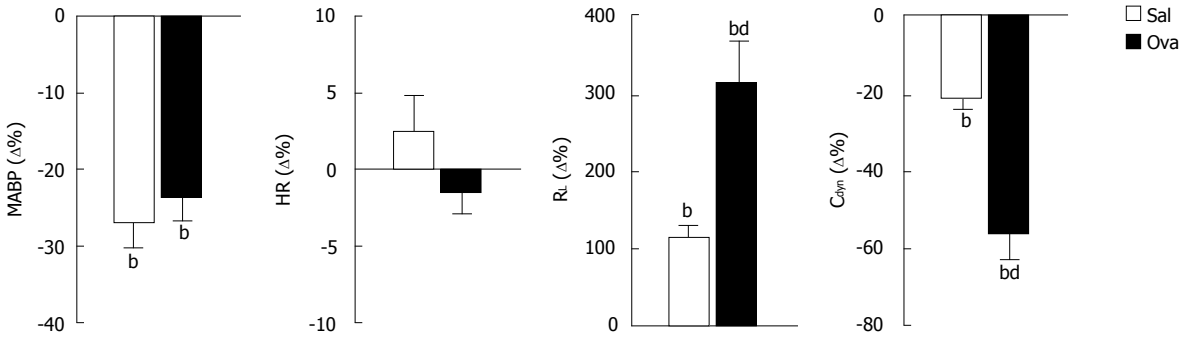

Figure 4 Peak airway responses to methacholine aerosol (100 μg/mL per minute for 2 min) and the associated cardiovascular changes. *n* = 9 and 8 in Sal- and Ova-treated guinea pigs respectively; mean ± SE. $^{b}P < 0.01$, *vs* the data of pre-methacholine (MCh) challenge presented as "0"; $^{d}P < 0.01$ between Sal- and Ova-treated groups. MABP: Mean arterial blood pressure; HR: Heart rate; R_L: Total lung resistance; C_{dyn}: Dynamic pulmonary compliance.

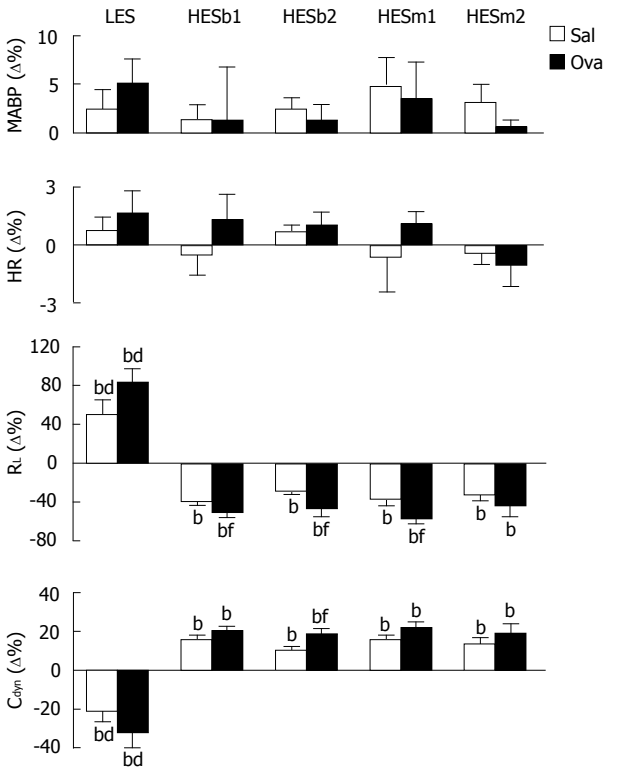

Figure 5 Airway and cardiovascular responses to the vagal electrical stimulations after exposure to methacholine aerosal (100 μg/mL per minute for 2 min). *n* = 8 and 9 in Sal- and Ova-treated guinea pigs; Mean ± SE. $^{b}P < 0.01$, compared to the data before methacholine (MCh) challenge; $^{d}P < 0.01$ LES *vs* HESs; $^{f}P < 0.01$ between Sal- and Ova-treated groups. MABP: Mean arterial blood pressure; HR: Heart rate; R_L: Total lung resistance; C_{dyn}: Dynamic pulmonary compliance.

cholinoceptors of smooth muscle. In addition, activation of vagal pulmonary afferent endings releases tachykinins, such as substance P (SP) and neurokinin A (NKA), causes smooth muscle contraction. The two others are inhibitory. Parasympathetic postganglionic fibers can release inhibitory nonadrenergic, noncholinergic (iNANC) neurotransmitters, such as nitric oxide (NO) and vasoactive intestinal peptide (VIP); and β_2 adrenoceptors mediate the muscle relaxation in response to circulating adrenaline. We assume that vagal HES leads to bronchodilation during MCh exposure *via* inhibiting or blocking vagal conduction to attenuate release of acetylcholine from parasympathetic fibers innervating airway smooth muscle for several reasons. Vagal HES-induced bronchodilation during MCh is similar to the blunted airway response to MCh after bivagotomy that was previously reported[5-8] and confirmed in this study. The similarity of vagal HES and bivagotomy was further confirmed by the evidence that vagal HES (Figures 1 and 2) and bivagotomy[5,6] failed to alter baseline R_L and C_{dyn}. These data suggest that R_L and C_{dyn} at rest state are largely determined by the mechanics of the airway and lung tissue independent of the basal cholinergic tone. In agreement with our data, the ability of HES to block nerve conduction was first found by Reboul[18] and subsequently confirmed by other investigators[11,19-21]. Particularly, a square-waveform HES, similar to that used in this study, has been shown to block the sciatic nerve in anesthetized cats[11]. The possibility of the involvement of SP and NKA release from pulmonary afferents in the HES-induced bronchodilation is discussed later. The HES-induced bronchodilation unlikely results from activation of the two inhibitory neural pathways. The iNANC pathway-mediated relaxation was evoked by low stimulating frequency (< 35 Hz) and often required several minutes to research the plateau[31]. Different from these, we used HES that induced a rapid bronchodilation. Electrical stimulation of airway sympathetic nerves could also evoke bronchodilation in the guinea pig[32,33]. Thus, one may ask whether the HES current spread could stimulate the adjacent airway sympathetic nerves, leading to the bronchodilation observed in our study. This opinion is strongly argued by lacking any significant cardiovascular response to vagal HES in this study. Taken together, our results demonstrate that vagal LES causes bronchoconstriction and bradycardia *via* activating the vagus nerve, while vagal HES leads to bronchodilation during MCh aerosol independent of stimulating waveforms, likely through inhibiting or blocking vagal conduction.

There are several concerns and limitations in this study. First, although vagal HES and bivagotomy share a similarity in inhibiting airway contraction in response to MCh aerosol, our data are not conclusive in proving vagal blockade by the HES. Technically, recording vagal afferent fiber signals is doable, but distinguishing these signals

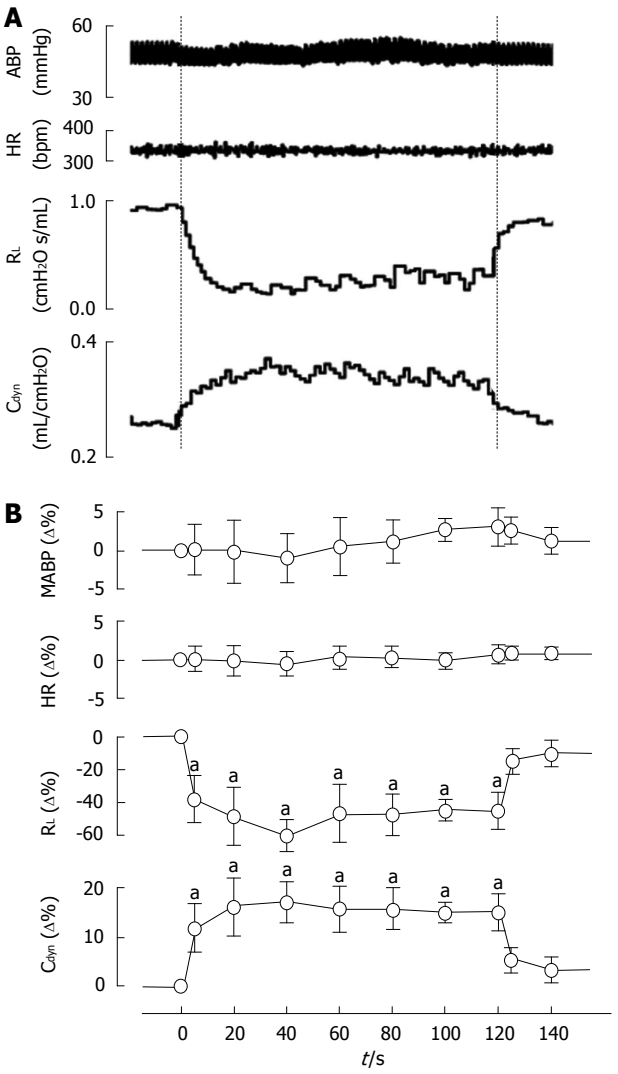

Figure 6 Cardiovascular and airway responses to 2 min vagal high-frequency electrical stimulation after exposure to methacholine aerosal (100 μg/mL per minute for 2 min). A: HESm1-evoked responses in an Ova-treated guinea pig about 2 min after initiation of methacholine (MCh) challenge, in which the baseline values of total lung resistance (R_L) and dynamic pulmonary compliance (C_{dyn}) were 0.1 cmH$_2$O s/mL and 0.4 mL/cmH$_2$O respectively; B: The averaged data of MABP, HR, R_L and C_{dyn} from three guinea pigs (five trials) are illustrated in panel. In both panels, the traces are arterial blood pressure (ABP/MABP), heart rate (HR), R_L, and C_{dyn}. n = 5 trials (3 guinea pigs); Mean ± SE; $^aP < 0.05$, high-frequency electrical stimulation (HES)-evoked responses *vs* the data before electrical stimulation presented as "0".

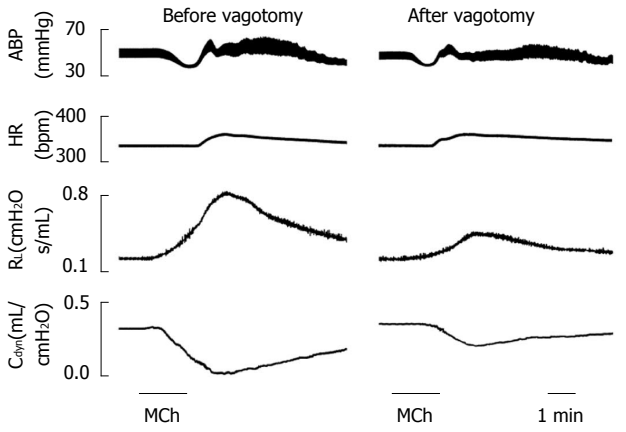

Figure 7 Comparison of the cardiovascular and airway responses to methacholine aerosol (100 μg/mL per minute for 2 min) before and after bivagotomy in an anesthetized and paralyzed Ova-treated guinea pig. The traces are arterial blood pressure (ABP), heart rate (HR), total lung resistance (R_L), and dynamic pulmonary compliance (C_{dyn}).

from the artificial signals of vagal HES is very difficult. Second, the cervical vagal trunk consists of both afferent (myelinated and unmyelinated) and efferent (myelinated) fibers[34]. Our data are unable to delineate which type(s) of vagal fibers are responsible for the HES-induced change. Previous results in cats have shown that A-fibers of the slow adapting receptors are not critical to the cholinergic-mediated airway responses because lung inflation has limited effects on the airway response to MCh administration[35]. On the other hand, bronchopulmonary C-fibers may be involved in modulating the airway response to cholinergic activaton *via* the local axonal- and/or the central-mediated reflexes. Nicotinic cholinergic receptors exist in bronchopulmonary C-fibers[36]. Degeneration of these fibers significantly diminishes the airway responsiveness to MCh aerosol in both rats and guinea pigs[37,38], while stimulation of these fibers causes airway constriction[34,39]. Thus, it is possible that vagal HES can induce bronchodilation during MCh challenge, presumably in part *via* inhibiting the conduction of bronchopulmonary C-fibers. Third, though our data failed to show a detectable fatigue and/or nerve damage induced by our HES applied within 2 min, it remains unknown whether HES applied in a prolonged period (> 2 min) would produce such pathophysiological changes. Fourth, our data cannot deny the plausible that pre-exposure to the conventional treatments clinically used may interfere with the effectiveness of HES denoted in the present study. Fifth, our study was performed in anesthetized preparation. Therefore, further experiments in conscious asthmatic animals are needed to rule out the possible interference of anesthetics in the vagal HES-induced bronchodilation.

COMMENTS

Background

High-frequency electrical stimulation (HES) of peripheral nerve is reportedly able to inhibit or block the nerve conduction, and thereby to inactivate the nerve-mediated responses. Airway hyperreactivity results predominantly from an increased acetylcholine release from vagal nerves innervating the airways in asthmatic patients and in animal models of asthma. Authors tested whether HES of vagal nerves could inhibit acetylcholine-induced airway hyperreactivity.

Research frontiers

The results showed that HES of vagus nerves greatly attenuated airway hyperreactivity in response to acetylcholine, suggesting an inhibitory effect of HES on vagus nerves.

Innovations and breakthroughs

The major innovation of authors' data is to ensure an inhibitory effect of HES on vagus nerves that leads to an attenuation of acetylcholine-induced airway hyperreactivity.

Applications

An inhibitory effect of HES on vagus nerves, as presented in this study, may provide a therapeutical approach to alleviate airway hyperreactivity in asthmatic patients.

Terminology

HES: low-frequency electrical stimulation; methacholine; ovalbumin; total lung resistance and dynamic pulmonary compliance.

Peer review

This is a very good study in which the authors demonstrated that HES of vagus nerves in guinea pigs attenuated the acetylcholine-induced airway hyperreactivity. The results are interesting and potentially translational from data to preclinical research.

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ORIGINAL ARTICLE

Effects of methyl palmitate and lutein on LPS-induced acute lung injury in rats

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Abstract

AIM: To investigate the effects of methyl palmitate and lutein on lipopolysaccharide (LPS)-induced acute lung injury (ALI) in rats and explore the possible mechanisms.

METHODS: Male Sprague-Dawley rats were divided into 4 groups: (1) control; (2) LPS; (3) Methyl palmitate; and (4) Lutein groups. Methyl palmitate (300 mg/kg, *ip*) was administered 3 times per week on alternating days while lutein (100 mg/kg, oral) was given once daily. After 1 wk of vehicle/methyl palmitate/lutein treatment, ALI was induced by a single dose of LPS (7.5 mg/kg, *iv*). After 24 h of LPS injection, animals were sacrificed then biochemical parameters and histopathology were assessed.

RESULTS: Treatment with methyl palmitate attenuated ALI, as it significantly decreased the lung wet/dry weight (W/D) ratio, the accumulation of the inflammatory cells in the bronchoalveolar lavage fluid (BALF) and histopathological damage. However, methyl palmitate failed to decrease lactate dehydrogenase (LDH) activity in BALF. On the other hand, lutein treatment produced significant anti-inflammatory effects as revealed by significant decrease in accumulation of inflammatory cells in lung, LDH level in BALF and histopathological damage. Methyl palmitate and lutein significantly increased superoxide dismutase (SOD) and reduced glutathione (GSH) activities with significant decrease in the lung malondialdehyde (MDA) content. Importantly, methyl palmitate and lutein decreased the level of the inflammatory cytokine tumor necrosis factor-α (TNF-α) in the lung. Lutein also reduced LPS-mediated overproduction of pulmonary nitrite/nitrate (NO_2^-/NO_3^-), which was not affected by methyl palmitate pretreatment.

CONCLUSION: These results demonstrate the potent protective effects of both methyl palmitate and lutein against LPS-induced ALI in rats. These effects can be attributed to potent antioxidant activities of these agents, which suppress inflammatory cell infiltration and regulated cytokine effects.

Key words: Acute lung injury; Lipopolysaccharide; Methyl palmitate; Lutein; Rats

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INTRODUCTION

Acute lung injury (ALI) is an acute inflammatory process in the air spaces and lung parenchyma with increased vascular permeability leading to impairment of respiratory

function. It is caused by an imbalance of pro-inflammatory and anti-inflammatory mediators. Pro-inflammatory cytokines, such as interleukin (IL)-8, IL-1, and tumor necrosis factor-alpha (TNF-α) cause neutrophils to adhere to pulmonary capillaries, extravasate into the alveolar space and undergo activation[1-3]. Activated neutrophils propagate inflammation and injury through the release a variety of factors, such as reactive oxygen species (ROS) and proteolytic enzymes, which contribute to local tissue damage, accumulation of edema fluid in the airspaces, and surfactant inactivation, thereby making the alveolar unit unable to expand. These destructive forces released by neutrophils can be counteracted by endogenous anti-proteases, antioxidants, and anti-inflammatory cytokines (*e.g.,* IL-10). The balance between the destructive and protective factors determines the degree of tissue injury and clinical severity of ALI[3,4].

Lipopolysaccharide (LPS) is a main constituent of the cell wall of the gram-negative bacteria[5]. When delivered into animals and humans, LPS displays major features of microvascular lung injury, including leukocyte accumulation in lung tissue, pulmonary edema, profound lung inflammation and mortality. It is a common cause of both direct lung injury (pneumonia) and indirect lung injury (sepsis)[6]. LPS produces acute injury to the endothelial and epithelial barriers in the lungs and acute inflammatory response in the air spaces either by inhalation or systemic (intravenous and intraperitoneal) administration[7]. LPS-induced injury is a very useful experimental *in vivo* model, closely resembling ALI in humans[8].

Methyl palmitate is an endogenous naturally occurring fatty acid methyl ester. Recently, methyl palmitate has been shown to possess potent anti-inflammatory and anti-fibrotic effects through multiple pathways, inhibiting the activation of isolated macrophages of alveolar origin (RAW cells)[9]. It can also attenuate the severity of oxidative stress and inflammatory response[10]. Methyl palmitate decreases secretion of IL-10, TNF-α, NO, and prostaglandin E_2. This effect is thought to occur by the inhibition of nuclear factor kappa-B (NF-κB)[11]. In the liver, methyl palmitate is reported to play an important role in regulating the inflammatory process as it inhibits the secretion of TNF-α and NO by its ability to inhibit Kupffer cells[12,13].

Lutein is a xanthophyll carotenoid which is usually consumed in the diet by humans and has good biovailability. Lutein is found in fruits and vegetables like spinach, broccoli, kale, egg yolk, zucchini, corn, peas and kiwi[14,15]. It has a strong antioxidant activity due to its chemical structure. Lutein not only has conjugated double bonds but also has hydroxyl groups at both ends making it a stronger antioxidant then other carotenoids[16]. It was reported that lutein can reduce the concentrations of NO, TNF-α in the aqueous humor. Lutein also suppressed the expression of inducible nitric oxide synthase (iNOS) and COX-2 in RAW cells[17]. The present study aimed to test the potential protective effects of methyl palmitate and lutein treatments against LPS-induced ALI and to investigate their possible mechanism(s).

MATERIALS AND METHODS

Drugs and chemicals

LPS (Escherichia coli serotype O111:B4), was purchased from Sigma-Aldrich, St. Louis, MO. LPS was dissolved in normal saline and prepared fresh on the day of the experiment. Methyl palmitate (Sigma-Aldrich, St. Louis, MO) was kindly provided by Dr. El-Demerdash E, and was dissolved in corn oil by vortex. Lutein capsules were manufactured by United States Nutrition Inc. (Bohemia, NY). All other chemicals and bio-chemicals used in this study were of high analytical grade.

Experimental animals

Male Sprauge Dawely rats (150-200 g) were purchased from the "Egyptian Organization for Biological Products and Vaccines", Giza, Egypt. The animals were housed (4/cage) in an air-conditioned room maintained at 25 ± 2 ℃ with regular 12 h light/12 h dark cycle. All procedures involving animals were conducted in accordance with the protocol approved by the committee of animal experimentation of the Faculty of Pharmacy, Mansoura University.

Experimental design

Animal groups: Male Sprague-Dawley rats were randomly divided into 4 groups each containing six animals (n = 6). These groups were (1) control; (2) LPS, (3) Methyl palmitate; and (4) Lutein groups. Methyl palmitate (300 mg/kg, *ip*) was administered three times per week on alternating days[9,18] while lutein (100 mg/kg, oral) was given once daily[17]. Rats of the control and LPS-treated groups received 1% (w/v) CMC orally as vehicle, once daily for one week. After one week of vehicle/methyl palmitate/lutein treatment, ALI was induced by a single dose of LPS (7.5 mg/kg) intravenously[19,20] in LPS, methyl palmitate and lutein groups while rats of the control group were treated with saline. Twenty-four hours after LPS injection, animals were killed under diethyl ether anesthesia. The lungs were lavaged, collected, perfused with ice-cold saline and taken for analysis. The remaining lung was flash frozen in liquid nitrogen and stored at -80 ℃ for further analysis.

Measurement of lung wet/dry weight ratio: The wet upper left lung was excised, blotted dry and weighed to calculate "wet" weight, and then placed in an oven at 80 ℃ for 24 h to obtain the "dry" weight. The lung wet/dry weight (W/D) ratio was calculated to evaluate tissue edema.

Bronchoalveolar lavage fluid: Bronchoalveolar lavage (BAL) was performed by cannulating the trachea and infusing the right lung with sterile 0.9% saline. The volume of saline used for BAL was 6 mL. Bronchoalveolar lavage fluid (BALF) fractions were centrifuged (4000 rpm,

10 min, 4 ℃) using a cooling centrifuge (Damon/IEC Division, Model: CRU-5000, Needham, MA) to collect the cell pellet for the total cell count determination. The supernatants of the BALF were stored at -80 ℃ until required for determination of protein content and lactate dehydrogenase (LDH) activity.

Measurement of lung injury in BALF: (1) Total cell count: The cell pellets obtained after centrifugation of the BALF were resuspended in 100 μL of saline, centrifuged onto slides and stained for 8 min with Wright-Giemsa staining. Differential cell count was determined through quantification of the slides for neutrophils and lymphocytes by counting a total of 200 cells/slide at 40 × magnification; (2) Protein content: The total protein concentration was measured in BALF using a commercial kit (Thermo Scientific, Rockford); and (3) LDH: LDH activity was assessed in BALF using a commercial kit (Human Gesllschaft fur Biochemica und Diagnostica, Germany). In brief, the reaction mixture consisting of NADH (0.8 mmol/L), sodium pyruvate (1.5 mmol/L) and TRIS buffer (50 mmol/L, pH 7.4) was added to the sample. The changes in absorbance at 340 nm were recorded and enzyme activity was calculated and expressed in U/L.

Histopathological examination of lung: The lower left pulmonary lobe was harvested and flushed with phosphate buffered saline then fixed in 10% neutral-buffered formalin for 24 h, embedded in paraffin wax, sectioned (6 μm) and stained with hematoxylin-eosin (HE). The tissues were examined under a microscope in a random order and without knowledge of animal or group. The structural alterations of tissue were assessed based on the degree of cellular proliferation, alveolar wall thickening, and inflammatory lesions. Such changes were graded in terms of severity and distribution. The grading system adopted was as follows and was utilized for each group of animals[21]. For severity of lesions: 0 = nothing/zero, 1 = marginal, 2 = slight, 3 = moderate, 4 = severe, 5 = very severe. For distribution of lesion over the tissue: 0 = absent, 1 = rare/occasional (10% of the lung area), 2 = sparse/limited (10%-25% of the lung area), 3 = moderate (25%-50% of the lung area), 4 = extensive/widespread (50%-75% of the lung area), 5 = very extensive/predominant (over 75% of the lung area).

Measurement of oxidative stress: (1) Measurement of malondialdehyde: malondialdehyde (MDA) concentration was determined as an indicator of lipid peroxidation in the lung tissue. The tissue samples of lung were weighed and homogenized (1:10, w/v) in 0.1 mol/L phosphate buffer (pH 7.4) in an ice bath. The homogenate was centrifuged at 3000 *g* for 20 min at 4 ℃. Subsequently, MDA content in the supernatants was measured according to the method described by Satoh[22] using a commercial kit purchased from Bio-diagnostic Company (Giza, Egypt); (2) Measurement of superoxide dismutase: The superoxide dismutase (SOD) activity was estimated according to the method described by Nishikimi *et al*[23] using a commercial kit purchased from Bio-diagnostic Company (Giza, Egypt); and (3) Measurement of reduced glutathione: The concentration of reduced glutathione (GSH) in the lung homogenate was measured according to the method described by Beutler *et al*[24] using a commercial kit purchased from Bio-diagnostic Company (Giza, Egypt).

Measurement of NO_2^-/NO_3^- concentration: NO_2^-/NO_3^- concentration was determined according to the method described by Green *et al*[25]. Briefly, 6 μL of lung homogenate were mixed with 44 μL of double distilled water, 20 μL 0.31 mol/L phosphate buffer (pH 7.5), 10 μL of 0.86 mmol/L NADPH, 10 μL of 0.11 mmol/L FAD and 10 μL of 1 U/mL nitrate reductase in individual wells of a 96-well plate. Samples, standards and controls were incubated for 1.5 h at room temperature. 200 μL of Griess reagent were added to each well. Absorbance at 540 nm was measured after 10 min of incubation at room temperature. Nitrite concentration (μmol/L) was determined for each sample based on a standard curve.

Assay of TNF-α: The level of TNF-α in the supernatant of the lung homogenate was determined by using a commercial enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (Bender Med. systems Gmbh, Vienna, Austria). TNF-α was determined from a standard curve.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's Kramer multiple comparisons Test. The values are means ± SE. for six rats in each group. P value < 0.05 was considered as significant.

RESULTS

Effect of methyl palmitate and lutein on lung W/D ratio and protein content in LPS-induced ALI rats

As shown in Figure 1A, there was a significant increase (P < 0.05) in lung W/D ratio in the LPS group as compared to the control group. Methyl palmitate pretreatment resulted in a significant decrease (P < 0.001) in lung W/D ratio as compared to the LPS group, while, lutein groups produced no significant change as compared to the LPS group. As shown in Figure 1B, LPS injection caused a significant increase (P < 0.01) in the level of total protein as compared to the control group. There was no significant change in the level of total protein between methyl palmitate and lutein groups and that of the LPS group.

Effect of methyl palmitate and lutein on total and differential cell counts in the BALF of LPS-induced ALI rats

The injection of LPS caused a significant elevation (P < 0.001) in the total and differential cell counts in com-

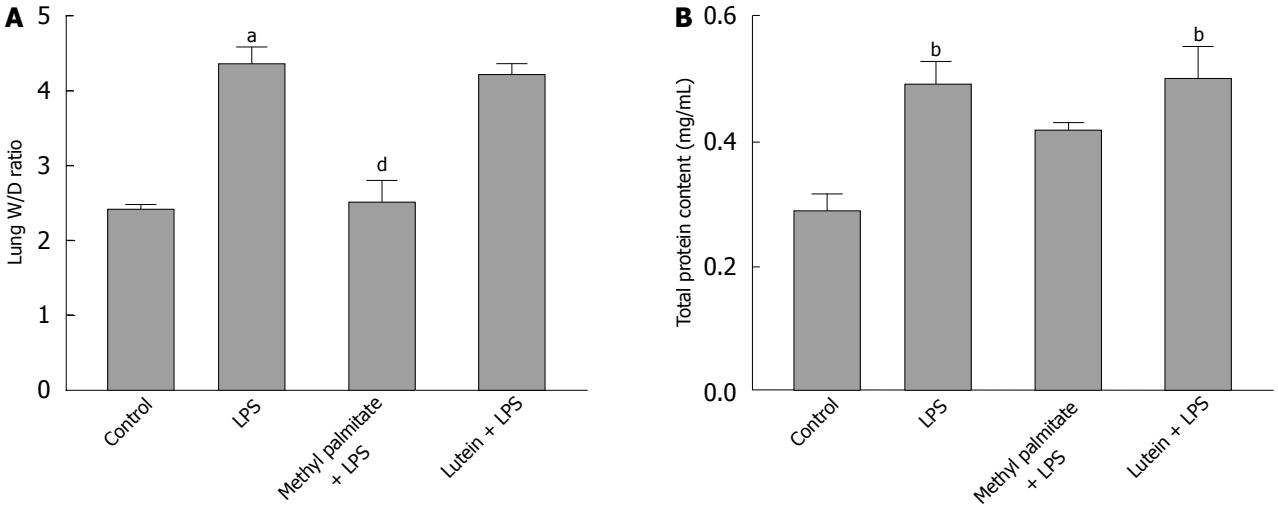

Figure 1 Effect of methyl palmitate and lutein on wet/dry ratio of the lung weight and total protein content in the bronchoalveolar lavage fluid of lipopolysaccharide-induced acute lung injury in rats. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the control group; $^{d}P < 0.01$ *vs* the lipopolysaccharide (LPS) group (one way ANOVA followed by Tukey-Kramer's multiple comparisons test). W/D: Wet/dry.

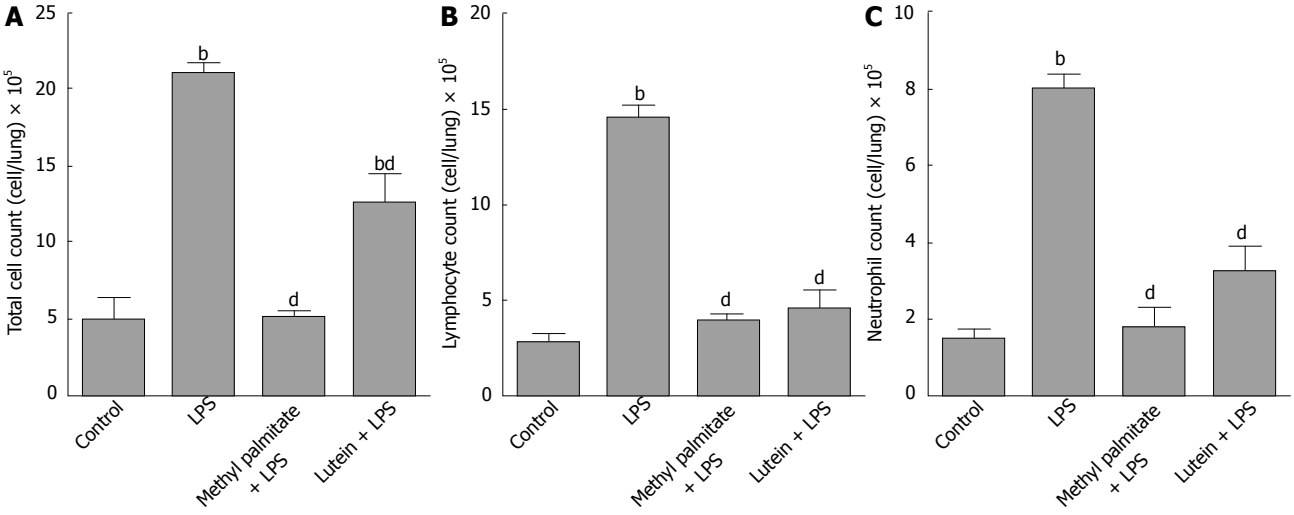

Figure 2 Effect of methyl palmitate and lutein on total and differential cell counts in the bronchoalveolar lavage fluid of lipopolysaccharide-induced acute lung injury in rats. $^{b}P < 0.01$ *vs* the control group; $^{d}P < 0.01$ *vs* the lipopolysaccharide (LPS) group (one way ANOVA followed by Tukey-Kramer's multiple comparisons test).

parison to those of the control group (Figure 2). The administration of methyl palmitate and lutein resulted in a significant decrease in the total ($P < 0.001$, $P < 0.01$ respectively) and differential ($P < 0.001$) cell counts as compared to the LPS group.

Effect of methyl palmitate and lutein on LDH activity in the BALF in LPS-induced ALI rats

LPS injection produced a significant increase ($P < 0.001$) in LDH activity as compared to the control group (Figure 3). The lutein group showed a significant decrease ($P < 0.001$) in LDH activity as compared to the LPS group. The methyl palmitate group did not show any significant decrease in the elevated LDH activity as compared to the LPS group.

Effects of methyl palmitate and lutein on lung histology of rats with LPS-induced ALI

As shown in Figure 4, rats of the control group showed normal histology while the lungs of rats of the LPS group showed severe inflammatory reaction, marked alveolar wall thickness with oedema and haemorrhage, decreased alveolar spaces and extensive (widespread) distribution affecting 50%-75% of the lung area (Figure 4B). As shown in Table 1, LPS injection caused a significant increase ($P < 0.001$) in the severity and the distribution grade of the lesions, as compared to the control group. Methyl palmitate pretreatment resulted in a significant reduction ($P < 0.001$) in the severity and the distribution grade of the lesions as compared to the LPS group. The specimen collected from the methyl palmitate group showed mild inflammatory reaction with thickened alveolar wall edema and hemorrhage, less alveolar space and the distribution of the lesions was sparse (limited), affecting 10%-25% of the lung area (Figure 4C).

The distribution as well as the severity of the lesions were significantly decreased ($P < 0.001$, $P < 0.01$) in the

Table 1 Effect of methyl palmitate and lutein on lung histopathology of the lung in lipopolysaccharide -induced acute lung injury in rats

Groups	Severity of lesion grade						Average severity grade	Distribution of lesion grade						Average distribution grade
	0	1	2	3	4	5		0	1	2	3	4	5	
Control	3	3	0	0	0	0	0.5 ± 0.2	3	3	0	0	0	0	0.500 ± 0.224
LPS	0	0	0	3	3	0	3.5 ± 0.2[b]	0	0	0	3	3	0	3.5 ± 0.2[b,d]
Methyl palmitate + LPS	0	1	5	0	0	0	1.83 ± 0.17[b,d]	0	0	6	0	0	0	2.0 ± 0.0[b,d]
Lutein + LPS	0	0	3	3	0	0	2.5 ± 0.2[b,d]	0	0	5	1	0	0	2.17 ± 0.17[b,d]

Values represent the mean ± SE of 6 rats/group. [b]$P < 0.01$ *vs* the control group; [d]$P < 0.01$ *vs* the lipopolysaccharide (LPS) group (one way ANOVA followed by Tukey-Kramer's multiple comparisons test).

Table 2 Effect of methyl palmitate and lutein on oxidative stress in the lung homogenate in lipopolysaccharide induced cute lung injury in rats

	Parameters		
	MDA (nmol/g tissue)	SOD (units/mg protein)	GSH (μmol/g tissue)
Control	114.036 ± 10.2	25.750 ± 0.629	6.559 ± 0.26
LPS	214.799 ± 9.932[b]	9.667 ± 0.882[b]	3.049 ± 0.309[a]
Methyl palmitate +LPS	164.200 ± 11.707[a,c]	22.108 ± 1.080[d]	7.352 ± 0.738[d]
Lutein + LPS	155.649 ± 6.852[d]	25.333 ± 1.333[d]	6.051 ± 0.315[a,c]

Values represent the mean ± SE of 6 rats/group. [a]$P < 0.05$, [b]$P < 0.01$ *vs* the control group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* the lipopolysaccharide (LPS) group (one way ANOVA followed by Tukey-Kramer's multiple comparisons test). MDA: Malondialdehyde; SOD: Superoxide dismutase; GSH: Glutathione.

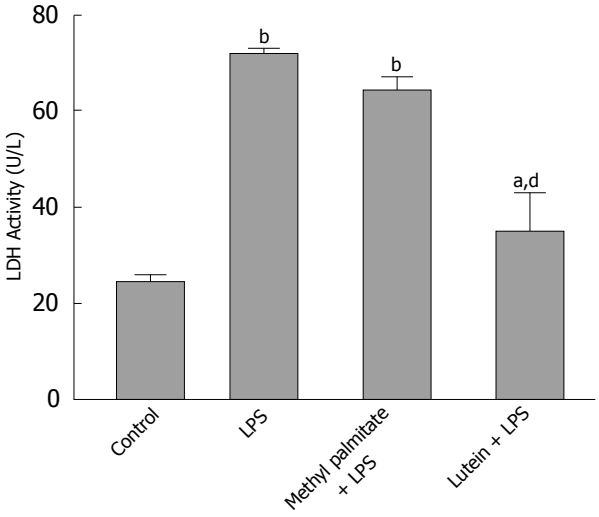

Figure 3 Effect of methyl palmitate and lutein on lactate dehydrogenase activity in the bronchoalveolar lavage fluid of lipopolysaccharide-induced acute lung injury in rats. [a]$P < 0.05$, [b]$P < 0.01$ *vs* the control group; [d]$P < 0.01$ *vs* the lipopolysaccharide (LPS) group (one way ANOVA followed by Tukey-Kramer's multiple comparisons test). LDH: Lactate dehydrogenase.

lutein group as compared to the LPS group. The specimen collected from the lutein group showed moderate inflammatory reaction, moderate alveolar wall thickness, oedema and hemorrhage, decreased alveolar spaces and the distribution of the lesions was moderate, affecting 25%-50% of the lung area (Figure 4D).

Effect of methyl palmitate and lutein on oxidative stress in lung homogenate in LPS-induced ALI rats

LPS injection resulted in a significant increase ($P < 0.001$) in MDA content in lung while GSH and SOD activities were markedly decreased ($P < 0.001$, $P < 0.05$ respectively), as compared to the control group. These changes were all blocked by methyl palmitate or lutein treatment for one week prior to LPS challenge (Table 2).

Effect of methyl palmitate and lutein on total NO_2^-/NO_3^- content in lung homogenate in LPS-induced ALI rats

As demonstrated in Table 3, the level of total NO_2^-/NO_3^- in the LPS group was significantly higher ($P < 0.001$) than that of the control group. Lutein group showed a significant decrease ($P < 0.05$) in total NO_2^-/NO_3^- content, as compared to the LPS group. Methyl palmitate failed to significantly decrease the elevated NO_2^-/NO_3^- level, as compared to the LPS group.

Effect of methyl palmitate and lutein on TNF-α level in lung homogenate in LPS-induced ALI rats

As shown in Figure 5, the level of TNF-α in the LPS group was significantly higher ($P < 0.001$) than that of the control group. Methyl palmitate and lutein groups showed a significant reduction ($P < 0.001$, $P < 0.05$ respectively) in TNF-α level, as compared to the LPS group.

DISCUSSION

The results of the present study indicate that pretreatment with methyl palmitate or lutein exerts potent anti-inflammatory effects against ALI induced by LPS in rats. Therefore, they may represent potential new therapeutic agents against lung inflammation. Previously, LPS was shown to induce the production of several inflammatory cytokines, tissue edema and injury[5,6] starting after 2-4 h and reaching a maximum at 24-48 h[3,26]. Therefore, in this

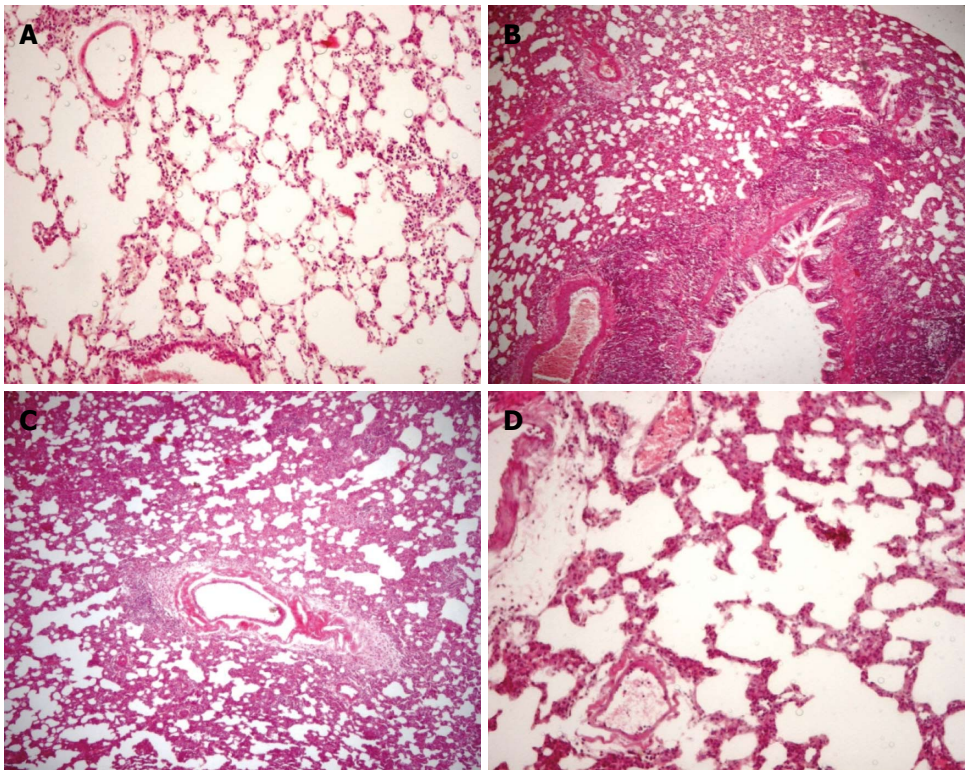

Figure 4 Effect of methyl palmitate and lutein on lung histopathological examination in lipopolysaccharide -induced acute lung injury in rats (HE, × 200). A: Control, no inflammatory infiltrate, edema or fibrosis (score 0); B: Lipopolysaccharide (LPS), severe interstitial inflammation, peribronchial inflammation, fibrosis and edema (score 4); C: Methyl palmitate, mild interstitial inflammation, edema and peribronchial inflammation (score 2); D: Lutein, mild interstitial inflammation, edema and peribronchial inflammation (score 1).

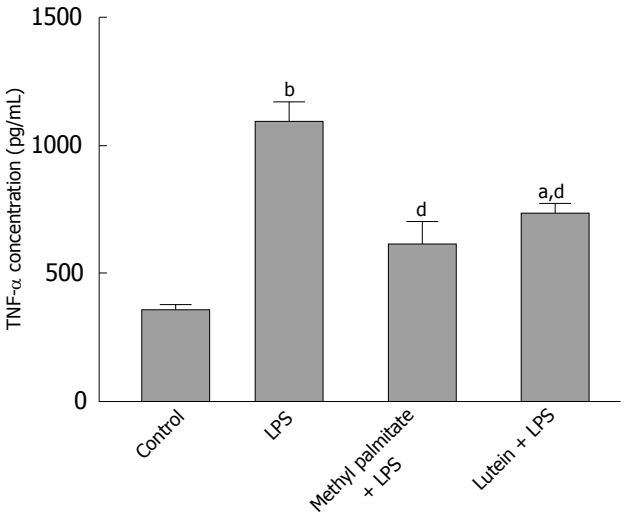

Figure 5 Effect of agmatine, methyl palmitate and lutein on tumor necrosis factor-α level in lung homogenate in lipopolysaccharide -induced acute lung injury in rats. $^{a}P < 0.05$, $^{b}P < 0.01$, *vs* the control group; $^{d}P < 0.01$ *vs* the lipopolysaccharide (LPS) group (one way ANOVA followed by Tukey-Kramer's multiple comparisons test). TNF-α: Tumor necrosis factor-α.

Table 3 Effect of methyl palmitate and lutein on total nitrite/nitrate content in lung homogenate in lipopolysaccharide induced cute lung injury in rats

Treatments	Total nitrite/nitrate (μmol/L)
Control	26.459 ± 0.665
LPS	69.129 ± 0.612[b]
Methyl palmitate + LPS	65.723 ± 2.4[b]
Lutein + LPS	58.018 ± 3.56[b,c]

$^{b}P < 0.01$ *vs* the control group; $^{c}P < 0.05$ *vs* the lipopolysaccharide (LPS) group (one way ANOVA followed by Tukey-Kramer's multiple comparisons test).

study BALF and tissue samples were collected 24 h after LPS exposure.

Initially, both W/D ratio of lung weight and total protein content were measured to estimate the integrity of the lung air/blood barrier and the extent of lung injury. LPS elevated the lung W/D ratio and total protein content in BALF, indicating pulmonary edema and an increase in the leakage of serous fluids into lung tissue, in agreement with previous reports[3,27]. Also, LDH activity was markedly elevated after LPS injection, indicating cell damage or cell death[28], in agreement with Shen *et al*[29] and Kung *et al*[30]. The inflammatory cells, which play a central role in the pathogenesis of ALI, were estimated in the BALF. Activated neutrophils adhered to lung endothelium, transmigrated across endothelial surfaces and into the tissues, where they exerted their toxic effects through the release of ROS, resulting in microvascular dysfunction and local inflammatory response[31,32]. Consistent with previous studies[27,33,34], rats exposed to LPS presented a massive infiltration of inflammatory cells in the lungs. These results were supported by the histopathological examination, which showed an increase in the alveolar wall thickness, severe edema and inflammatory reaction in lung specimens.

Pretreatment with methyl palmitate or lutein resulted, to a varying degree, in marked protection against LPS-induced lung injury. Methyl palmitate reduced lung W/D ratio, total and differential cell count, and histopathological damage (mainly the severity and distribution scores

of the lesions), while it failed to attenuate the elevated total protein level and LDH activity. These results are in accordance with the previous studies[35,36] that proved the anti-inflammatory effects of methyl palmitate both in the suppression of inflammatory cell infiltration and in the management of paw edema. On the other hand, the histopathological results showed that lutein decreased LDH activity and inflammatory cell counts as well as the severity and the distribution of the inflammatory lesions. However, lutein failed to attenuate the increased W/D ratio of lung weight and the total protein level, indicating its inability to counteract pulmonary edema.

To explore the possible mechanism(s) of the protective action of methyl palmitate and lutein against LPS-induced ALI, oxidative stress and inflammatory factors were evaluated in the lung tissue.

One of the earliest manifestations of ALI is the activation of free radical generation by the pulmonary endothelium and neutrophils[37]. During the inflammatory response, neutrophils undergo a respiratory burst and produce superoxide. Overproduction of ROS is highly toxic to host tissues, and their interactions with various cellular macromolecules can have severe pathophysiological consequences[38,39]. GSH acts as a major cellular antioxidant defense system by scavenging free radicals and other ROS. LPS-induced oxidative stress can lead to GSH depletion[40] which aggravates LPS toxicity, probably *via* diminution of the antioxidant defense. SOD is the only antioxidant enzyme that can scavenge superoxide and it has been reported to be markedly decreased in LPS-induced ALI[41]. Certain antioxidants and some synthetic drugs can control the LPS-induced inflammation either by directly scavenging free radicals or by enhancing the endogenous antioxidant defense system[42,43].

Results of the present study showed that LPS injection increased oxidative stress and inflammatory cytokines in the lungs, as seen from the significant increase in MDA content, which is commonly regarded as a marker of oxidative stress and antioxidant status[44]. This was accompanied by a significant decrease in SOD and GSH activities. These results are in accordance with the previous study of Bhavsar *et al*[40]. Pretreatment with methyl palmitate or lutein controlled the levels of SOD and GSH to near control values with resultant decrease in MDA content. Previous investigations have shown the ability of methyl palmitate to attenuate liver fibrosis by decreasing oxidative stress and hence the inflammatory response that leads to fibrosis[10]. Lutein showed a decrease in MDA level and an increase in GSH and SOD levels[45]. Furthermore, Miki[16] stated that lutein enhances the antioxidant enzyme system in blood and liver tissue, proving that lutein has a profound effect on the antioxidant defense system. Therefore, the present study showed that both methyl palmitate and lutein may effectively reduce oxidative burden during the inflammatory response to LPS.

Additionally, it was essential to evaluate the effect of methyl palmitate and lutein on LPS-induced production of inflammatory cytokines which induce, enlarge and facilitate the entire or focal inflammatory reaction.

LPS is known to stimulate various cell types within the lung, including bronchial epithelia, pulmonary artery smooth muscle cells, macrophages, and neutrophils, to over-express iNOS[46]. Results presented here revealed an increased level of NO_2^-/NO_3^- in lung after LPS injection, which is consistent with previous studies that showed the overproduction of NO after LPS injection due to activation of iNOS[47,48]. Excessive production of NO by iNOS mediates increased protein leakage as well as hemodynamic and vascular permeability changes[49]. Furthermore, NO up-regulates inflammatory cytokines, such as TNF-α, and amplifies the inflammatory response during inflammation[50]. TNF-α is considered the first multifunctional cytokine produced from LPS-stimulated monocytes and macrophages. It elicits the inflammatory cascade and contributes to the severity of lung injury[51]. As expected, LPS caused significant increase in TNF-α production in lung. This result is in agreement with previous studies which reported a significant increase in TNF-α after LPS exposure[3,8,52].

Results for methyl palmitate are in agreement with a previous study[36] which reported that methyl palmitate decreased TNF-α level in the systemic LPS injection model. However methyl palmitate failed to reduce elevated NO level indicating that it protective effect against LPS-induced ALI is mainly mediated through its antioxidant activity and it ability to suppress TNF-α production. In addition, lutein has shown ability to suppress elevated NO level and TNF-α production. This is in accordance with the previous investigation of Sasaki *et al*[53] which showed the effect of lutein in decreasing TNF-α level and quenching ROS in LPS-induced retinal inflammation. Furthermore, the previous study of Jin *et al*[17] showed that the expression of iNOS in LPS-stimulated RAW cells was inhibited by lutein. Thus, it appears that lutein suppresses NO production by blocking iNOS protein expression. However, the intracellular mechanisms by which lutein exerts its effects on NO expression are still to be explored.

In conclusion, the present study shows that methyl palmitate and lutein can protect against LPS-induced ALI. Pretreatment with these agents reduced inflammatory changes as well as histological damage during LPS-induced ALI. The protective effect of these agents may be related to their ability to depress ROS generation, enhance antioxidant status and regulate proinflammatory cytokine production. Therefore, methyl palmitate and lutein may possibly represent a novel therapeutic strategy for lung inflammatory diseases.

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COMMENTS

Background

The lack of effective pharmacological interventions remains a major impediment in the treatment of inflammatory diseases of the lung. Lipopolysaccharide (LPS), a bacterial cell wall component, is known to induce the production of several inflammatory cytokines, tissue edema and injury. It is considered to be the most important pathogen that leads to the development of acute lung injury (ALI) in rats.

Research frontiers

Methyl palmitate and lutein are natural compounds that have been shown to possess different beneficial activities against induced organ damage. The research issue is how to use these compounds against ALI. The observed protective effects of these agents suggest their possible medical uses in different inflammatory lung diseases.

Innovations and breakthroughs

The present study demonstrates the protective effects of methyl palmitate and lutein against LPS-induced ALI. Pretreatment with these agents reduced inflammatory changes as well as histological damage during LPS-induced ALI. Furthermore, the present study found that the protective effect of these agents may be related to their ability to depress reactive oxygen species generation, enhance antioxidant status and regulate proinflammatory cytokine production.

Applications

The present study reveals that use of methyl palmitate and lutein possibly represent a novel therapeutic strategy for lung inflammatory diseases.

Terminology

ALI is characterized by the abrupt onset of significant hypoxemia and diffuse pulmonary infiltrates in the absence of cardiac failure. There is also an inflammation-associated increase in pulmonary vascular permeability, and epithelial and endothelial cell death.

Peer review

The study explored the effects of methyl palmitate and lutein on ALI in rats. The results are interesting and comprehensive. The authors should be congratulated on their comprehensive assessment of ALI parameters *i.e.*, wet/dry weight, cellular infiltration, histological scoring and a cytokine of choice, in this case tumor necrosis factor-α.

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BRIEF ARTICLE

Seasonal variation and living alone are related to pulmonary rehabilitation non-completion

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Abstract

AIM: To identify baseline characteristics that independently predict pulmonary rehabilitation non-completion and compare these findings against the participant's reasons for non-completion.

METHODS: Participants with chronic obstructive pulmonary disease (COPD) who attended a standardised twice weekly, eight week pulmonary rehabilitation program (located in the sub-tropics, latitude 27°29′ South) between 2010 and 2012 were recruited. The baseline characteristics of program completers and non-completers were compared in a case-controlled design. Participants who attended < 12/16 sessions were classified as a non-completer. Non-completers (those who missed > 4 sessions of the program) were asked by one independent investigator to participate in a survey about their pulmonary rehabilitation experience. Baseline characteristics were assessed for differences between program completers and non-completers. The baseline characteristics included disease severity, exercise capacity, smoking history, participant's social support and the season when each participant commenced rehabilitation. Non-completers that agreed to participate in the survey were asked to indicate what personal factors or external factors contributed to their program non-completion. Comparisons of completers and non-completers baseline characteristics were performed using cross-tabulations and t-tests, with significant measures analysed in a multivariate binary logistic regression model. Non-completers survey responses were compared to the identified independent predictors using cross-tabulations.

RESULTS: Twenty-six participants (23.4%) of the 111 participants with COPD [(mean ± SD) age was 67.4 ± 9.2 years and FEV1 54.6% ± 22.3%)], were classified as non-completers. Forty-five participants (40.5%) commenced pulmonary rehabilitation during winter. Thirty-six participants (32.4%) were living alone at program commencement. In the multivariate analysis (n = 111), only programs that commenced in winter (Exp B: 0.255, 95%CI: 0.090-0.727, P = 0.011) and participants that lived alone (Exp B: 2.925, 95%CI: 1.039-8.229, P = 0.042) were identified as independent predictors of program non-completion. Twenty participants of the twenty-six non-completers agreed to participate in the survey about their pulmonary rehabilitation experience. The reasons given for non-completion were grouped into: medical reasons (75%), other personal reasons (30%) and external barriers (45%), with ten non-completers reporting more than one reason.

No participant reported living alone or that the program commenced during winter as a reason for non-completion. There was no relationship between illness being the participant's reason for non-completion and the programs that commenced in winter (P = 0.135).

CONCLUSION: Despite winter commencing programs and participants who lived alone being independent predictors of program non-completion, neither measure was reported by participants as a reason for non-completion.

Key words: Chronic obstructive pulmonary disease; Pulmonary rehabilitation; Predictive factors; Program completion; Program non-completion

Core tip: The study's purpose was to identify baseline characteristics that independently predict pulmonary rehabilitation non-completion and compare these findings against the participant's reasons for non-completion. Twenty-six of the 111 participants with chronic obstructive pulmonary disease were classified as non-completers. Only programs that commenced in winter (P = 0.011) and participants that lived alone (P = 0.042) were identified as independent predictors of program non-completion. Twenty non-completers were interviewed about their pulmonary rehabilitation experience with their reasons grouped into: medical reasons (75%), other personal reasons (30%) and external barriers (45%). No participant reported living alone or that the program commenced during winter as a reason for non-completion.

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INTRODUCTION

Pulmonary rehabilitation is an effective intervention in managing chronic obstructive pulmonary disease (COPD), with level one evidence demonstrating that these programs can improve participant's exercise capacity, quality of life and breathlessness[1,2]. Despite these benefits, recent studies have reported non-completion rates for pulmonary rehabilitation programs between 20%-30%[3-6]. Additionally, poor pulmonary rehabilitation attendance has been associated with less improvement in quality of life and exercise capacity[7,8]. With less than 1.5% of individuals with COPD estimated to be able to access pulmonary rehabilitation each year[9-11], it is important to improve the understanding of factors associated with program non-completion to ensure optimal use of this limited health care resource.

Previous studies have described common themes related to pulmonary rehabilitation non-completion including illness[4,12], transport difficulties[12], and a lack of social support at home[13]. A number of participant factors have been associated with non-completion including decreased quadriceps strength[5], smoking status[3,14], depression[5,15], living alone[3] and having a greater distance to travel[15]. The weather was also described by participants as a reason for non-completion[4], with seasonal variation shown to affect COPD patient's physical activity[16]. However, it is unclear if programs located in different climates have the same influence on non-completion. In a recent systematic review, Keating *et al*[17] suggested that the best model for predicting pulmonary rehabilitation non-completion included quadriceps weakness, depression and smoking but this model only explained 45% of the variation[5]. Conversely, there is considerable variation in the demographic and clinical factors associated with predicting non-completion[3,4,14,15]. Possible reasons for this variability, include the inconsistency in defining non-completion[3,4,14] and differences in the program length[3,4,15]. Furthermore, only the study by Fischer *et al*[3] has compared, in the same dataset, the independent factors associated with pulmonary rehabilitation program non-completion against the participant's described reasons for non-completion. This study suggested that no baseline socio-demographic, clinical or psychological variables predicted program non-completion[3]. However, the pulmonary rehabilitation programs investigated by Fischer *et al*[3] were ⩾ three supervised days per week for a twelve week period which may be more intensive than the typical pulmonary rehabilitation program[18,19].

Given that the models used to date to assess non-completion have explained so little of the variance, further investigation of factors affecting non-completion in a standardised pulmonary rehabilitation program is warranted. Hence the purpose of this study was to compare the independent factors associated with non-completion in a standardised eight week program against the participant's reasons for non-completion. The factors investigated include measures of disease severity, comorbidities, exercise capacity, quadriceps strength, quality of life, self-efficacy, smoking history, social support and the season when each participant commenced rehabilitation. Of these factors, the influence of comorbidities and the Body-mass-index, Airflow Obstruction, Dyspnea, and Exercise Capacity (BODE) index[20] on non-completion is not known. Therefore, the study aims were to identify participant characteristics at baseline assessment that independently predict pulmonary rehabilitation program non-completion; and to compare these characteristics against the participant's reported reasons for non-completion. We hypothesized that the reasons for program non-completion, including medical reasons and external barriers, would be related to the participant's baseline characteristics identified.

MATERIALS AND METHODS

Participants with COPD who attended the tertiary hospital's pulmonary rehabilitation program (located in the sub-tropics, latitude 27° 29' South), between 2010 and 2012 were considered for inclusion. All participants with COPD who completed the pulmonary rehabilitation program's baseline assessment were included in the study. Participants who declined to participate in the survey or were unable to be contacted by telephone were excluded from the survey component of the study. The characteristics of program completers and non-completers were compared in a case-controlled design using the baseline measures from a concurrent study. There is no clear consensus in classifying program non-completion with criteria ranging between: participant were classified as "poor attenders" with < 67% attendance of the total sessions[14], and participants being classified as a non-completer if one session was missed[15] or by participants not completing the final program assessment[3]. Therefore for the purpose of this study, a non-completer was arbitrarily classified as a participant who attended < 12/16 of the program's sessions. Once an individual missed > 4 sessions, the participants were contacted as soon as possible by a single investigator (who was independent of the pulmonary rehabilitation program) to ask them to take part in a survey about their pulmonary rehabilitation experience. Institutional ethics committee approval (HREC/08/QPCH/116-EC28116 and 2009000403) and each participant's informed consent were obtained prior to study commencement.

Measurements

Demographic information including social support and medical history were collected for each participant. The season when each participant commenced pulmonary rehabilitation was recorded. Lung function was measured pre-program according to standard methods[21]. The participant's comorbidities were classified into the categories of musculoskeletal, cardiac and metabolic diseases as described previously by Crisafulli *et al*[22] The influence of multiple comorbidities was assessed with the number of participant's comorbidities categorised into: zero (no associated comorbidity), one, two or three comorbidity categories and by using the Charlson Comorbidity index[23]. The BODE index was used as an indicator of disease severity[20]. Six minute walk distance (6MWD) assessed exercise capacity as per the recognised guidelines[24]. Quadriceps strength was assessed using hand-held dynamometry (the Lafayette Manual Muscle Test System) as previously described[25], with an adjustable strap added to ensure an isometric contraction. In order to compare between participants, quadriceps strength [adding together the participant's best attempt on each leg (kilograms)] was divided by the participant's body weight to calculate a percentage. Quality of life was assessed using the Chronic respiratory questionnaire (CRQ) and its four domains of dyspnea, fatigue, emotional function and mastery[26,27]. Participant's self-efficacy was measured using the COPD self-efficacy scale[28] and calculated by dividing the aggregate score by the number of questions answered[5].

A survey consisting of both closed response and open-ended questions was developed by the study's investigators, the pulmonary rehabilitation program's clinical staff and from the previous literature. Prior to study commencement, the face and content validity of the questionnaire was tested on participants from two pulmonary rehabilitation courses at program completion. The participants were asked to indicate possible factors that affected program completion and feedback was sought regarding the readability of the survey. The final version of the survey included specific questions asking if personal factors (illness, musculoskeletal injury, family commitments, and other commitments) or external factors (the weather, transport difficulties, and program location) contributed to program non-completion. Other possible reasons for program non-completion, such as lack of social support at home[13], were incorporated into more generic questions asking if there was any other personal reason affecting program completion or if there was any other external factors affecting program completion. To better interpret all questions related to non-completion, participants were also asked to provide additional comments explaining any reported reasons that affected non-completion. Participants were also asked for: general comments about the program, if the participant believed that the program could help manage their lung condition and if the participants noticed any benefits from their program. The survey also asked participants if they liked the program's assessment, education and exercise sessions.

Pulmonary rehabilitation program

The pulmonary rehabilitation program was a standardised twice weekly, eight week program[18,19], with five separate programs completed each year. Participants were referred to the program by respiratory physicians, respiratory nurses, or allied health professionals. Car parking was available for a fee (approximately $15/session). The program consisted of one hour each of multi-disciplinary education and exercise per session. Staff ratio during the exercise training sessions was two to three staff to supervise twelve to fifteen enrolled participants with the same staff supervising all programs. The supervised one hour exercise sessions consisted of lower limb endurance training which was individualised for intensity[19] and an upper and lower limb strengthening program. The training intensity of the participant's walking program was commenced at 80% of the average six minute walk test speed[19] and the cycle ergometry program was determined from 80% of the 6MWD as per the published protocol[29]. The lower limb endurance training intensity was progressed as tolerated for each participant throughout the program. All participants were encouraged to complete at least one additional unsupervised exercise session of lower limb endurance and strength training each week[18].

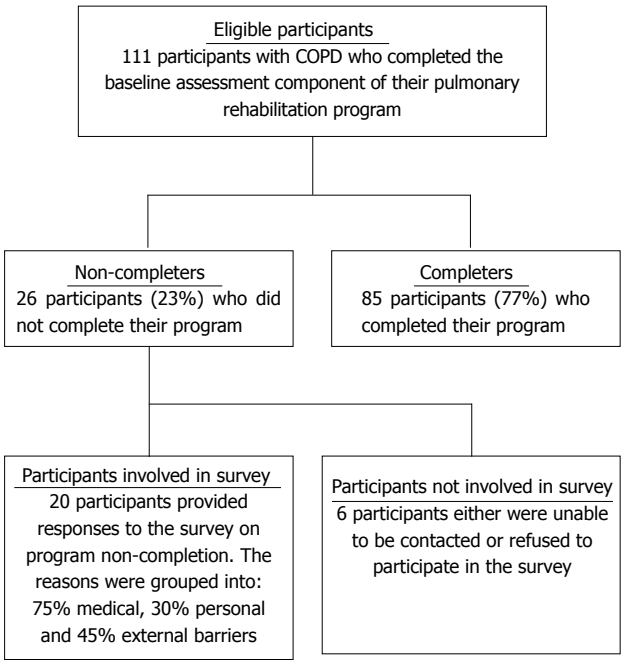

Figure 1 Study flow chart. COPD: Chronic obstructive pulmonary disease.

Statistical analysis

Comparisons of completers and non-completers baseline characteristics were performed using cross-tabulations and *t*-tests. Participant's baseline characteristics including respiratory function, Charlson Comorbidity index, BODE index, CRQ, six minute walk distance, quadriceps strength, self-efficacy, smoking history, if a participant lived alone and the season when each participant commenced rehabilitation were analysed in a univariate model to determine measures that were associated with non-completion. Significant measures ($P < 0.1$) were further analysed using a multivariate binary logistic regression model to identify independent predictors of non-completion. The non-completers survey responses used descriptive statistics to group individual responses and the responses were compared to the identified independent predictors using cross-tabulations.

RESULTS

One hundred and eleven pulmonary rehabilitation participants with COPD, (mean ± SD) age was 67.4 ± 9.2 years and FEV_1 54.6% ± 22.3%, were included in the study. Twenty-six of the participants (23.4%) were classified as a non-completer (attended < 12/16 sessions) with none of this group completing the end of program assessment (Figure 1). Seventy-one participants (64.0%) were categorised with ≥ one comorbid category. Thirty-three participants were categorised with musculoskeletal disease, 29 participants were categorised with cardiac disease and 36 participants were categorised with metabolic disease. Participants from eleven separate programs were included in this study, with four programs commenced during winter, three during summer, and two each during autumn and spring. Forty-five participants (40.5%) commenced pulmonary rehabilitation during winter. Thirty-six participants (32.4%) were living alone at program commencement and the remaining participants were living with their partner (*n* = 59), other family member (*n* = 15) or a friend (*n* = 1).

The participant's baseline characteristics were assessed for differences between program completers and non-completers. Non-completers had a lower baseline 6MWD ($P = 0.030$), lower total CRQ score ($P = 0.012$), and more non-completers lived alone ($P = 0.015$) when compared to program completers (Table 1). A higher percentage of non-completers also commenced pulmonary rehabilitation during winter when compared to program completers ($P = 0.006$). There was no relationship between program non-completion and participants with musculoskeletal ($P = 0.469$), cardiac ($P = 0.205$), metabolic disease ($P = 0.238$), the participant's number of comorbidity categories ($P = 0.257$) or any other participant characteristics.

In order to identify independent predictors of non-completion, the baseline participant characteristics were evaluated using univariate and multivariate logistic regression models (*n* = 111) with non-completers (Yes = 1, No = 0) as the dependent variable. In the univariate analysis, the significant factors associated with non-completion were lower baseline 6MWD, lower CRQ total score, and a greater percentage of people who lived alone and commenced pulmonary rehabilitation in winter. In the multivariate analysis, only programs that commenced in winter (Exp B: 0.255, 95%CI: 0.090-0.727, $P = 0.011$) and participants that lived alone (Exp B: 2.925, 95%CI: 1.039-8.229, $P = 0.042$) were identified as independent predictors of non-completion (Table 2).

Twenty-six participants were identified as non-completers. Six individuals either could not be contacted despite multiple attempts (*n* = 4) or refused to participate in the survey (*n* = 2). Therefore, the following survey results were from the remaining twenty participants (20/26) classified as non-completers. Most of the participants provided positive general comments, liked the program structure and reported benefits from the program despite non-completion (Table 3). The reported benefits included improved understanding of the lung condition (*n* = 6), given an exercise program (*n* = 4) and improved breathing control (*n* = 3).

From the survey results, the participant's reasons for non-completion were grouped into: 75% medical reasons (illness, musculoskeletal injury, medical investigations and slow to recover after illness), 30% other personal factors (family commitments, work commitments and hard to motivate themselves to leave the house), and 45% external factors (transport difficulties, the weather and program location, Table 4). Ten non-completers (50%) reported more than one reason for non-completion. Although, programs that commenced in winter was identified as independent predictors of non-completion, only the heat and/or humidity (*n* = 3) and air pollution (*n*

Table 1 Participant's baseline demographic data *n* (%)

	Completers (*n* = 85)	Non-completers (*n* = 26)	*P* vaule
Age (yr)	67.4 ± 9.1	67.2 ± 9.5	0.93
Sex (female)	36 (42.3)	12 (46.2)	0.822
Social support (living alone)	22 (25.9)	14 (53.8)	0.015
Currently smoking	6 (7.1)	4 (15.4)	0.24
Programs commencing in Winter	28 (32.9)	17 (65.4)	0.006
FEV1% predicted	55.4 ± 22.4	51.4 ± 22.5	0.452
FVC% predicted	76.8 ± 18.5	71.4 ± 19.5	0.243
Charlson Co-morbidity index	1.9 ± 1.1	1.9 ± 1.0	0.851
BODE index	2.6 ± 1.9	3.1 ± 2.1	0.256
BMI (kg/m^2)	27.6 ± 5.2	26.6 ± 4.2	0.383
Quadriceps strength (%)	62.3 ± 22.5	60.1 ± 21.8	0.681
6MWD (m)	406 ± 107	350 ± 134	0.03
COPD self-efficacy score (mean score/question)	2.8 ± 0.8	2.8 ± 0.8	0.924
Chronic respiratory Questionnaire	86.7 ± 21.5	73.2 ± 29.9	0.012
CRQ-dyspnea domain (mean score/question)	4.2 ± 1.4	3.9 ± 1.3	0.279
CRQ -fatigue domain (mean score/question)	3.6 ± 1.3	3.1 ± 1.0	0.064
CRQ-emotional domain (mean score/question)	4.6 ± 1.2	4.3 ± 1.3	0.179
CRQ-mastery domain (mean score/question)	4.7 ± 1.3	4.4 ± 1.4	0.456

Continuous data expressed as the mean ± SD. FEV1: Forced expiratory volume in one second; FVC: Forced expiratory volume; BODE: Body-mass-index, Airflow Obstruction, Dyspnea, and Exercise Capacity; BMI: Body mass index; 6MWD: Six minute walk distance; COPD: Chronic obstructive pulmonary disease; CRQ: Chronic respiratory questionnaire.

= 1) were the reasons given. There was no relationship between illness being the participant's reason for non-completion during the programs commenced in winter [70% (7/10)] when compared to programs commenced during the remaining seasons [60% (6/10); P = 0.135]. Transport barriers in attending the program, including parking costs (n = 10), limited disabled parking (n = 3) and limited public transport (n = 1), were discussed by thirteen non-completers (65%) including 70% (7/10) of the non-completers who lived alone. However, despite transport being a barrier, only seven participants indicated that transport difficulties was a reason for program non-completion with four of these seven respondents living alone.

DISCUSSION

The current study assessed significant differences in the baseline characteristics between pulmonary rehabilitation program completers and non-completers. Non-completers were shown at baseline to have reduced exercise capacity, decreased quality of life, and increased fatigue in the CRQ domain, and a greater percentage of non-completers were living alone and commenced the program in winter. However of these factors, living alone and programs that commenced in winter were the only baseline characteristics shown to independently predict non-completion. Participant's baseline characteristics of smoking status (self-reported), lung function, quadriceps strength, the Charlson Comorbidity index, BODE index, and self-efficacy were also not related to non-completion. Interestingly, neither living alone nor programs that commenced in winter were reported by participants in the survey responses as a reason for program non-completion. The survey results showed that medical reasons were the most common participant reasons for program non-completion. In addition, non-completers reported other barriers to program non-completion including personal factors of work and family commitments and external factors including transport difficulties and the weather.

Participants who lived alone or participants that commenced pulmonary rehabilitation in winter were identified, from baseline assessment measures, as the only independent predictors of program non-completion. Despite, no surveyed participant directly indicating that living alone was a reason for program non-completion, living alone has been previously related to participants with poorer motivation and an increased challenge of getting to the pulmonary rehabilitation program[3,4]. In the present study, this relationship is also relevant, with 70% of the non-completers who lived alone indicating that there were transport difficulties in attending the program. Programs that commenced in winter (4/11 programs) accounted for 65.4% (17/26) of the participants who were classified as non-completers. Although programs commencing in winter was identified as an independent predictor, the heat and/or humidity (n = 3) and air pollution (n = 1) were the only weather related reasons reported by the participants for non-completion. Furthermore, our result of programs commencing in winter being an independent predictor was somewhat surprising considering the program's location has a relatively mild winter (most winter days are sunny with an average temperature of around 17 ℃) when compared to other parts of the world. Despite illness being more commonly reported as a reason for program non-completion during winter (70%) when compared to the remaining seasons (60%), this findings was not statistically significant (P = 0.135). It is possible that there are other factors, including the

Table 2 Binary logistic regression model for a non-completer in pulmonary rehabilitation

	β	SE	Wald χ^2	*P*	Odds ratio (Exp β)	95%CI for Exp β
Univariate analysis						
Six minutes walk distance	-0.004	0.002	4.435	0.035	0.996	0.992–1.000
CRQ fatigue domain	-0.351	0.192	3.357	0.067	0.704	0.483-1.025
CRQ total score	-0.024	0.01	5.662	0.017	0.976	0.957-0.996
Social support-lives alone	1.19	0.465	6.548	0.01	3.288	1.321-8.182
Programs commenced in Winter	-1.347	0.472	8.127	0.004	0.260	0.103-0.656
Multivariate analysis						
Social support-lives alone	1.073	0.528	4.134	0.042	2.925	1.039-8.229
Programs commenced in Winter	-1.366	0.534	6.541	0.011	0.255	0.090-0.727

Only variables with $P < 0.1$ are shown in the table. There was a strong correlation between the chronic respiratory questionnaire (CRQ) total score and the CRQ fatigue domain ($r = 0.783$), therefore only the CRQ total score was assessed in the multivariate analysis.

Table 3 Non-completers responses about their pulmonary rehabilitation experience

	Yes	No	Undecided/no comment
Did you like the initial assessment?	15	2	3
Did you like the education sessions?	18	0	2
Did you like the exercise sessions?	12	2	6
Did you notice any benefits from participating in the program?	16	1	3
Do you believe pulmonary rehabilitation can help you manage your condition?	17	0	3

presence of depression as a comorbidity, not investigated during the current study that may have influenced this relationship between winter programs and non-completion. However, both hot and cold weather have been reported previously as barriers to program completion[4]. Further investigation is needed to determine if seasonal variation in different climates also affects pulmonary rehabilitation program non-completion.

The non-completion rate of 23% in the present study was similar to the published findings in other pulmonary rehabilitation programs[3-6]. Using a similar criteria for non-completion as the current study, Garrod *et al*[5] reported that 31% of participants were non-completers of a twice weekly seven week pulmonary rehabilitation program. Although the present study's criteria [< 75% (< 12/16) attendance] was arbitrary, no participant who attended < 75% of the sessions completed the final program assessment.

In the present study, participants personal and disease related characteristics, with the exception of living alone, did not discriminate between individuals who may or may not complete pulmonary rehabilitation. In our cohort, non-completers who were still smoking at program commencement (15.4%) were not significantly different to the completers group (7.1%, $P = 0.240$). While smoking status has been previously associated with non-completion, our study had a lower rate of participants still smoking at program commencement (9.0%), when compared to the previous studies (14%-17%)[3,14]. The smaller cohort of current smokers in our study may explain why smoking status was not an independent predictor. Baseline quadriceps strength was not significantly different between completers and non-completers ($P = 0.681$) in our results despite being previously shown to be an independent predictor of non-completion[5]. Our results also suggest that self-efficacy, CRQ and its domains of dyspnea, self-mastery and emotional function do not identify participants likely to have poorer program completion. Furthermore, we have shown that there are multiple reasons for non-completion, including medical reasons, other personal factors and external barriers. In the current study, 75% of surveyed non-completers reported a medical reason, including illness and musculoskeletal injury, as the most common reason for program non-completion reflecting the findings of Fischer *et al*[3]. The study by Fischer *et al*[3] also found that program non-completion was not related to medical and psychosocial variables, including illness perception.

In previous studies a lack of perceived benefit from pulmonary rehabilitation programs has been associated with poor adherence and non-completion[12,30]. However, despite the multiple reported challenges to program non-completion, the majority of the surveyed non-completers expressed favourable comments about the program, reported benefits from the program and believed that pulmonary rehabilitation could help manage their lung condition. When considering the participants reasons for non-completion, these positive responses from the participants could suggest that program non-completion was largely unexpected due to unforeseen circumstances on program commencement. This may reflect why it remains difficult to identify participants' characteristics which identify likely program non-completion. However, it is important to note that transport barriers in attending pulmonary rehabilitation were discussed by thirteen of the surveyed non-completers with seven of these participants indicating that transport difficulties were a reason for non-completion. Participants with greater resources such as social and emotional support have previously been shown to have better adherence[5], with increased

Table 4 Reasons given for pulmonary rehabilitation non-completion

	Yes	No	Undecided/no comment
Personal factors			
Did an illness affect you completing the program?	13	5	2
Did an injury affect you completing the program?	3	16	1
Did family commitments affect you completing the program?	3	15	2
Did other commitments affect you completing the program?	5	13	2
Did any other personal factors affect you completing the program?	3	15	2
External factors			
Did the weather affect you completing the program?	4	15	1
Did transport difficulties affect you completing the program?	7	12	1
Did the program location affect you completing the program?	1	18	1
Did any other factor affect you completing the program?	0	19	1

The other commitments affecting program completion were: work (n = 3) and medical investigations (n = 2). The other personal factors affecting program completion were: hard to motivate self to leave the house (n = 2) and slow to recover after being hospitalised (n = 1).

social support probably assisting participants to overcome the reported challenges such as transport difficulties and poor motivation required to complete a pulmonary rehabilitation program. Therefore, better recognition and support for participants who are living alone may reduce the reported challenges of transport difficulties and poor motivation, and increase program adherence[31]. Further research needs to investigate different strategies to increase support for participants living alone and to minimise other barriers to non-completion.

Study limitation

Some study limitations need to be acknowledged. While living alone was identified as an independent predictor of program non-completion, there was no corresponding question in the survey tool that specifically asked participants if living alone affected their program completion. However, participants were able to discuss if living alone was a reason for non-completion in the more generic questions that asked if there was any other personal reason affecting program completion. Similarly, the present study did not assess the influence of psychological factors, such as depression or anxiety, on program non-completion despite these comorbidities being prevalent in people with COPD. Therefore it is unknown what influence these psychological factors have on the current study's findings. However, there was no significant difference between program completers and non-completers in the other psychological measures including self-efficacy, self-mastery and emotional function. Lastly, while the sample size in the present study is relatively small (n = 111), it is similar to previous studies by Garrod *et al*[5] (n = 74) and Steele *et al*[6] (n = 146). However, it is possible that with a larger sample size there may be a relationship between program non-completion in winter and illness as the reason reported for non-completion.

Despite programs commencing in winter and participants who lived alone being identified as characteristics that independently predicted program non-completion, neither measure was reported by participants as a reason for non-completion. More support provided to people who live alone may limit the number of participants who do not complete pulmonary rehabilitation programs.

COMMENTS

Background

Pulmonary rehabilitation is an effective intervention in managing chronic obstructive pulmonary disease (COPD). However, non-completion rates have been reported between 20%-30%. With less than 1.5% of individuals with COPD estimated to be able to access pulmonary rehabilitation each year, it is important to improve the understanding of factors associated with program non-completion to ensure optimal use of this limited health care resource.

Research frontiers

Previous studies have reported considerable variation in the demographic and clinical factors associated with predicting pulmonary rehabilitation non-completion due in part to differences in program structure. Given that the models used to date to assess non-completion have explained so little of the variance, further investigation of factors affecting non-completion in a standardised pulmonary rehabilitation program is warranted. Therefore, the purpose of this study was to compare the independent factors associated with non-completion in a standardised eight week program against the participant's reasons for non-completion.

Innovations and breakthroughs

Prior to this study, there was only one study that compared, in the same dataset, the independent factors associated with pulmonary rehabilitation program non-completion against the participant's described reasons for non-completion. In the current study, participants who lived alone or participants that commenced pulmonary rehabilitation in winter were the only baseline characteristics that independently predicted program non-completion. However, no surveyed participant indicated that living alone or that program commenced during winter was a reason for non-completion. The surveyed non-completers reported a medical reason, including illness and musculoskeletal injury, as the most common reason for program non-completion. Furthermore, there was no relationship between illness being the participant's reason for non-completion and the programs that commenced in winter (P = 0.135).

Applications

The study investigated pulmonary rehabilitation program non-completion in a tertiary hospital's clinical program located in a sub-tropical climate. Non-completers reported many barriers as the reasons for non-completion including medical reasons (*e.g.,* illness or musculoskeletal injury), other personal factors (*e.g.,* work or family commitments, poor motivation) and external barriers (*e.g.,* transport difficulties, weather). Although programs commencing in winter was identified as an independent predictor, the heat and/or humidity (n = 3) and air pollution (n = 1) were the only weather related reasons reported by the participants for non-completion. Further investigation is needed to determine

if seasonal variation in different climates also affects pulmonary rehabilitation program non-completion. Despite participants who lived alone being an independent predictor of program non-completion, no surveyed participant indicated that living alone was a reason for program non-completion. Better recognition and support for participants living alone may assist these individuals to overcome the reported challenges such as transport difficulties and poor motivation required to complete a pulmonary rehabilitation program.

Peer review

This is an interesting study to explore the reasons why some participants who joined an 8-wk pulmonary rehabilitation program could not complete the course. Authors investigate a total 111 COPD patients and analyzed baseline characteristics. Authors concluded that despite winter commencing programs and participants who lived alone being independent predictors of program non-completion, neither measure was reported by participants as a reason for non-completion.

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CASE REPORT

EGFR mutation identifies distant squamous cell carcinoma as metastasis from lung adenocarcinoma

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Abstract

Lung cancer metastasis is typically determined by histologic similarity between distant and primary lesions. Herein, we present a 70-year-old Japanese woman with an adenocarcinoma in her lung and a squamous cell carcinoma in her femur; both tumors had an identical epidermal growth factor receptor mutation, G719S. This indicated that both tumors had a common origin, despite their histologic dissimilarity. The tumor in the femur was thus identified genetically as a lung cancer metastasis. This case suggests that genetic analysis can determine whether a distant lesion is a lung cancer metastasis, particularly when the histology differs from that of the primary lesion.

Key words: Non-small cell lung cancer; Adenocarcinoma; Squamous cell carcinoma; Epidermal growth factor receptor mutation; G719; Metastasis

Core tip: A tumor in the femur was identified genetically as a lung cancer metastasis. This case suggests that a matching epidermal growth factor receptor (EGFR) mutation in a distant lesion can validate a diagnosis of lung cancer metastasis, even if it differs histologically from the primary lesion. To the best of our knowledge, this is the first report showing the use of EGFR genetic analysis to identify a distant lesion as a lung cancer metastasis. In the era of molecularly-targeted treatments for non-small cell lung cancer, combined pathological diagnosis and genetic analysis could lead to more precise diagnoses and better understanding of histogenesis and more appropriate therapeutic selections.

Kanaji N, Bandoh S, Hayashi T, Haba R, Watanabe N, Ishii T, Kunitomo A, Takahama T, Tadokoro A, Imataki O, Dobashi H, Matsunaga T. *EGFR* mutation identifies distant squamous cell carcinoma as metastasis from lung adenocarcinoma. *World J Respirol* 2013; 3(2): 38-43 Available from: URL: http://www.wjgnet.com/2218-6255/full/v3/i2/38.htm DOI: http://dx.doi.org/10.5320/wjr.v3.i2.38

INTRODUCTION

Non-small cell lung cancer (NSCLC) can be classified into several histologic subtypes, including adenocarcinoma and squamous cell carcinoma. Rarely, two histologic subtypes exist within one tumor, such as adenosquamous

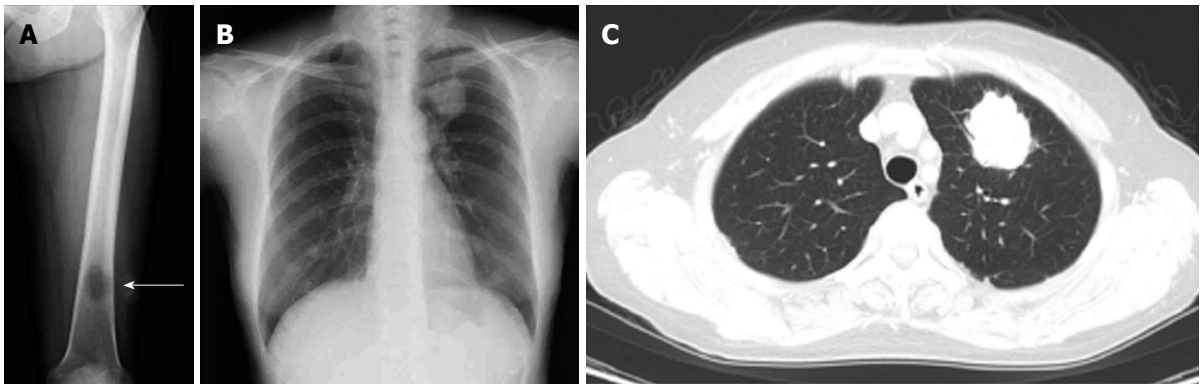

Figure 1 Images of the patient on admission. A: An X-ray of the left femur showed an osteolytic change (arrow); B: Chest X-ray showed a tumor shadow; C: Chest computed tomography showed a 4-cm tumor, which was considered to be the primary lesion.

carcinoma. Metastasis of NSCLC is typically clinically diagnosed based on symptoms, a physical examination, and imaging, such as X-rays and computed tomography (CT). For tissue samples obtained from distant lesions, the diagnosis of metastasis is typically determined by histologic similarity between the distant lesion and the primary lung lesion. Mutations of the epidermal growth factor receptor (*EGFR*) vary among histologic NSCLC subgroups, and among Asian patients, the incidence is approximately 40%-60% in adenocarcinoma and 3% in squamous cell carcinoma[1-6]. To the best of our knowledge, this is the first report showing the use of an *EGFR* genetic analysis to identify a distant lesion as a lung cancer metastasis.

CASE REPORT

A 70-year-old Japanese woman was admitted to our hospital in July 2012 for further evaluation of pain in her left femur, which had gradually progressed over 6 mo. She had never smoked, and had no past history of illness. A physical examination disclosed tenderness on her left femur. Lymphadenopathy and clubbing were not present. SpO_2 was 97%. Her vesicular sound was normal, and no crackles were auscultated. A left femur X-ray showed an osteolytic lesion, suggestive of metastasis (Figure 1A). A chest X-ray showed a mass shadow in the left lung field; CT showed a 4-cm tumor in the left upper lobe, suggesting that the primary lesion was lung cancer (Figure 1B and C). Among tumor markers, carcinoembryonic antigen (CEA; 23.4 ng/mL; normal range: < 5.0 ng/mL), cytokeratin 19 fragment (CYFRA; 11.6 ng/mL; normal range: < 3.5 ng/mL), and squamous cell carcinoma antigen (SCC; 1.9 ng/mL; normal range: < 1.5 ng/mL) showed values consistent with NSCLC. Shortly after a diagnostic bronchoscopic examination was planned, the patient's left femur fractured. Under general anesthesia, a biopsy was taken from under the osteosynthesis. The pathological findings included carcinoma, with single cell keratosis, tadpole cells, and intercellular bridges (Figure 2A and B). The cancer cells were positive for cytokeratin (CK) 5/6 and p63 (Figure 2C and D) and negative for thyroid transcription factor-1 (data not shown). Based on these findings, the tumor in the femur was diagnosed as a poorly differentiated squamous cell carcinoma; we observed no pathological findings that suggested adenocarcinoma.

Because squamous cell lung cancer in never-smokers is relatively rare, a transbronchial tumor biopsy was also performed. The pathologic findings showed poorly differentiated adenocarcinoma including glandular formation (Figure 3A). These cancer cells were positive for D-periodic acid-Schiff, suggesting mucin production (Figure 3B), and negative for CK5/6 and p63 (Figure 3C and D). No findings suggestive of squamous cell carcinoma were observed in the lung biopsy sample. Therefore, the pathologic diagnosis of the lung tumor was completely different from that of the femur. No primary lesions other than the lesion found in the lung were detected in any other part of her body.

We analyzed the *EGFR* mutation status in tumor samples from the lung and the femur as described previously[4]. Briefly, RNA was extracted from cell samples, and cDNA was synthesized. Reverse transcriptase-polymerase chain reaction (PCR) for *EGFR* was performed, and the PCR products were sequenced. The G719S mutation in exon 18 was detected in both samples (Figure 4). This mutation was not detected in circulating white blood cells (data not shown). Based on this genetic analysis, we determined that the lung and femur tumors had the same origin. She was thus diagnosed as having lung cancer with metastasis to the femur (cT2aN2M1b, stage Ⅳ). Chemotherapy with carboplatin (area under the curve 5, day 1) and docetaxel (60 mg/m^2, day 1) was administered, and the fractured left femur was irradiated (total 30 Gy). After four cycles of chemotherapy, the primary lesion markedly regressed, and all tumor markers, including CEA, CYFRA, and SCC, decreased to within the normal ranges. She exhibited a partial response to chemotherapy.

DISCUSSION

In general, EGFR mutations have been reported to be almost exclusively found in carcinomas of the lung, although they are observed with a low frequency in other

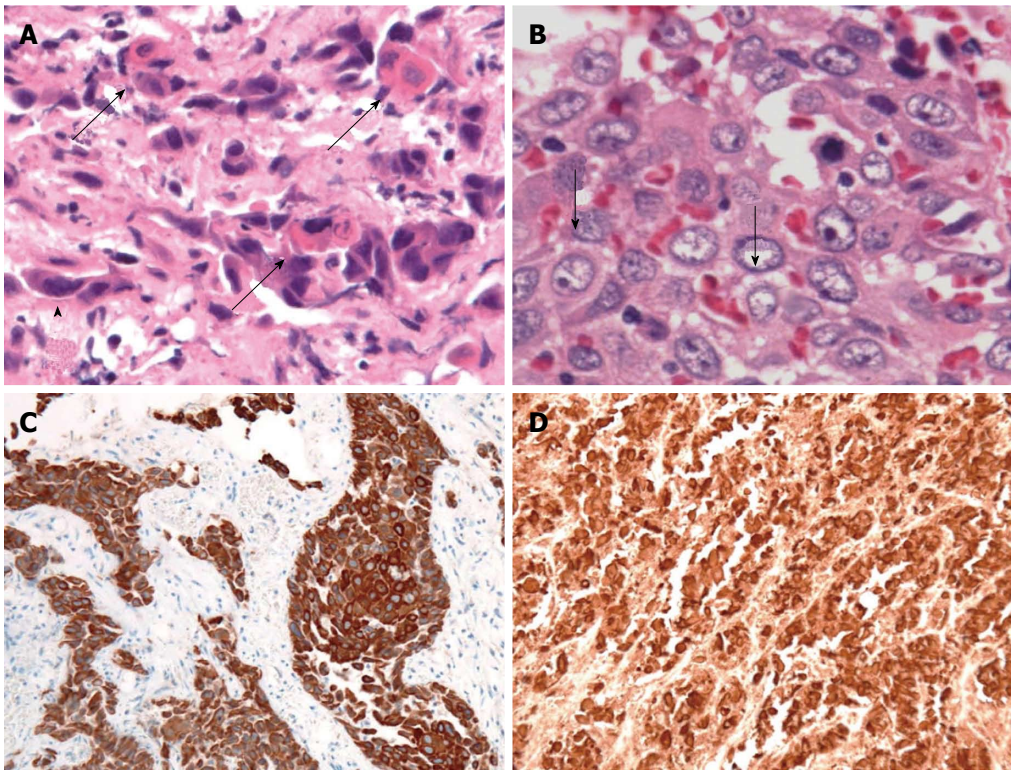

Figure 2 Histology of squamous cell carcinoma in the femur tumor. A: Single cell keratosis (arrows); tadpole cells (arrowhead; HE staining, × 40); B: Intercellular bridges (arrows; HE staining, × 40); C: Cytokeratin 5/6 immunostaining (× 4) (monoclonal mouse anti-human cytokeratin 5/6 antibody from clone D5/16 B4; DakoCytomation, Copenhagen, Denmark); D: p63 immunostaining (× 4) (Monoclonal mouse anti-human p63 antibody from Clone 4A4; Nichirei Biosciences Inc., Tokyo, Japan).

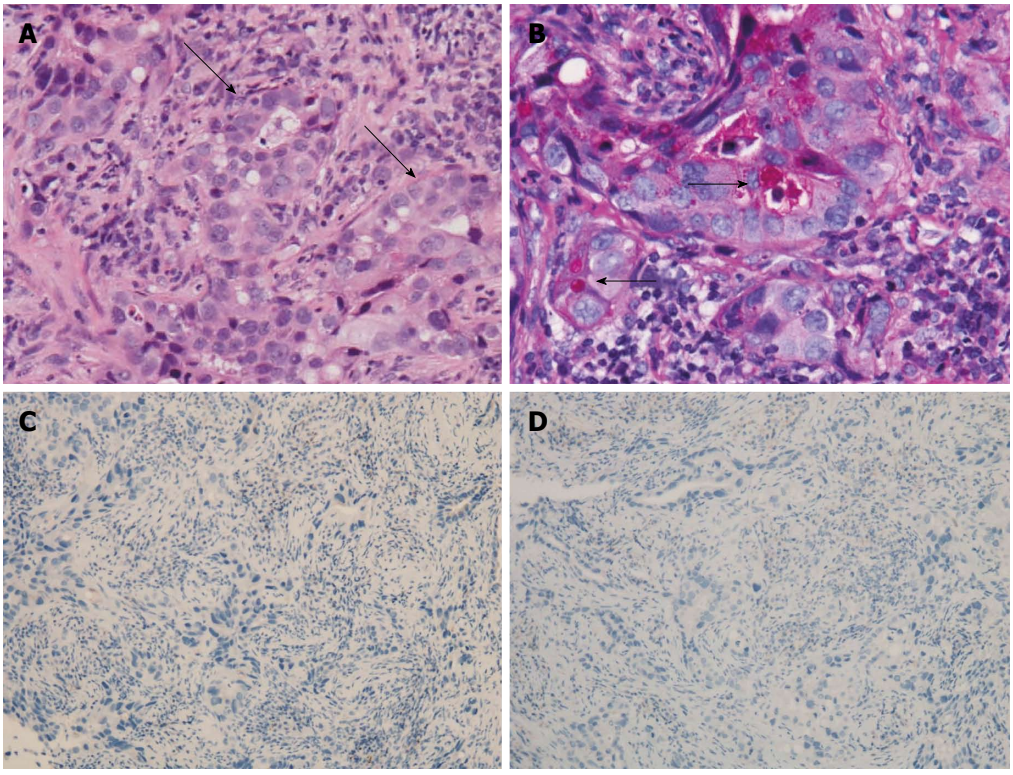

Figure 3 Histology of adenocarcinoma in the lung tumor. A: Glandular formations (arrows; HE staining × 40); B: D-periodic acid–Schiff suggests mucin production (arrows; × 40); C: Cytokeratin 5/6 immunostaining (× 4); D: p63 immunostaining (× 4).

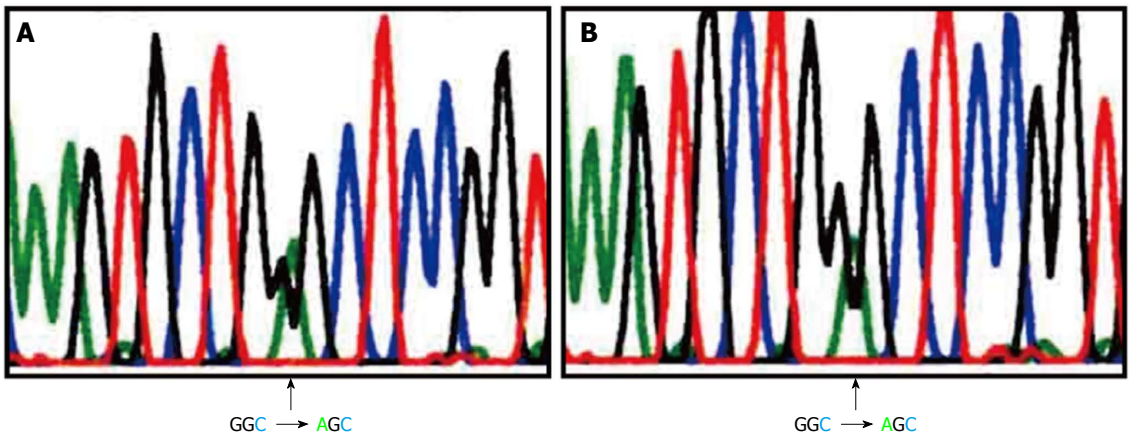

Figure 4 Sequencing of epidermal growth factor receptor showing G719S in both the lung and femur samples. A: Tumor of the lung; B: Tumor of the femur. Nucleotides: Green; A: Black; G: Blue; C: Red.

solid tumors[7]. In the present case, (1) primary lung cancer did exist; and (2) the *EGFR* mutation G719S was identified in both the primary and distant lesions, despite their different histology, adenocarcinoma and squamous cell carcinoma, respectively. This finding proved that these histologically different tumors had the same origin, facilitating a diagnosis of lung cancer and its metastasis.

Adenosquamous carcinoma has components of both adenocarcinoma and squamous cell carcinoma in one tumor and is a rare NSCLC subtype detected in 0.4%-4% of lung cancers[8,9]. A study of Korean patients found *EGFR* mutations in 11 (44%) of 25 adenosquamous carcinomas[9], which is in agreement with their frequency in Asian patients with adenocarcinoma[1-4,6]. Interestingly, in all 11 adenosquamous carcinomas, when the two tissue types were manually microdissected from each tumor, the adenocarcinoma and squamous cell carcinoma components had the same *EGFR* mutation[9]. This finding indicates that adenosquamous carcinoma components are more likely to originate from common monoclonal progenitor cells than from a polyclonal pathway[9].

In the present case, the lung biopsy sample showed adenocarcinoma with no squamous cell carcinoma. However, the lung tumor might have contained a squamous cell component because the tissue sample obtained by bronchoscopy was a small part of the total tumor. Possible lung tumor histology includes adenocarcinoma, adenosquamous carcinoma, and adenocarcinoma with squamous differentiation. However, the femur tumor was a squamous cell carcinoma with no adenocarcinoma component. The most likely course is that a squamous cell component of the lung tumor spread to the femur; alternatively, this specific bone environment might favor squamous cell differentiation from undifferentiated progenitor cells. Regarding the mechanisms of histological differentiation, some interesting studies have been reported. Lung adenocarcinoma phenotypes have been reproduced by introducing an active form of PIK3CA, cyclin-D1, or a dominant-negative form of liver kinase B1 in combination with genetic alterations including human telomerase overexpression, inactivation of the pRB and p53 pathways, and KRAS activation[10]. p63 regulates cell proliferation and differentiation[11] and is a well-known marker of squamous differentiation[12]. Although the actual factors involved in squamous and adenomatous differentiation in the current case are unknown, these molecular mechanisms may have been involved.

Currently, the clinical diagnosis of lung cancer metastasis is typically based on symptoms, a physical examination, and imaging such as X-ray and CT; pathologists' assessments of tissue samples are fundamental to this process, as they can confirm histological similarities between distant and primary lesions, using, for example, immunostains specific to tissue types. However, a distant tumor with a histology that differs from the primary lesion can be difficult to diagnose as a metastasis. In the present case, the presence of an identical *EGFR* mutation facilitated the identification of a distant lesion as a metastasis, despite its completely different histology.

We previously reported the significance of detecting an *EGFR* mutation in the cerebrospinal fluid of a patient with carcinomatous meningitis, whose primary lung lesion was not approached because of his poor performance status; detection of an *EGFR* mutation contributed to the diagnosis of lung cancer and facilitated the determination of an appropriate therapeutic protocol[13]. We found several previous reports showing the usefulness of p53 analysis in primary and metastatic lesions. In one study, a *p53* mutation analysis determined that the ovarian tumor was a metastasis from the sigmoid colon[14]. Similarly, an identical *p53* mutation confirmed that a neck nodal metastasis originated from oral squamous cell carcinoma that had been treated over five years prior[15]. The present case supports the use of genetic analysis in diagnosing distant lesions and suggests that it could be expanded to include mutations other than those in *EGFR* and *p53*, such as anaplastic lymphoma kinase[16], ret proto-oncogene[17,18], and c-ros oncogene 1, receptor tyrosine kinase[18]. Moreover, genetic analyses could help determine the origin of tumors of so-called unknown origin.

A short deletion in exon 19 and L858R in exon 21 are two major *EGFR* mutations that constitute approximately 80% of total *EGFR* mutations[3]. A mutation on codon G719 is an uncommon *EGFR* mutation that was detected only in 15 (2.4%) of 627 cases with *EGFR* mutations[3]. This amino acid substitution mutation includes G719A, G719C, G719D, and G719S, the last of which was detected in the present case. The efficacy of EGFR-tyrosine kinase inhibitor (TKI) treatment for tumors with G719 mutations was previously evaluated, and the response rate was 50% (4 of 8 cases without complex mutations)[3]. Although few cases were evaluated, progressive disease was observed in 3 cases (38%), suggesting a relatively lower effectiveness of EGFR-TKIs[3].

The frequency of *EGFR* mutations in squamous cell carcinoma is reportedly approximately 3%, which is much lower compared to adenocarcinoma or adenosquamous carcinoma[5]. Therefore, pulmonologists might wonder whether testing for an *EGFR* mutation found in only 3% of samples is cost-effective. In this regard, smoking history could be considered. The frequency of *EGFR* mutations is lower in smokers than in never-smokers (approximately 14% *vs* 60%)[19]. However, in one study of 87 patients with squamous cell carcinoma, all 3 patients with *EGFR* mutations were smokers[5]. We have been analyzed *EGFR* mutation status in all squamous cell carcinomas as well as adenocarcinomas.

In conclusion, a matching *EGFR* mutation in a distant lesion can validate a diagnosis of lung cancer metastasis, even if it differs histologically from the primary lesion. In the era of molecularly-targeted treatments for NSCLC, a combined pathological diagnosis and genetic analysis could lead to more appropriate therapeutic selections, more precise diagnoses, and a better understanding of histogenesis.

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EDITORIAL

Growth factors and fetal lung development mediated by mechanical forces

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Abstract

Incomplete development of the lung secondary to extreme prematurity or pulmonary hypoplasia causes significant morbidity and mortality during the neonatal period. Currently, the management is primarily supportive with no specific treatment to stimulate the growth and development of the lung. Mechanical forces generated inside the fetal lung by constant distention pressure and "breathing-like movements" are a major determinant of fetal lung development. However, the mechanisms by which lung cells sense these mechanical signals to promote lung development are not well-defined. Tracheal ligation has been used not only experimentally but also in human fetuses affected by severe congenital diaphragmatic hernia to stimulate lung growth and decrease the degree of pulmonary hypoplasia. Past investigations suggested that the increase of intratracheal pressure after tracheal ligation releases soluble factors that are critical for lung development. Studies from our laboratory have shown that mechanical strain of fetal type Ⅱ epithelial cells, simulating mechanical forces in utero, promotes differentiation *via* release of epidermal growth factor receptor ligands heparin binding epidermal growth factor-like growth factor and transforming growth factor alpha. The identification of growth factors released by mechanical forces that are important for normal lung development could lead to novel treatments to accelerate lung development.

Key words: Mechanical forces; Lung development; Tracheal ligation; Growth factors

Core tip: Identification of soluble factors released to the lumen of the lung after tracheal occlusion could lead to new therapeutic opportunities to accelerate lung development in newborns affected by extreme prematurity or pulmonary hypoplasia.

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INTRODUCTION

Pulmonary hypoplasia secondary to congenital diaphragmatic hernia, oligohydramnios, *etc.*, is an important cause of neonatal morbidity and mortality. Indeed, pulmonary hypoplasia is the most common finding (up to 26%) in neonatal autopsies[1]. Furthermore, more than 20000 babies are born every year in the United States before 27 wk of gestation (canalicular stage of lung development). These disorders have in common an incomplete development of the lungs. In addition to the risk of death, these conditions can also cause severe respiratory distress at birth and serious long-term morbidities[2]. Currently, the management is primarily supportive and there is not specific treatment to stimulate the growth/development of the lungs.

The lungs are unique in that their growth and development depends primarily on extrinsic factors and spe-

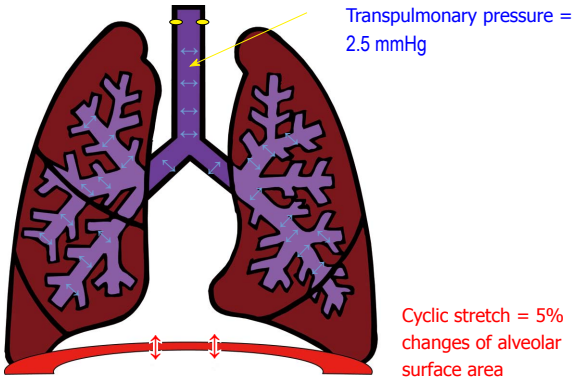

Figure 1 Mechanical forces generated by constant distension pressure (blue) and episodic breathing-like movements (red) are critical for normal fetal lung development.

cifically on mechanical forces[3-7]. During gestation, the epithelium of the lung secretes fluid creating a constant distension pressure in the lumen of the lung of approximately 2.5 mmHg[8]. Moreover, the fetus makes episodic breathing movements (FBM) starting in the first trimester and increasing in frequency up to 30% of the time by birth[9] (Figure 1). It is clear from experimental animals that drainage of lung fluid volume[10] or abolition of FBM[11,12] lead to lung hypoplasia. Therefore, both tonic hydrostatic distension and cyclic mechanical deformation provide physical signals necessary for normal fetal lung development. However, the mechanisms by which lung cells sense these mechanical signals to promote lung development are not well-defined.

Tracheal ligation has been used experimentally[13] and in humans fetuses affected by congenital diaphragmatic hernia[14] as a mechanism to increase the intraluminal pressure of the lung, accelerate development and minimize the degree of pulmonary hypoplasia. However, this approach has a high rate of complications such as preterm labor, premature rupture of membranes and even death[15] and limitations and inability to be used in other forms of pulmonary hypoplasia. Therefore, a different approach to this problem is to investigate how mechanical forces promote lung development and use that information to stimulate lung development.

Past investigations in fetal lambs have shown that lung fluid composition after tracheal ligation was critical to promote lung development, since acceleration of growth and differentiation was not observed when lung fluids were replaced with normal saline[16,17]. These studies suggest that increased intratracheal pressure after tracheal ligation releases soluble factors that are important for lung development. This hypothesis is supported by previous *in vitro* studies from our laboratory in which fetal type II epithelial cells isolated during the canalicular stage of lung development were exposed to mechanical strain mimicking mechanical forces in lung development. Our data showed (Figure 2) that mechanical strain cleavages and releases the soluble, mature forms of epidermal growth factor receptor (EGFR) ligands heparin binding epidermal growth factor-like growth factor and transforming growth factor alpha (TGF-α)[18,19]. Release of these soluble factors bind and activate the EGFR *via* autocrine or paracrine signaling and promote differentiation of type II cells *via* the extracellular signal-regulated kinase signaling pathway (Figure 3).

The identification of soluble factors released by mechanical forces that are important for normal lung development could lead to novel avenues to accelerate lung development. Potential translational research applications would be prenatal administration to fetuses affected by pulmonary hypoplasia secondary to congenital diaphragmatic hernia or oligohydramnios or fetuses with borderline viability (22-24 wk) and at risk for delivery. Another theoretical application would be postnatal administration *via* the endotracheal tube. This is just an example on how the information obtained from these *in vitro* mechanistic studies could have the potential for clinical applicability. However, the therapeutic applicability of TGF-α in human neonatal and adult lung diseases is questionable since animal studies have demonstrated that transgenic overexpression of TGF-α disrupts neonatal lung development[20] and induces adult lung fibrosis[21]. In addition, increased epithelial EGF receptor signaling mediates airway hyperreactivity and remodeling in a mouse model of chronic asthma[22]. Therefore, before considering their use in humans, rigorous experiments in animal models are required first to demonstrate the effectiveness of this therapy and the lack of untoward side effects.

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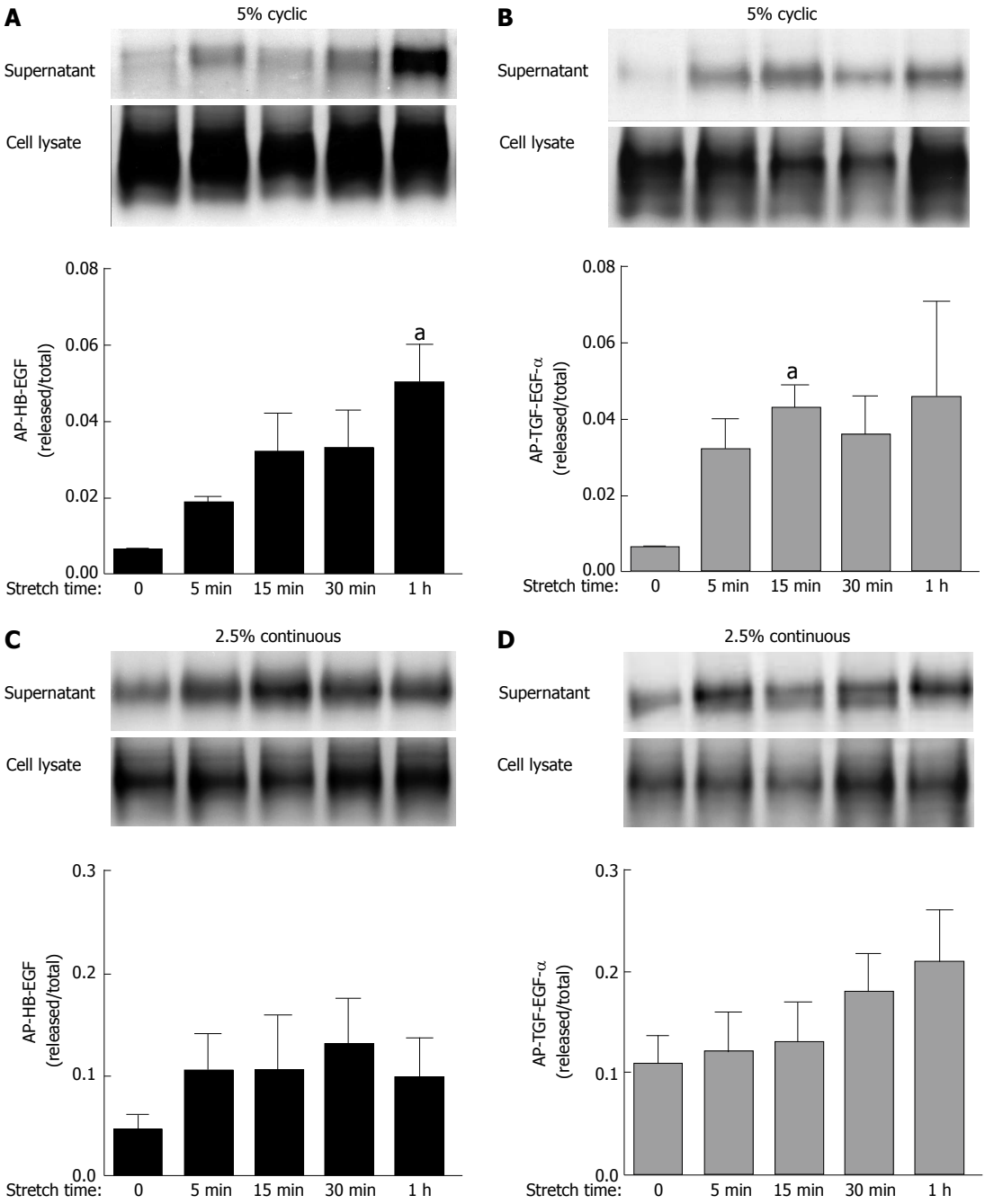

Figure 2 Cyclic mechanical strain releases heparin binding epidermal growth factor-like growth factor and transforming growth factor alpha ligands. Fetal type Ⅱ cells were transfected by electroporation with cDNA constructs encoding alkaline phosphatase-tagged heparin binding epidermal growth factor-like growth factor (HB-EGF) and transforming growth factor alpha (TGF-α) ligands. 24 h later, cells were exposed to 5% cyclic strain or 2.5% continuous strain for the indicated periods of time. Samples were processed to assess ligand-release into the supernatant using the alkaline phosphatase shedding assay protocol. Data are expressed as the intensity of each alkaline phosphatase (AP) supernatant band divided by total AP (supernatant + cell lysate). Upper panels shown representative blots. Data in the lower panels are from three independent experiments. $^{a}P < 0.05$ *vs* unstretched samples for 1 h.

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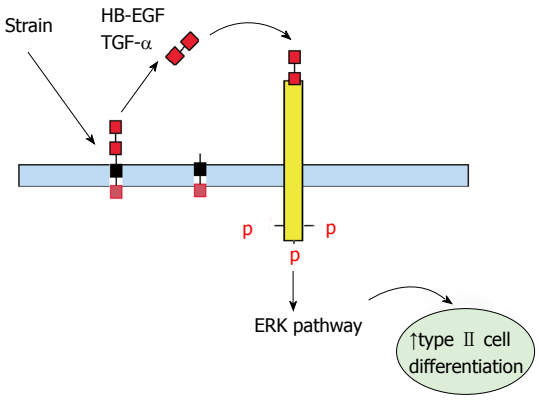

Figure 3 Mechanistic model. How mechanical forces promote differentiation of fetal type Ⅱ epithelial cells *via* release of soluble growth factors heparin binding epidermal growth factor-like growth factor (HB-EGF) and transforming growth factor alpha (TGF-α) with subsequent binding and activation of the epidermal growth factor receptor and extracellular signal-regulated kinase (ERK) signaling pathway.

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REVIEW

Von Hippel-Lindau protein and respiratory diseases

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Abstract

Von Hippel-Lindau protein (pVHL) was first identified as a tumor suppressor gene as mutations in the *VHL* gene predispose individuals to systemic benign or malignant tumors and cysts in many organs, including renal cell carcinoma of the clear-cell type and hemangioblastoma. Although pVHL is best known to act as a component of ubiquitin protein ligase for the proteasomal degradation of hypoxia inducible factor (HIF)-α, pVHL also interacts with extracellular matrix proteins and cytoskeleton, regulating extracellular matrix assembly, cell signaling, and many other cellular functions. Recent studies suggest that pVHL contributes to many lung diseases, including pulmonary arterial hypertension, lung cancer, pulmonary fibrosis, and acute respiratory distress syndrome. Mutation or loss of function of pVHL activates HIF and induced expression of vascular endothelial growth factor, endothelin-1, and FoxM1, leading to pulmonary arterial hypertension. Loss of pVHL in lung cancer cells promotes epithelial-mesenchymal transition and cancer migration and invasion while decreasing lung cancer cell proliferation and colonization. In patients of idiopathic pulmonary fibrosis, elevated expression of pVHL induces expression of fibronectin/integrin α5β1/focal adhesion kinase signaling, resulting in fibroproliferation and fibrosis. In alveolar epithelial cells, pVHL mediates Na, K-ATPase degradation in an HIF independent pathway, causing decreased edema clearance during hypoxia. These studies suggest that pVHL plays key roles in the pathogenesis of many lung diseases, and further investigations are warranted to elucidate the underlying molecular mechanisms.

Key words: Von Hippel-Lindau protein; Lung cancer; Pulmonary fibrosis; Pulmonary hypertension

Core tip: Although von Hippel-Lindau protein (pVHL) was first identified as a tumor suppressor and is best known as a component of ubiquitin protein ligase for the proteasomal degradation of hypoxia inducible factor (HIF)-α, recent studies suggest that pVHL contributes to many lung diseases in both HIF-dependent and HIF-independent pathways. Loss of pVHL promotes pulmonary arterial hypertension *via* activation of HIF. In lung cancer, loss of pVHL promotes epithelial-mesenchymal transition and cancer migration and invasion while decreasing cell proliferation and colonization. pVHL also induces fibronectin/integrin α5β1/focal adhesion kinase signaling to facilitate fibrogenesis. pVHL mediates Na,K-ATPase degradation to cause decreased edema clearance during hypoxia.

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VON HIPPEL-LINDAU PROTEIN

The von Hippel-Lindau (*VHL*) gene was first identified in patients with von Hippel-Lindau syndrome, an autosomal dominant disease with an incidence of one in 36000

births[1]. Germline mutation of the *VHL* gene, which is located in human chromosome 3p25[2], predisposes individuals to benign or malignant tumors and cysts in many organ systems and causes clear-cell renal cell carcinoma, hemangioblastoma, pheochromocytoma in adrenal glands, pancreas tumors, endolymphatic sac tumors of middle ear, and epididymal cystadenomas in testes. There are also findings that VHL contributes to liver and pulmonary hemangiomas[3]. Individuals with VHL disease usually have an inherited mutant copy of the *VHL* gene and an inactivated wild-type allele through somatic mutation or hypermethylation.

pVHL regulates HIF activity

The *VHL* gene, cloned in 1993[4], consists of three exons and produces two alternatively spliced mRNAs, translating into two proteins pVHL^{30} and pVHL^{19}[5,6]. The larger protein contains 213 amino acids and the shorter is internally translated and is missing the first 53 amino acids. VHL^{30} is found in the nuclear, cytosolic and membrane fractions while VHL^{19} localizes mostly in the nucleus. The VHL protein contains two domains, α and β domain. The α domain serves as an elongin C binding site while the β domain acts as a substrate-recognition/docking site. VHL is found in a multiple-protein complex with Elongin B, Elongin C, Cullin (Cul) 2, and Rbx 1 (also known as ROC1/Hrt1), forming an ubiquitin protein ligase (E3) complex named VEC[7,8]. Elongin C brings VHL and Cul2 together. Cul2 is associated with Rbx1, which recognizes a cognate ubiquitin conjugating enzyme (E2).

pVHL is best known as a component of E3 for the proteasomal degradation of hypoxia inducible factor (HIF)-1/2α[9,10]. In normoxic conditions, HIF-α is hydroxylated at the conserved proline residues within the oxygen-dependent degradation domain by the prolyl-hydroxylase domain proteins[9-11]. The prolyl-hydroxylated HIF-α is recognized by pVHL, leading to poly-ubiquitination and degradation[9,10]. During hypoxia, the prolyl -hydroxylases are inhibited, HIF-α is not hydroxylated and is unable to bind pVHL, leading to its stabilization. Stabilized HIF-α forms a heterodimer with a HIF-β family member and acts as a transcriptional factor to activate expression of downstream genes that contain the HIF-responsive elements in their promoters[12]. Many of these genes are involved in promoting adaption to hypoxic conditions, including the angiogenic genes vascular endothelial growth factor (VEGF) and erythropoietin (EPO), glycolysis-involving gene phosphoglycerate kinase, and glucose transporter Glut-1, *etc.*[12,13].

pVHL was identified as a tumor suppressor because the *VHL* gene mutation was shown to be associated with tumors in the kidney[3,14]. Iliopoulos *et al*[14] and Kondo *et al*[15] demonstrated that reintroduction of the wild-type VHL into the VHL-mutated renal carcinoma cell line 768-O inhibited the tumor formation in nude mice after injection of the renal carcinoma cells. HIF activation is a crucial for carcinogenesis in absence of pVHL since HIF induces expression of angiogenic factors VEGF, EPO and platelet-derived growth factor[3,12,16]. pVHL also regulates a proliferative response to hypoxia since the loss of pVHL leads to constitutively elevated Cyclin D1 and abnormal proliferation rate of the renal carcinoma cells[17,18]. Additionally, the pVHL mutant is known to increase Akt-mTOR signaling[19], induce fibroblast growth factor receptor signaling[20,21], disrupt cilia formation[22], and downregulate p53[23] to promote cancer initiation and progression.

pVHL regulates extracellular matrix assembly

In the other characterized pVHL pathway, fibronectin interacts with pVHL in cells and co-localizes with a fraction of pVHL on the endoplasmic reticulum (ER)[24]. Moreover, renal carcinoma cells with loss of pVHL have a defective assembly of extracellular fibronectin matrix[24]. These data support a direct role of pVHL in fibronectin matrix assembly[24]. Although pVHL null cells have intact extracellular matrix (ECM) expression and secretion levels[25,26], pVHL deficient 786-O renal cancer cells are unable to organize an adequate matrix even in the presence of an excess of exogenous fibronectin, suggesting that proper assembly of fibronectin matrix requires adequate interaction between fibronectin and its receptor[27]. Further studies demonstrated that association of fibronectin with αvβ1 integrin is crucial for the fibronectin matrix assembly. In cells with pVHL expression, αv integrin forms "patch-like" focal contacts and relocates to the intercellular junctions where αv and β1 integrin form large fibrillar adhesions and anchor firmly to the fibronectin substrate. In the absence of pVHL, αv integrin focal contacts remain unchanged; however, they are unable to assemble β1 fibrillar adhesions[27]. Activation of β1 integrin by exogenous divalent cations or activating antibodies partially restores the capability of VHL null cells to assemble β1 fibrillar adhesions and fibronectin fibers[27]. These studies suggest that pVHL is an important regulator of αvβ1 integrins and is essential for the formation of β1 fibrillar adhesions and the organization of extracellular fibronectin.

The interaction with pVHL is also necessary for collagen to be assembled into the ECM. Failure of pVHL to interact with collagen correlates with the loss of collagen network and collagen remodeling *in vitro* and *in vivo*[24,28,29]. Although pVHL can interact with type Ⅰ, Ⅱ, Ⅳ, and Ⅴ collagen in denatured conditions, the most specific binding occurs with type Ⅳ collagen, specifically the collagen Ⅳ α2 (COL4A2)[28,29]. The pVHL-COL4A2 interaction occurs on the ER where the N-terminal tail of COL4A2 protrudes from the ER lumen into the cytosol to associate with pVHL. This association requires a collagenous domain of Gly-*X*-*Y* triplets in the full-length α-chains of collagen (*X* and *Y* can be any amino acid residue but are commonly proline or hydroxyproline). However, hydroxylation of the N-terminal domain leads to disassociation of collagen from pVHL, and collagen folds into the matured triple helical conformation[29]. Taken together, these findings suggest that pVHL can bind directly to a variety of collagen chains and that this

association is critical for collagen matrix assembly.

pVHL and cytoskeleton dynamics and epithelial cilia maintenance

Accumulating evidence suggests a novel function of pVHL in cytoskeleton dynamics and epithelial cilia maintenance[30,31]. Hergovich *et al*[32,33] reported that pVHL associates with microtubules and protects them from depolymerization. Amino acids 95-123 of pVHL, particularly point mutations such as pVHL (Y98H) and pVHL (Y112H), are critical for the pVHL-microtubule interaction[32]. The pVHL-microtubule interaction appears to be indirect and is mediated by microtubule motor Kinesin-2, which is responsible for the transport of pVHL to the cell periphery along microtubules, which also stabilizes them[34,35]. Accordingly, pVHL affects the normal function of primary cilium, a microtubule-based cellular sensory organelle in the kidney. pVHL functions to stabilize microtubules in the axoneme and localizes to primary cilia, but loss of pVHL alone does not affect primary cilia structure in primary cells[36]. However, inactivation of both the pVHL and GSK3β leads to loss of cilia, suggesting that mutation of pVHL may sensitize cells to lose their primary cilia and promote the formation of cysts in the kidney[36]. In absence of functional pVHL, loss of GSK-3β activates Akt and extracellular signal-regulated kinase, inducing epithelial cell proliferation and kidney cyst formation[37]. Conversely, mice with the double deletion of VHL and phosphatase and tensin homolog, which mimics Akt activity, display cilia loss and cyst formation in the kidney[37]. These findings indicate that, although loss of pVHL function alone is insufficient to cause uncontrolled cellular proliferation and cyst formation, additional signaling such as activation of Akt and inactivation GSK-3β may allow cyst formation.

pVHL also regulates actin cytoskeletal organization and cell motility. Overexpression of pVHL translocates vinculin from the cytoplasm to the cell membrane and induces focal adhesion formation and adhesion[38]. In contrast, overexpression of pVHL also increases stress fibers which exhibit a spreading morphology, leading to reduced cell motility[38]. These results suggest that the loss of pVHL may promote cancer progression *via* destabilized actin organization and increased motility in cancer cells. Additional studies suggest that loss of pVHL results in the loss of Brick1 (Brk1), a component of the Wave/Scar pathway that regulates branched nucleation of actin fibers. Consistently, suppression of Brk1 causes abnormal vinculin distribution, loss of Arp2/3 and Wave proteins at the cellular protrusions, and abnormal actin stress fiber formation. Furthermore, suppression of Brk1 decreases proliferation, migration, and invasion in renal carcinoma cells[39]. A recent genetic study shows that germline and somatic mutations in VHL is associated with loss of *HSPC300* gene, a regulator of actin dynamics and cytoskeleton organization. Depletion of *HSPC300* causes cytoskeleton abnormalities and cytokinesis arrest in tumor cells[40]. The underlying mechanism of how pVHL regulates Brk1 and HSPC300, however, remains unclear.

VHL AND PULMONARY ARTERIAL HYPERTENSION

Pulmonary artery hypertension (PAH) is a devastating disease that results in a progressive increase in pulmonary vascular resistance, right ventricular failure, and ultimately death[41,42]. Despite recent advances in management of PAH, there is currently no cure for PAH. PAH is characterized by pulmonary arterial remodeling that includes vascular cell proliferation[41,42]. Hypoxia is a well established stimulus for the induction of pulmonary hypertension (PH) in several animal models that exhibit pulmonary artery smooth muscle cell proliferation and de-differentiation after exposure to hypoxia[43]. HIF is a master transcription factor that regulates cellular adaptation in hypoxia[44,45] and has been implicated in PH[46,47]. Furthermore, inhibition of HIF by Digoxin prevents and reverses hypoxia-induced PH[48].

Distinct from the classic VHL-associated inherited cancer syndrome, Chuvash polycythemia is defined as a new form of VHL-associated disease which is caused by the homozygosity for the C598T mutation of the VHL gene[49]. The C598T VHL mutation increases the HIF-α level and the expression of several HIF target genes including EPO and VEGF, leading to the development of polycythemia[49]. Smith *et al*[50] reported that a small group of patients with Chuvash polycythemia showed striking abnormalities in the respiratory and pulmonary vascular systems. Compared to control individuals, Chuvash polycythemia patients displayed elevated basal ventilation and pulmonary vascular tone as well as increased ventilatory, pulmonary vasoconstrictive, and heart rate responses to acute hypoxia, suggesting a role of pVHL/HIF signaling in calibration and homeostasis of the respiratory and cardiovascular system[50]. Other studies have also shown that patients with Chuvash polycythemia had a higher systolic pulmonary artery pressure and plasma concentration of endothelin-1 and VEGF than control individuals[51,52], suggesting that, in Chuvash polycythemia patients, the VHL mutation may activate HIF and upregulate endothelin-1 and VEGF, leading to pulmonary hypertension.

In a strain of mice harboring the C598T VHL mutation (VhlR/R mice) as a model for Chuvash disease, Hickey *et al*[53] found that lungs from VhlR/R mice showed pulmonary vascular remodeling, hemorrhage, edema, and macrophage infiltration resembling pulmonary hypertension. Interestingly, they found that the C598T VHL mutation in mouse lungs did not change the expression of HIF-1α and its targets whereas the expression of HIF-2α protein and HIF-2α-regulated genes such as Serpine1 were induced[53]. Moreover, heterozygosity of HIF-2α (HIF-2α+/-), but not HIF-1α (HIF-1α+/-), suppressed both polycythemia and pulmonary hypertension in the VhlR/R mice and attenuated vascular remodeling in VhlR/R lungs, suggesting a selective and critical role for HIF-2α in the pulmonary pathology in

C598T VHL mutants[53]. This concept is strengthened by the study of Formenti *et al*[54] in which they showed that patients with HIF-2α gain-of-function mutations developed pulmonary hypertension with increased cardiac output, heart rate, and pulmonary ventilation. Recently, we found that HIF-2α, but not HIF-1α induced expression of FoxM1, leading to the induction of Aurora A kinase and Cyclin D1 and cell cycle progression which resulted in the proliferation of pulmonary artery smooth muscle cells[55]. A recent study reported novel VHL mutations in exon2 (G376A) and exon3 (C548T) of the lungs in a 2-month-old boy with severe polycythemia and pulmonary arterial hypertension[56]. Together, these studies suggest that, in Chuvash polycythemia, VHL mutations activate HIF, particularly HIF-2α, and contribute to the development of pulmonary arterial hypertension.

VHL AND LUNG CANCER

Lung cancer is the most common cause of cancer-related death in the United States[57]. There are two main types of lung cancer, small cell lung cancer (SCLC), and non-small cell lung cancer (NSCLC)[57]. SCLC is more aggressive and is strongly associated with cigarette smoking. SCLC arises in the larger airways: the primary and secondary bronchi. NSCLC accounts for 80% of lung cancer cases and is divided into three subcategories: squamous cell carcinoma, large cell carcinoma, and adenocarcinomas. Adenocarcinomas account for 30% of NSCLC cases and usually arise from epithelial cells in peripheral lung tissue. Recently, the population of adenocarcinomas cases in nonsmokers has been rising[58,59]. As in other solid tumors, lung cancer cells proliferate at a rate that exceeds the oxygen supply, resulting in regions of low oxygen tension (hypoxia)[60,61]. Tumor cells adapt to hypoxia by inducing genes involved in angiogenesis or glucose metabolism *via* HIF[61-63]. Hypoxia induces epithelial-mesenchymal transition (EMT) in tumor cells. EMT is a molecular and cellular process during which epithelial cells lose cell-cell interactions and apico-basal polarity and acquire mesenchymal and migratory properties[64-67]. EMT is featured with changes on cell morphology and genetic markers including the disappearance of an epithelial marker such as E-cadherin and acquisition of mesenchymal markers such as α-smooth muscle actin (α-SMA) and vimentin[68]. The significance of EMT in tumor metastasis is recently evidenced in a few tumor models[69]. Many researchers observed the loss of epithelial characteristics paired with the gain of mesenchymal markers in the invasive front of various cancers, pointing to a possible contribution of EMT in tumor metastasis[70]. EMT leads to increased tumor cell invasiveness and metastatic potential and resistance to radiation-induced cell death, which are the main causes of cancer death[61-63,66,71].

It is well established that in renal carcinoma, mutation of pVHL leads to stabilization of HIF, which drives angiogenesis and cancer development. However, the role of pVHL in lung cancer is less known. Lungs express levels of pVHL comparable to kidneys[72,73], and loss of *VHL* allele frequently occurs in patients with NSCLC[74-76], suggesting that pVHL likely contributes to lung cancer development. Indeed, we showed that suppression of pVHL in lung cancer cells induced EMT and increased migration and invasion[77], which is consistent with the observation that loss of pVHL promotes EMT and invasion in renal carcinoma cells[78-80]. We have shown that hypoxia induces lung cancer cell EMT and invasion and migration *via* a HIF-dependent pathway[81,82]. Therefore, loss of pVHL may mediate EMT through HIF signaling. However, we have found that HIF activation alone is not sufficient to induce EMT in lung cancer cells[81], indicating that a HIF independent pathway is also contributing to EMT when pVHL is absent. Surprisingly, our study suggests that loss of pVHL repressed lung cancer cell proliferation *in vitro* and decreased colonization *in vivo*[77], suggesting that loss of pVHL may limit lung cancer development. Consistently, loss of pVHL also reduces cell proliferation in fibroblasts, mammary epithelial cells, and chondrocytes and inhibits fibrosarcoma[83-85]. Since loss of pVHL induces HIF, we investigated the role of HIF in lung cancer cell proliferation and colonization. We found that constitutively active HIF increased lung cancer cell colonization whereas dominant negative HIF inhibited lung cancer colonization[77]. Thus, loss of pVHL causes reduced lung cancer cell proliferation and colonization in a HIF-independent pathway. We showed that suppression of pVHL decreased integrin and phosphorylated focal adhesion kinase (FAK) levels, suggesting that knockdown of pVHL represses lung cancer cell proliferation and colonization *via* decreased integrin/FAK signaling. Taking these together, we conclude that the role of pVHL/HIF in lung cancer development and progression may be determined by the status of HIF activity and pVHL status and stage of the lung cancer. Further studies with spatially and temporally controlled pVHL expression are warranted.

VHL AND PULMONARY FIBROSIS

Idiopathic pulmonary fibrosis (IPF) is another devastating lung disease with poor patient survival. It is characterized by progressive scarring of the lungs and elevated respiratory failure[86-89]. The etiology of IPF is unknown, and currently there is no effective treatment[90,91]. Pulmonary fibrosis is characterized by proliferation of lung fibroblasts and exaggerated deposition of ECM proteins, especially collagen and fibronectin[86,89]. ECM proteins interact with integrins, which act as their cell membrane receptors and thus initiate FAK signaling cascades involved in cell proliferation[92,93]. The proper assembly of fibronectin and collagen matrix requires the presence of pVHL, and loss of pVHL prevents fibroblast proliferation[24,28,29,83], suggesting that pVHL may play a role in the pathogenesis of pulmonary fibrosis.

A microarray study showed that lungs of IPF patients expressed higher levels of pVHL mRNA than lungs of

control individuals[94]. We have reported that lungs of fibrotic patients expressed elevated levels of pVHL in fibroblastic foci. In an experimental lung fibrosis model induced by Bleomycin, pVHL expression is also elevated in mouse lung fibroblasts but not in alveolar type Ⅱ cells. Ectopic overexpression of pVHL in lung fibroblasts increased the expression of fibronectin, collagen, and the α5 integrin subunit as well as lung fibroblast proliferation[95]. In addition, overexpression of pVHL induced FAK phosphorylation and activation[95]. Consistently, inhibition of the fibronectin/integrin α5β1/FAK signaling pathway diminished pVHL mediated cell proliferation while activation of α5 and β1 integrin fibronectin/integrin α5β1/FAK signaling pathway increased proliferation of fibroblasts. Moreover, pVHL is necessary for fibroblast proliferation after treatment of TGF-β1, a potent pro-fibrotic cytokine. These results suggest that elevated expression of pVHL results in the aberrant fibronectin expression and activation of integrin/FAK signaling, leading to fibroblast proliferation and fibrosis[95]. Although fibroblast activation (increased expression of collagen) and differentiation to myofibroblast (elevated levels of α-smooth muscle actin) are critical steps in fibrogenesis, we found that pVHL increased collagen expression but not the expression of α-smooth muscle actin, indicating pVHL may participate in earlier events in the formation of the fibroblastic foci, such as fibroblast proliferation[95]. Interestingly, this gain of function of pVHL did not alter HIF activity, suggesting that pVHL plays a HIF-independent role in the pathogenesis of pulmonary fibrosis. However, Hickey and colleagues have recently reported that the mutation of pVHL at codon 200 (R200W) causes Chuvash disease with pulmonary vascular remodeling and hypertension[53]. Older mice with this mutation contain elevated ECM deposition and develop fibrosis. In terms of the mechanism underlying the fibrosis in R200W mutation mice, the authors speculated that fibrosis is partially secondary to hemorrhage, edema, and impaired endothelial integrity and partially due to HIF2 activity[53]. Thus, additional studies are warranted to elucidate the HIF-dependent and HIF-independent roles of pVHL in pulmonary fibrosis.

VHL AND ACUTE RESPIRATORY DISTRESS SYNDROME

The acute respiratory distress syndrome (ARDS) is a key cause of acute respiratory failure. ARDS can be caused by pneumonia, sepsis, aspiration of gastric contents, and major trauma, and it is characterized by pulmonary edema and severe hypoxemia[96]. Most patients with ARDS who cannot clear alveolar edema efficiently have worse outcomes[97-99]. Alveolar fluid clearance is effected by active Na^+ transport across the alveolar epithelium through apical Na^+ channels and basolateral Na, K-ATPase[100]. Na^+ enters alveolar epithelial cells *via* the apical Na^+ channels and is extruded by the basolateral Na, K-ATPase, with water following the osmotic gradient into the interstitium and pulmonary circulation, leading to the clearance of edema[100-103]. Over-expression of Na, K-ATPase has been shown to increase alveolar fluid clearance[104-107]. In contrast, in several models of lung injury, Na, K-ATPase activity in alveolar epithelial cells is decreased, resulting in decreased lung fluid clearance[97,102,108-111].

Hypoxia is common in the lungs of patients with ARDS and inhibits Na, K-ATPase activity, thereby decreasing the rate of alveolar fluid reabsorption and worsening the outcomes of ARDS patients[110-112]. Dada *et al*[113] reported that hypoxia increases mitochondrial reactive oxygen species (ROS) production, which activates protein kinase C (PKC). PKC phosphorylates Ser18 of Na-K-ATPase α1 subunit, triggering the endocytosis of Na-K-ATPase[113]. Although short-term effects of hypoxia appear to be reversible upon reoxygenation with no significant degradation of total Na-K-ATPase in the whole cell lysate, prolonged hypoxia results in degradation of alveolar epithelial Na-K-ATPase *via* the ubiquitination/proteosome pathway[113,114].

We found that alveolar epithelial Na-K-ATPase is downregulated in a pVHL-dependent manner[115]. In the presence of pVHL, hypoxia promoted the degradation of plasma membrane Na-K-ATPase; in the absence of pVHL, hypoxia is unable to degrade plasma membrane Na-K-ATPase. pVHL mutants and dominant-negative Ubc5 (an ubiquitin conjugating enzyme, E2) prevented Na-K-ATPase from degradation, suggesting a functional pVHL E3 ligase is essential for Na-K-ATPase degradation during hypoxia[115]. Interestingly, HIF overexpression is not sufficient to induce Na-K-ATPase degradation, and loss of HIF does not prevent hypoxia-mediated degradation of Na-K-ATPase. Therefore, pVHL-mediated Na-K-ATPase degradation is likely HIF independent[115]. Moreover, generation of reactive oxygen species was necessary for pVHL-mediated Na-K-ATPase degradation during hypoxia[115].

CONCLUSION

Although pVHL was first identified to be a tumor suppressor in renal carcinoma, accumulating evidence suggests that pVHL has a much broader spectrum of functions and is key to many organ dysfunctions. In this review, we summarized recent evidence that pVHL plays an essential role in a few respiratory diseases, including pulmonary arterial hypertension, lung cancer, pulmonary fibrosis, and ARDS. As in other tissues, pVHL functions in two distinct pathway in the lungs: HIF independent (fibrosis, lung cancer, and ARDS) and HIF dependent (lung cancer and pulmonary arterial hypertension). Certainly, this is only the beginning of uncovering the complex role of pVHL in lung diseases, as evidenced by a recent report which suggests pVHL expression is elevated in skeletal muscles of patients with chronic obstructive pulmonary disease (COPD)[116]. However, the challenge to dissect the function of VHL disease is to investigate the role of pVHL in a cell-type-specific, context-specific,

and temporal fashion. A more challenging task is to elucidate in detail the molecular mechanisms by which pVHL regulate these functions in the lungs as an E3 ligase and adaptor protein.

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REVIEW

Early chronic obstructive pulmonary disease: Beyond spirometry

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Abstract

The significant healthcare burden associated with chronic obstructive pulmonary disease (COPD) is driving us to improve our understanding of the natural history of this disease. Historically, the focus has been largely centred on diagnosing and treating individuals with moderate and severe disease. However, it is now recognised that the speed of decline in lung function as measured by forced expiratory volume in 1 s occurs faster in the earlier stages of the disease process. As a result, a clearer understanding of the potential benefits of treatment in early COPD is needed. It is recognised that many patients with COPD remain undiagnosed in the community which has prompted global case-finding initiatives. In this review we discuss the difficulties in diagnosing COPD in its early stages, examine the role of case-finding and look at the evidence for early intervention with therapeutic agents. There is a growing interest in the phenotypic variation amongst patients with COPD and we explore the role of phenotyping in early COPD and its potential benefits in providing a more individualised approach to COPD management. The majority of patients with COPD are known to die from non-respiratory causes such as cardiovascular disease. The mechanistic link is thought to relate to systemic inflammation, causing us to question whether earlier interventions could have a beneficial impact on the burden of co-morbidities for patients with COPD.

Key words: Chronic obstructive pulmonary disease; Chronic bronchitis; Pulmonary emphysema; Early disease; Mild; Case-finding; Phenotypes; Treatment

Core tip: In this review article we outline the difficulties in diagnosing chronic obstructive pulmonary disease (COPD) in its early stages and examine the role of case-finding initiatives. In addition we explore the evidence for early intervention with therapeutic agents and consider the impact of phenotyping in early disease, highlighting the potential benefits to a more individualized approach to COPD management.

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major global public health problem with a significant associated economic burden on both developing and higher

income countries. In 2004, COPD was the fourth leading cause of death worldwide[1] and due to projected increases in tobacco use, it has been predicted by the World Health Organisation that it will become the third leading cause by 2030[2]. The Global Initiative for COPD (GOLD) has defined it as "a common preventable and treatable disease which is characterised by persistent airflow limitation that is usually progressive and associated with enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases"[3]. The pathophysiology of COPD is complex resulting from a variety of gene-environment interactions and there is considerable phenotypic heterogeneity expressed amongst disease sufferers[4].

There remains a lack of therapeutic interventions with strong evidence of disease modifying or curative potential, so early diagnosis and prevention strategies including smoking cessation are essential in our approach to COPD healthcare. Within the last decade studies have shown the prevalence of COPD to be much higher than previously realised[5,6] and the proportion of patients with COPD that remain undiagnosed has been reported to be as high as 66%-73%[7,8]. This has stemmed interest in "case-finding" initiatives by screening high risk individuals with the potential to diagnose COPD early and in some cases even before symptoms develop. As a result, it is likely that the number of patients diagnosed with "early" or "mild" disease is likely to increase, amplifying the need for clear management strategies for this cohort of COPD patients. In this article we will explore the definition of "early COPD" and examine the emerging concepts with respect to disease classification, monitoring of disease progression and therapeutic interventions, highlighting current controversies within these fields.

DIAGNOSING COPD

Current guidelines and potential pitfalls

Spirometry is the primary tool utilised by respiratory physicians worldwide in the diagnosis of COPD. The GOLD and joint American Thoracic Society and European Respiratory Society guidelines advise physicians to consider spirometry in patients presenting with symptoms of chronic cough, dyspnoea or sputum production with a history of exposure to a risk factor such as smoking or occupational dust. In the context of such symptoms, the presence of a post-bronchodilator FEV_1/FVC (forced expiratory volume in 1 s/forced vital capacity) ratio < 0.7 demonstrates incompletely reversible airflow limitation and hence a diagnosis of COPD[3,9]. The FEV_1 as a percentage of its predicted value for the patient's sex, age and height is used to sub-classify patients with respect to the severity of their airflow obstruction (mild $FEV_1 \geqslant 80\%$, moderate $\geqslant 50\%$ $FEV_1 < 80\%$, severe $\geqslant 30\%$ $FEV_1 < 50\%$, very severe $\leqslant 30\%$)[3]. Advances in technology including the advent of simple to use hand held spirometers makes it a convenient test which can be easily performed in the outpatient clinical setting however, it is important to also recognise the potential drawbacks of using the fixed "FEV_1/FVC ratio < 0.7" approach to diagnosing COPD.

Although this fixed cut-off is easy to remember it does not take in to consideration the fact that the FEV_1/FVC ratio reduces with age[10] resulting in the potential over-diagnosis of COPD in the elderly population. Hardie *et al*[11] found 35% of healthy elderly never smokers to have a pre-bronchodilator FEV_1/FVC ratio of less than 0.7, increasing to 50% of those over 80 years of age. Other approaches to interpreting spirometric values have therefore been advocated, in particular the use of statistically derived "lower limit of normal (LLN)"reference values[12]. In comparison to the fixed ratio method this has been shown to reduce the number of people potentially misclassified as having significant airflow obstruction[13-15]. The FEV_1/FVC fixed ratio cut-off has also been found to underestimate airflow obstruction in younger adults[16]. This has the potential to create missed opportunities for early intervention and may impact on the success of smoking cessation[17].

Another potential pitfall of diagnosing COPD on the basis of spirometry alone is the recognition that emphysema and airflow obstruction do not necessarily go hand in hand, particularly in the early stages of disease. In a study of 80 current smokers who underwent high resolution computed tomography (HRCT) scanning and lung function tests, 20 were found to have radiological emphysema but only 5 of these subjects had evidence of airflow obstruction (defined by the authors as a low FEV_1 and/or a low MEF_{50} using LLN cut-off)[18]. A more recent study followed up current and heavy smokers who had participated in a lung cancer screening trial. 1391 individuals had no evidence of airflow obstruction at baseline ($FEV_1/FVC > 0.7$) but 21.9% progressed to developing obstruction over a mean period of 3 years. More severe baseline radiological emphysema (quantified by a lower Perc15 value) was found to be a risk factor for developing airflow obstruction at follow up[19]. The radiation and cost involved in performing HRCT scanning are likely to limit its utility in the early investigation of patients in clinical practice and many of the patients in these studies were asymptomatic. However, it does serve to highlight the point that radiological evidence of smoking related lung damage and lung function parameters can be discordant particularly in "early COPD" and this could potentially cause smokers with normal spirometry to be falsely reassured if no other investigations are undertaken.

Role of case finding

The recognition that the majority of individuals with COPD remain undiagnosed has focused attention on methods of identifying these so called "missing millions" through case finding strategies[20-25]. There is a growing body of literature looking at different methods including utilisation of questionnaires to identify "at risk" patients and other screening tools such as peak flow (PEF) and spirometry, however at present there is no consensus of

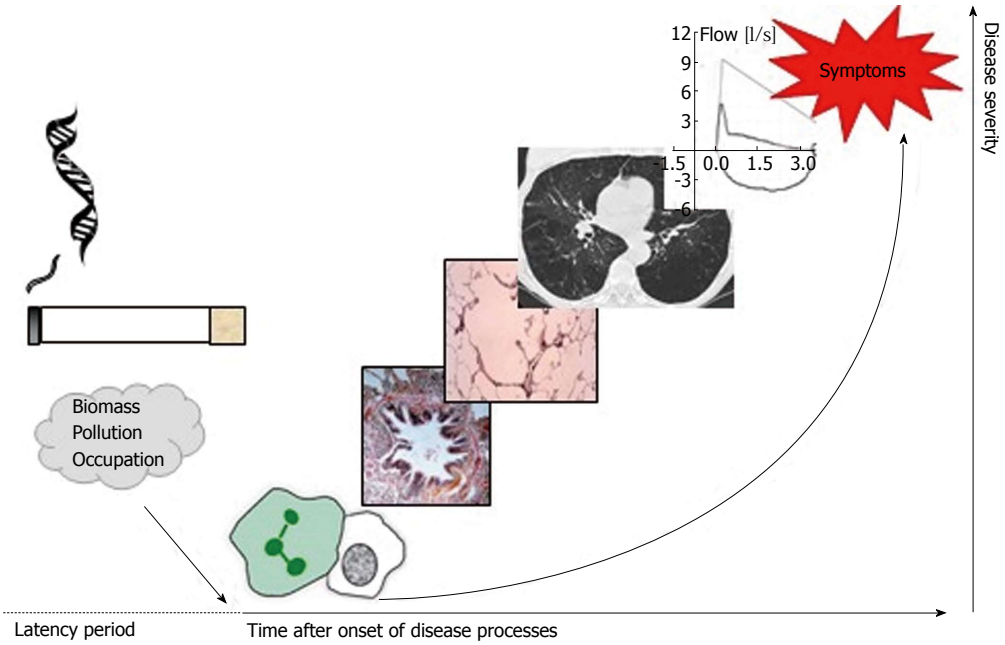

Figure 1 Disease progression in chronic obstructive pulmonary disease. Genetic factors interact with exposures, predominantly cigarette smoke, but also others such as biomass smoke to cause cellular changes in the lung, including inflammation. These cause changes that can be detected first at a pathological level - for instance subtle small airway changes or early emphysema. Later these are seen radiologically, and finally physiologically. Symptoms may occur after physiological changes occur, or in some cases earlier in the disease process. Indeed by the time symptoms develop it is likely the disease is established and may no longer be true "early" chronic obstructive pulmonary disease.

opinion on the best approach. Jithoo *et al*[22] used data from the Burden of Obstructive Lung Disease Study to compare combinations of questionnaire, PEF and spirometry strategies and concluded the pre- bronchodilator PEF followed by confirmatory spirometry to be the most cost effective approach. However this study was aimed only at the detection of moderate/severe COPD. Their reasoning for this was that the management of milder disease is only smoking cessation which should be offered to all smokers and the lack of proven benefit of pharmacological interventions in mild disease. Indeed one of the recommendations from a National Heart, Lung and Blood Institute workshop created to address case finding strategies in the United States was to target those with moderate to severe COPD[26]. Focus of attention on those with more severe disease is often highlighted due to the higher morbidity and mortality, the greater proven benefit of treatments and interventions for these groups and hence improved cost-effectiveness of a targeted approach. However, are we forgetting that all these individuals will previously have had mild disease? Could a more aggressive approach at an earlier stage not impact on the disease burden later on?

A previous review looking at the future for COPD made the important point that the terms "mild" and "early" COPD are not necessarily interchangeable given the vast variability in lung function decline in different individuals. The author highlighted the point that mild airflow obstruction in an older individual does not necessarily mean they have "early" disease[27]. The complexities of the pathophysiology of COPD and wide phenotypic variation means that at present we have no clear way of easily identifying patients early in the disease course, however a growing interest in developing biomarkers may serve to solve this problem in the future[28-30]. Decramer *et al*[31] proposed a concept of disease progression in COPD highlighting that physiological abnormalities are not necessarily present in the early stages of the disease process and that to truly identify patients with "early" COPD we need alternative diagnostic methods. This concept is shown in Figure 1.

The potential benefits and role of case finding in diagnosing patients with mild COPD does appear to be splitting opinion amongst healthcare professionals[32,33]. Some feel strongly that putting emphasis on diagnosing and treating mild COPD has the potential to divert limited resources away from interventions such as smoking cessation and from providing care for patients with "clinically-important" COPD[32]. Unfortunately we lack evidence from large randomized controlled studies (RCTs) that show efficacy of treatment in patients with mild COPD, and to meet the basic principles of a screening test we need to question "Does treatment of the developed clinical condition at an earlier stage than normal affect its course and prognosis?"[25]. As yet, we cannot prove that treatment of symptoms earlier in the disease prolongs life or quality adjusted life years conclusively, nor has any of the major drug trials proven reduced FEV_1 decline in milder disease. Nevertheless appropriate interventional studies in milder disease are likely to occur over the next few years, such that the concept of earlier treatment may become better supported by RCT evidence, and ultimately support a screening based approach.

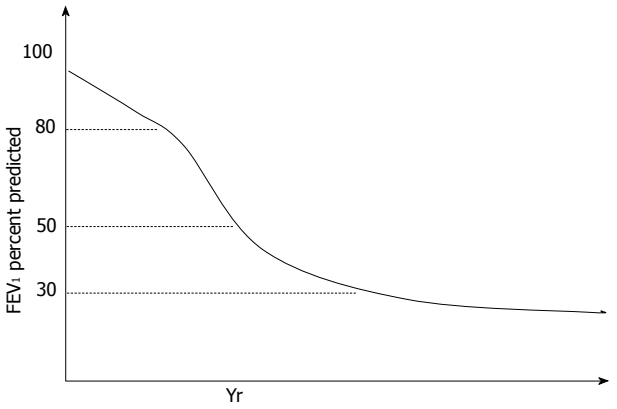

Figure 2 Lung function decline in chronic obstructive pulmonary disease. Forced expiratory volume in 1 second (FEV_1) declines at a rate of about 40 mL/year when FEV_1 is 80% or more; in some at risk populations such as those with AATD it may be much higher. When FEV_1 is between 50%-80% FEV_1 declines fastest, ranging 50-80 mL/year, then declines to a rate of 35 mL/year or less once FEV_1 reaches 30% predicted.

Identifying subgroups of patients with early COPD

Symptoms, exacerbations and disease progression: There is considerable variability between individuals with respect to the timing of onset and nature of symptoms in early COPD. There can also often be discordance between symptomatic burden and severity of airflow limitation. A study specifically designed to explore whether symptoms predict the presence of COPD found that 92% of the smokers with airflow obstruction reported symptoms such as cough, dyspnoea, sputum production and wheeze. However, symptoms were also reported in 76% of smokers with normal spirometry[34]. The study concluded that the presence of symptoms added little to the predictive value of spirometry beyond age and smoking history but didn't address the interesting question of whether the symptomatic smokers with normal spirometry are more likely to go on to develop airflow obstruction. The initial GOLD guidelines classified these individuals as GOLD stage 0 "at risk" however this classification has been removed from the more recent versions of the guidelines[3,35]. Interestingly, Miravitlles *et al*[8] found that these individuals have a significantly impaired health-related quality of life (QOL) compared to control subjects without COPD. A study aimed at validating whether individuals classified as GOLD stage 0 do go on to develop COPD, found that stage 0 was associated with a risk of excess FEV_1 decline, but multivariate logistic regression analyses found the effect of GOLD stage 0 on the likelihood of developing COPD to be small[36]. One of the likely reasons for this was that it was found to be an "unstable" feature, as after 15 years follow up many individuals with stage 0 at baseline had subsequent resolution of symptoms and only 49.4% were still classified as having at least stage 0 disease. This study highlighted the need for us to be able to identify smokers "at particular risk" as assessing symptoms alone appears to be an inadequate predictor.

Large case finding studies are also highlighting the presence of undiagnosed, asymptomatic smokers with airflow obstruction. One such study in China reported that 35.5% of those diagnosed with COPD were asymptomatic and unsurprisingly over 90% of these individuals had no previous diagnosis[37]. The authors discussed the concept that common symptoms in COPD-cough, wheeze, sputum production and dyspnoea may represent different pathological processes such as airway hyperresponsiveness, mucus hypersecretion and emphysema. An individual's perception of breathlessness in particular may be difficult to assess as those with early disease may unknowingly adapt to their dyspnoea symptoms or attribute this to being "unfit" or ageing. For these reasons some studies advocate that we should be screening all smokers over the age of 40 irrespective of symptoms[34,37]. It is also not clear whether certain symptoms are more likely to make patients seek healthcare advice - for example we might question whether "asymptomatic" COPD patients are more likely to have a "emphysema predominant" phenotype and therefore not be troubled by chronic sputum production and wheeze.

The ABCD assessment approach in the most recent GOLD guideline update recognises the importance of symptoms and exacerbation history when assessing a patient with established COPD and examining their future risk of exacerbations and decline[3]. Bridevaux *et al*[38] demonstrated in a population based cohort study that the presence of symptoms in individuals with GOLD stage 1 airflow obstruction has a significant impact on FEV_1 decline, healthcare utilisation and quality of life measures. FEV_1 decline is the most commonly used method for assessing COPD disease progression, and contrary to the classical belief that FEV_1 decline accelerates with more advanced disease, it is now recognised that FEV_1 decline is in fact faster in the earlier stages particularly GOLD stage 2 (Figure 2)[39]. It is therefore logical that we should be focusing more on investigating treatment at the earlier stages of COPD to impact on the natural history of the disease. The data available on FEV_1 decline in mild disease is limited and further large scale prospective trials are needed to better evaluate this.

It is also apparent that there is a wide variation amongst individuals with respect to the rate of FEV_1 decline stemming interest in finding ways to identify the so-called "rapid decliners" early, including the use of biomarkers and investigation for specific genetic variations[40]. However further evidence is needed to establish if early interventions can have a positive impact on disease progression in these individuals.

More advanced phenotyping methods: The concept of COPD as a heterogeneous disease is now well established, and assessing patients by simply examining the degree of airflow limitation is inadequate in reflecting this[41]. The concept of COPD "phenotypes" is not new, however there has been difficulty in creating clear, clinically relevant definitions. Han *et al*[42] emphasised that phenotyping patients should ideally provide prognostic information and

defined a COPD phenotype as "a single or combination of disease attributes that describe individuals with COPD as they relate to clinically meaningful outcomes (symptoms, exacerbations, response to therapy, rate of disease progression or death)". This provokes the interesting question of whether we should be aiming to phenotype patients with early COPD and by doing so whether we may be able to impact on their prognosis by having a more individualised approach to therapeutic strategies and interventions. They, and others, advocated the use of more detailed physiological tests as well as potentially CT scanning and biomarkers to define subgroups.

Initial work looking at proportions of COPD phenotypes and degrees of "overlap" focused on the three main subgroups of chronic bronchitis, emphysema and asthma[43]. For many Respiratory Physicians the "asthma overlap" group can often cause a diagnostic and therapeutic quandary and the true prevalence of this mixed phenotype is unknown. The fact that these patients are frequently excluded from both asthma and COPD studies means we may be doing these patients a disservice by blindly following COPD therapeutic guidelines based on evidence that may not be generalizable to this particular subgroup[44]. They will therefore be an important group for future RCTs to focus on, or provide as pre-specified subgroup analyses.

Other commonly discussed COPD phenotypes include the "frequent exacerbators"[45] and so-called "systemic inflammatory response"[46] patients. Frequent exacerbators are usually defined as those with more than 2 exacerbations per year, as used in the ECLIPSE study. The systemic inflammation group are of particular interest due to potential links with the development of comorbidities which will be discussed later. The validity of these subgroups will require prospective longitudinal studies and further research is needed to establish if they relate to differing underlying pathological processes. However, it is also realised that this method of defining phenotypes does rely on "a priori assumptions" with the potential for introducing bias[47]. This has spurred on a recent move towards utilising statistical methods such as principal component and cluster analyses as alternative approaches of identifying potential candidate phenotypes[47-50], although this has not been conducted specifically in a mild or early disease population.

The evidence for treating early COPD

Smoking cessation is an essential part of the treatment for early COPD, and is advocated unreservedly due to the strength of evidence for many health outcomes. Successful smoking cessation has been shown to halve the rate of FEV_1 decline in patients with mild to moderate COPD returning it to a level comparable with never smokers[51]. However, it has also been demonstrated that inflammation can persist within the lung many years after smoking cessation causing speculation of an autoimmune or immune activating component to COPD[52-54]. Other treatments may also be worth considering; the evidence base for these is considered below.

Inhaled therapy: There is a paucity of large RCTs aimed specifically at effects of drug treatment in early COPD. Much of the evidence of potential benefit has been extrapolated from sub-group analyses of patients from large trials aimed primarily at patients with moderate to severe disease.

Bronchodilators: In early COPD, the mainstay of pharmacological treatment advised by the current guidelines for symptomatic patients is inhaled bronchodilator therapy in the form of short and long acting β2-agonists (LABA) and antimuscarinics (LAMA)[3]. The UPLIFT study examined the long-term effects of treatment with the LAMA tiotropium compared to placebo and found it reduced exacerbations and hospitalisations, improved lung function parameters and QOL but did not significantly reduce the rate of decline in FEV_1[55]. However subsequent analyses looking specifically at the subgroup of patients with GOLD stage 2 disease found the rate of decline in the post-bronchodilator FEV_1 to be lower in the tiotropium group compared to placebo[56]. The effect was small, calling into question the clinical relevance however it did raise the possibility that early intervention with LAMAs may have the potential to affect the course of disease in patients with moderate COPD. One limitation of this post-hoc analysis was that it only looked at a subcategory of GOLD stage 2 patients with an FEV1 of $\leqslant$ 70%. When a further analysis of individuals with an FEV_1 of 60%-78% was undertaken, a trend for reduction in post-bronchodilator FEV_1 decline was still seen (41 mL/year in tiotropium group, 49 mL/year in placebo group) but this no longer met statistical significance (P = 0.07)[57]. Significant improvements in symptoms, a reduction in exacerbation frequency and mortality were demonstrated however, supporting the use of tiotropium in patients with moderate airflow obstruction.

A small RCT specifically looking at efficacy in mild to moderate disease ($FEV_1 \geqslant 60\%$) found that over a 12 wk period tiotropium significantly improved FEV_1 and FVC compared to placebo[58]. Significant improvements in dyspnoea and QOL were not seen, perhaps due to study limitations such as the questionnaires used, sample size and length of study. Bronchodilator therapy may also benefit lung function in symptomatic GOLD stage 1 patients: in response to the administration of nebulised ipratropium bromide, O'Donnell *et al*[59] demonstrated improvements in forced expiratory flow rates, reduction in residual volumes and specific airway resistance and found a reduction in end expiratory lung volumes which was associated with less severe dyspnoea during exercise.

LABAs became a widely used treatment for COPD following studies that demonstrated their ability to improve lung function and provide symptomatic benefit to patients[60,61]. The most commonly used agents are salmeterol and formoterol, which are both twice daily LABAs with a 12 h duration of action. However, none

of the major RCTs examining their efficacy in COPD included patients with an FEV_1 of greater than 70% predicted[60-69]. It therefore remains unknown whether they could provide any benefit to patients with symptomatic GOLD stage 1 disease. Combining different classes of bronchodilators is also advocated in the guidelines and deemed preferential to increasing doses of a single agent due the potential for side effects. In recent years new 'Ultra-LABAs' such as indacterol and vilanterol have been developed which have a 24 h duration of action[70]. Clinical trials investigating their use in combination with once daily LAMAs are currently underway[71]. One small RCT published in 2010 assessed the efficacy of QVA149 (a once daily combination inhaler containing the LABA indacterol and LAMA glycopyrronium) and demonstrated significant improvements in FEV_1 compared to monotherapy with indacterol or placebo. Again, GOLD stage 1 individuals were not included in this trial. However, it is interesting to speculate whether in the future these once-daily combination bronchodilators could play a role for patients with mild disease who remain symptomatic despite monotherapy with LAMAs or LABAs. The once daily administration is likely to be advantageous in terms of compliance and there may be less concern over the side effect profile in comparison to using inhaled corticosteroids.

One question that remains unanswered is whether patients found through case-finding initiatives with asymptomatic airflow obstruction should be commenced on bronchodilator therapy. There is no evidence to support this approach at present and issues of compliance with therapy and cost-effectiveness are likely to have an impact on treatment decisions for this cohort. However if future studies demonstrate the reduction in exacerbation frequency and mortality seen in the post-hoc analyses from the UPLIFT trial[57] then a more aggressive treatment approach may be warranted.

Inhaled corticosteroids: The current guidelines do not advocate the use of monotherapy with inhaled corticosteroids (ICS) in the treatment of COPD[3,9,72]. The Lung Health Study demonstrated that treatment with the ICS triamcinolone is associated with a reduction in airway reactivity and respiratory symptoms in patients with an FEV_1 of 30%-90% predicted, however, there was no impact on FEV_1 decline[73]. Similarly another placebo controlled trial examining the efficacy of inhaled budesonide in patients with an $FEV_1 \geqslant 50\%$ (corresponding to GOLD stage 1 and 2) who continued to smoke found that ICS use was associated with an initial increase in FEV_1 but no effect on long term decline[74].

Combination inhalers containing ICS and LABA are in widespread use for COPD patients with more advanced disease, and evidence of their potential benefit at earlier stages appears to be building. The TORCH study was a large multicentre, randomised, placebo controlled trial examining the effect of combination LABA/ICS inhaler therapy (salmeterol and fluticasone proprionate) on survival in COPD[65]. One of the inclusion criteria was a pre-bronchodilator FEV_1 of $\leqslant 60\%$ however a post-hoc analysis established the benefits of a reduction in exacerbations, improved health status and FEV_1 applied to patients across all the GOLD stages included in the study and therefore promoted it as an effective treatment for GOLD stage 2 disease[75]. However it is important to note that only GOLD stage 2 patients with an FEV_1 of 50%-60% predicted were included in this study. A subgroup analysis from a smaller RCT comparing "triple therapy" with fluticasone/salmeterol and tiotropium *vs* monotherapy with tiotropium revealed that triple therapy was associated with a significant improvement in FEV1 and QOL scores with no increase in adverse events in GOLD stage 2 patients[76]. Neither of these studies included GOLD stage 1 patients but a recent study specifically aimed at examining the physiological derangements and impact of combination LABA/ICS treatment in mild to moderate COPD found there were significant improvements in airway function as measured by FEV_1, functional residual capacity, inspiratory capacity and specific airways resistance both at rest and during exercise[77]. However these improvements did not translate into any symptomatic benefit in terms of dyspnoea or improved exercise tolerance.

One important consideration with ICS and ICS/LABA combination therapies is concern over the potential side effects such as reduction in bone mineral density, bruising and an increased risk of pneumonia[65,78,79]. In weighing up the risks and benefits for patients with mild disease we are hampered by the lack of firm evidence of benefit.

Future directions for early COPD treatment

Will phenotyping alter our treatment approach?

Proof of differential responses of COPD subgroups to therapeutic interventions is emerging. For example, in a subset of patients with chronic bronchitis and severe COPD, Roflumilast (a phostphodiesterase-4 inhibitor) has been shown to reduce exacerbation frequency and improve lung function[80]. Another study demonstrated that there was a greater reduction in the odds of exacerbations (45% *vs* 25%) in stable chronic bronchitis patients following the use of pulsed moxifloxacin if they had purulent or mucopurulent sputum at baseline. The presence of chronic bronchitis is known to be associated with a higher risk of exacerbations[81] which in turn puts patients at risk of a more rapid decline in lung function[82] which emphasises the clinical relevance of these findings. A subgroup of COPD patients that have been shown to gain greater benefit from higher dose ICS therapy are those with sputum eosinophilia[83-85], a controversial phenotype for many, and one which is key to differentiate from asthma. A recent article promoting the concept of treating by phenotype discusses the fact that COPD-asthma overlap patients should be treated with ICS in addition to LABAs irrespective of the severity of airflow obstruction[86]. Circulating eosinophils have also been

found to predict response to steroid treatment during COPD exacerbations[87]; this marker may be more practical for many centres to use than sputum eosinophilia.

As with the majority of therapeutic studies in COPD, patients with mild disease were generally excluded from the studies discussed above, however the results should certainly cause us to question whether early characterisation of COPD patients with respect to phenotype using relatively simple methods such as history taking for chronic bronchitis symptoms, sputum colour charts and sputum induction for cell count analysis could allow for an earlier targeted approach to management with the potential to make a significant impact on the clinical course of the disease.

Systemic inflammatory response and comorbidities in early COPD: The systemic consequences of the chronic inflammatory response in COPD, means that it can no longer be considered a disease that only affects the lungs[88]. Similar to other chronic conditions such as diabetes, we should take a multisystem, holistic approach to assessing patients[89]. Previous studies have shown that the majority of patients with COPD die from non-respiratory causes[90,91]. Mannino *et al*[90] found that the increased risk of all-cause mortality spans all GOLD stages including individuals with respiratory symptoms but no airflow obstruction (previously GOLD stage 0). Indeed the majority of individuals with mild to moderate COPD died from cardiovascular or other causes. This study did not account for smoking, however there is evidence that demonstrates the link between COPD and co-morbidities, in particular cardiovascular disease goes beyond such common aetiological factors[92]. The mechanistic link is thought to relate to systemic inflammation[93]. Although the prevalence of systemic effects such as skeletal muscle dysfunction, osteoporosis and other co-morbidities increase along with the severity of airflow obstruction, it is recognised that they can occur even in the earlier stages of disease[94]. It remains unknown whether targeting treatment of systemic inflammation has the potential to influence the natural history of COPD[42] or indeed prevent the development of systemic complications. Some anti-inflammatory treatments, such as corticosteroids, have the potential to cause or worsen co-morbidities such as osteoporosis, diabetes and obesity. However new treatment paradigms are evolving with the development of novel agents and a growing interest in drugs with anti-inflammatory properties that are already licensed for other indications such as statins and macrolides[95]. Whether early treatment with anti-inflammatory drugs could improve outcomes for patients with COPD remains unknown but is an interesting prospect for future research.

CONCLUSION

To make a significant impact on the high global morbidity and mortality statistics related to COPD, it seems logical that we should move our focus to better understanding the early course of the disease. Studies in the last decade have changed our perspective of the natural history of COPD, and it is now evident that the rate of decline in lung function in the earlier stages is greater than previously realised. The early development of co-morbidities, impaired QOL and increased risk of mortality of patients with $FEV_1 > 50\%$ predicted means we should not simply prescribe salbutamol and wait for these individuals to deteriorate. Symptoms may be inadequate to assess these mild patients, some of whom may also be early in their disease course; ideally we need biomarkers or genetic markers that can identify individuals at risk before significant physiological abnormalities become apparent. This information could be an additional tool in driving smoking cessation and lifestyle modification[96].

To date the majority of evidence relating to treatment in early COPD is from retrospective post-hoc analyses and there is a clear need of large scale prospective trials adequately powered to look at phenotypic subgroups. This would be in line the general move towards a more 'individualised' approach to treating patients with COPD, which requires a detailed assessment of disease severity, disease activity and impact on the individual. This can then allow us to tailor our therapeutic approach to maximise the benefit to the patient and hopefully impact on disease progression rather than taking a "one size fits all" approach to prescribing in early disease.

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REVIEW

Role of chronic obstructive pulmonary disease in lung cancer pathogenesis

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Abstract

Chronic obstructive pulmonary disease (COPD) and lung cancer are two important smoking related conditions. However, COPD has been shown to be an independent risk factor for lung cancer regardless of smoking history, suggesting that COPD and lung cancer may share a common pathogenesis. This review summarizes the epidemiology of lung cancer and COPD briefly, as well as discussing the potential for shared genetic risk, and shared genomic mechanisms, such as epigenetic changes or DNA damage induced by smoking. How key areas of COPD pathogenesis, such as inflammation, oxidative stress and protease imbalance may contribute to subsequent development of cancer will also be covered. Finally the possibility that consequences of COPD, such as hypoxia, influence carcinogenesis will be reviewed. By understanding the pathogenesis of COPD and lung cancer in detail it is possible that new treatments may be developed and the risk of lung cancer in COPD may be reduced.

Key words: Chronic obstructive pulmonary disease; Non-small cell lung carcinoma; Smoking; Oxidative stress; Inflammation

Core tip: Chronic obstructive pulmonary disease (COPD) has been shown to be an independent risk factor for lung cancer regardless of smoking history, suggesting that COPD and lung cancer may share a common pathogenesis. Chronic inflammation and oxidative stress are the most likely mechanistic links between COPD and lung cancer. Further analysis and elucidation of the molecular mechanisms involved in the pathogenesis of COPD and lung cancer should provide us with new treatment modalities and perhaps a key to understanding how the risk of lung cancer in COPD patients may be reduced.

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a condition characterized by airflow obstruction which is not normally fully reversible with the use of bronchodilators and is progressive over time[1]. The airflow obstruction usually occurs as a result of smoking but genetic factors (*e.g.*, alpha 1 antitrypsin deficiency), the burning of biomass fuels in developing countries and occupational factors also play a role[1,2]. Damage in both the airways and

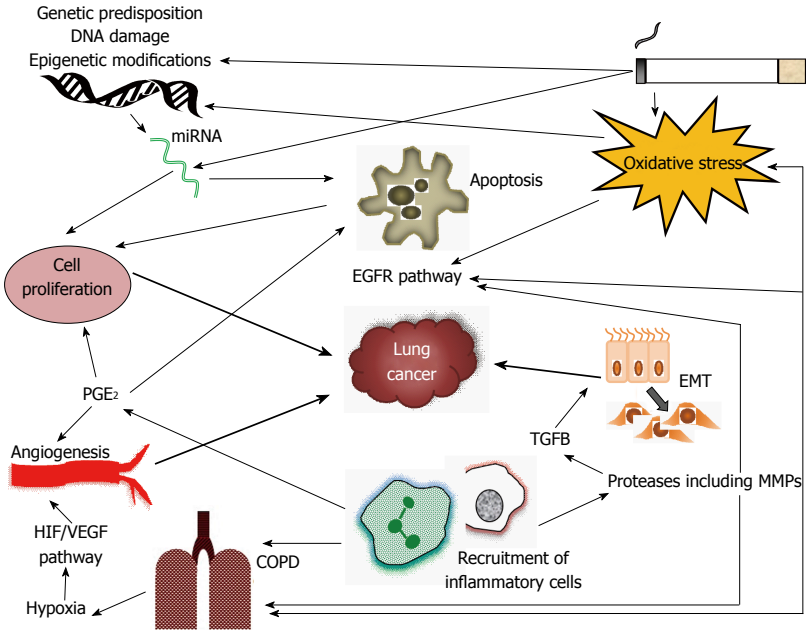

Figure 1 Pathogenic processes linking chronic obstructive pulmonary disease and lung cancer. The figure shows some of the key pathways leading to both chronic obstructive pulmonary disease (COPD) and cancer, and demonstrates the complexity of the interactions between the diseases. Cigarette smoke causes oxidative stress which can both drive inflammation and occur due to inflammation; both processes lead to COPD. Inflammation may in turn lead to activation of matrix metalloproteases (MMPs) and the transforming growth factor β (TGFβ) pathway, which by way of epithelial mesenchymal transition can promote lung cancer growth. Oxidative stress may also directly activate the epidermal growth factor receptor pathway which is involved in lung cancer growth. Cigarette smoke also interacts with pre-existing genetic predisposition and causes changes in DNA and miRNA which lead to processes relevant to cancer growth, such as cell proliferation and apoptosis, as well as to COPD. Finally, COPD may cause hypoxia which may augment angiogenesis, thereby interacting with prostaglandin based pathways to influence cell proliferation further, with the potential to influence cancer risk. EGFR: epithelial growth factor; EMT: epithelial cell mesenchymal transition; PGE2: Prostaglandin E2; HIF: Hypoxia inducible factor; VEGF: Vascular endothelial growth factor.

lung parenchyma contribute to the airway obstruction[1,3]. COPD is common. One study found a 10.1% prevalence of COPD (with forced expiratory volume in one second (FEV_1) < 80%) in adults over 40 years in 12 different countries worldwide[4]. COPD is an important cause of morbidity and mortality and resulted in over 3 million deaths in 2005 globally[5].

Lung cancer is the leading cause of cancer morbidity in North America, and is amongst the top 5 cancers ranked by disability adjusted life years lost (DALYs) in all regions of the world, except Sub-Saharan Africa[6]. It causes 626 DALYs/100000 heads of population worldwide[6] and is the most common cause of cancer death accounting for 1.18 million deaths in 2002[7]. 90% of lung cancers are caused by smoking, although other environmental factors such as asbestos exposure also play a role[8]. Lung cancers are classified by histology into small cell and non-small lung cell cancers (NSCLC); the latter are the more common and include squamous and adenocarcinomas. Generally small cell tumours have the poorest prognosis, with overall survival being 9 mo when managed by chemotherapy[9]. Even in NSCLC prognosis is generally poor unless surgery can be offered, and a recent systematic review reported mean survival of just over 7 mo if left untreated[10].

The relative risk of lung cancer in COPD is over twice that of the general population[11]. Longitudinal studies have shown that after approximately 15 years up to 33% of patients with COPD will develop lung cancer[12-14]. COPD is also a common co-morbidity in lung cancer patients occurring in 40%-70% of lung cancer cases[15]. As both diseases are related to smoking it could be assumed that the relationship between COPD and lung cancer might be related to smoking alone. However, in recent years it has been shown that patients with COPD, or CT diagnosed emphysema, have a higher risk for lung cancer even when adjusting for smoking history[15-18]. One study demonstrated that patients with COPD were six times more likely to develop lung cancer when compared to matched smokers without COPD[15]. Furthermore increased severity of airway obstruction is associated with a correspondingly elevated risk of lung cancer[18]. Emphysema in non-smokers is also a risk for lung cancer further reinforcing that smoking, alone, cannot explain the relationship between COPD and lung cancer[19,20]. In addition stopping smoking does not completely reduce the risk of lung cancer in patients with COPD[12]. Taken together this evidence suggests that there is a link between COPD and lung cancer other than smoking; this article will review the current evidence for common pathogenesis of the two conditions. The key concepts are summarized in Figure 1.

SHARED GENETIC AND GENOMIC MECHANISMS

Genetic epidemiology

Only 20%-30% of smokers develop COPD and 10%-15% develop lung cancer[21]. This epidemiological evidence, together with familial patterns of disease, and many subsequent genetic association studies have demonstrated that for both diseases a proportion of the risk of developing them is due to genetic factors. In fact, there is evidence that COPD has a heritability of 40%-77% and lung cancer a heritability of 15%-25%[21]. Familial linkage studies and genome-wide association studies (GWAS) have been performed for COPD, lung function and lung cancer and there is overlap in the chromosome areas identified, which demonstrates shared genetic risk[21,22]. Most of the primary studies have been in patients with either COPD or lung cancer and others have then sought areas of commonality. Key regions of shared susceptibility are shown in Table 1.

Gene expression studies

In common with genetic epidemiology work, most of

Table 1 Examples of genetic regions relevant to increased susceptibility to both chronic obstructive pulmonary disease and lung cancer

Chromosomal region	Possible gene(s)	Ref.
2q	*GSS*	[23]
4q31	*HHIP, GYPA*	[21]
5q33	*HTR4, ADAM19*	[21]
6p	*GCLCL*	[23]
6q	*SMOC2*	[22,24,25]
10q	*GSTO2*	[23]
11p	*MMP1, MMP12, RRM1*	[23,26]
12p	*GSTM1*	[27,28]
15q25	*CHRNA3-5, IREB2*	[21]
19q	*ERCC1*	[23]

the studies that have linked COPD and lung cancer at a genomic level have been in one disease or the other, and similarities or common themes have been noted later. Some studies have taken advantage of resections performed for lung cancer in patients who have COPD to get data on both diseases. For example, Wang *et al*[29] looked at the gene expression pattern in lung samples from COPD patients and demonstrated that genes involved in extracellular membrane synthesis and apoptosis were up-regulated, whilst genes involved in the anti-inflammatory response were down-regulated. It was also shown that urokinase plasminogen activator, its receptor and thrombospondin were expressed which are involved in transforming growth factor-β1 (TGF-β) and matrix metalloprotease (MMP) activation[29]. Growth factors and MMPs may be responsible for promoting malignant transformation of the bronchial epithelium[30-32]. Another study looking at gene expression in squamous cell carcinoma found that in patients with co-existing COPD there was a more frequent loss of 5q or a low expression of genes on 5q than in patients without co-existing COPD[33]. Cystatin A (*CSTA*), an intracellular protease inhibitor, has been shown to be up-regulated in NSCLC. *CSTA* is also expressed to a greater level in patients with COPD compared to smokers with normal lung function[34]. This suggests that pathways leading to lung tumorigenesis may vary between COPD patients and smokers with normal lung function.

Smoking has also been shown to alter gene expression - specifically it increases expression of xenobiotic genes, antioxidants, electron transport and oncogenes. Reduced levels of inflammatory regulator genes and tumor suppressor genes have also been seen in smokers[35,36]. Some of these genetic changes reverse on smoking cessation, but others including changes in oncogenes and tumor suppressor genes do not[35]. Wistuba *et al*[37] also demonstrated genetic alterations in the form of microsatellite deletions and loss of heterozygosity in normal epithelium of both current and ex-smokers. Irreversibility of genetic alterations or gene expression may explain why ex-smokers remain at an increased risk of developing lung cancer. It has also been shown that these changes in gene expression are associated with changes in protein expression[38].

DNA damage and repair

Smoking, occupational toxins and air pollution may result in damaging mutations which have potential to induce dysplastic and neoplastic changes in the lung parenchyma due to alterations in cell differentiation, growth and death[19]. Cigarette smoke contains many carcinogens which can be activated by cytochrome P450 enzymes; inhalation of these, pollutants and micro-organisms can cause damage directly or due to oxidative stress[19]. Repeated insults such as in COPD results in increased amounts of reactive oxygen species (ROS) which interact with DNA in the epithelium causing mutations. This results in DNA adducts which in turn may cause mutations if they are not repaired[39]. Commonly these mutations are in oncogenes, but they may also affect inflammatory pathways[40]. Mostly these mutations are repaired, but when there is a high rate of damage due to ROS cells are likely to be transformed to a malignant phenotype[40]. Studies have shown that there is impairment in DNA repair in COPD due to low levels of Ku 86, a protein involved in DNA repair[41]. This suggests that oxidant induced damage in COPD patients is more likely to result in carcinogenesis.

It is also possible that processes which control DNA repair may influence a patient's risk of developing lung cancer[42] and that such processes may be altered in COPD. For example, there is evidence that acetylation of histone H3 on lysine 56 (*H3K56*) is important in DNA repair[43]. Deacetylation of *H3K56* is controlled by histone deactylases (HDACs) 1 and 2 and sirtuin (SIRT) 1[44]. As there are low levels of HDAC2 and SIRT1 in COPD[45] this may reduce the protection against DNA breakage caused by environmental factors further increasing lung cancer risk.

Epigenetics

Epigenetics is the regulation of gene expression by heritable mechanisms that do not make direct changes to DNA itself. Examples of epigenetic mechanisms include histone acetylation and methylation; these may silence genes without changing their coding sequence and regulate pro-inflammatory gene expression in COPD and lung cancer[19]. The degree of histone acetylation in promoters of pro-inflammatory genes in COPD is related to disease severity and reversed by HDACs[46]. Since HDAC2 levels are low in COPD this could result in hyperacetylation of histones. SIRT1 acts similarly to HDACs and there are variable SIRT1 levels in lung cancer. However tumor suppressor genes, including *p53* may be rendered inactive in patients with low SIRT1 levels, including COPD patients[47,48]. Mouse models suggest this is a relevant pathway leading to lung tumors[48].

Genome-wide demethylation with site-specific hypermethylation is seen in lung cancer[19]. NA methylation is usually associated with gene silencing[49] and is associated with tumorigenesis and recurrence of NSCLC[50]. Methylation of the promoter of *p16* (a tumor suppressor gene) is seen in the sputum of COPD patients and aberrant methylation of *p16* can be also seen in the sputum of patients with NSCLC suggesting this too may be a

Table 2 Oxidative stress genes with good evidence that they contribute to both chronic obstructive pulmonary disease and lung cancer

Gene	Polymorphism	COPD risk	Lung cancer risk	Ref.
EPHX1	Rs2234922 (A139G) and haplotypes	↑Asians ↔ Caucasians	↑	[88,89]
GSTM1	Null genotype	↑	↑	[28,90]
SOD3	Rs1799896	↑	*In vitro* evidence of SOD3 ↑risk	[90-92]

COPD: Chronic obstructive pulmonary disease; EPHX1: Epoxide hydrolase; GSTM1: Glutathione S-transferase M1; SOD3: Superoxide dismutase 3.

shared mechanism of disease[51]. Hypermethylation of *p16*, *CDH13, RASSF1A* and *APC* have been associated with recurrence in lung cancer[50], but have not yet been reported in COPD.

Micro-RNA

These are small non-coding RNAs which act to regulate protein expression and immune response by acting on mRNA synthesis or translation. They can act as oncogenes or tumor suppressor genes and promote a range of functions including cell proliferation and apoptosis, both of which are relevant to tumor growth[52]. The function of micro-RNA (miRNA) may be altered by single nucleotide polymorphisms (SNPs), some of which are associated with poor survival in NSCLC[52].

miRNA expression is down-regulated with smoke exposure in both animal lungs and bronchial epithelium in man. In the rat model it was seen that many of the miRNAs involved in the activation of the NF-κB pathway were down-regulated[53]. Other studies have also demonstrated unique miRNA profiles in COPD[54] and lung cancer[55]. In COPD miR-223 and miR-1274a were the most markedly different from healthy smokers, and there were smaller changes in some of the miRNA associated with cancer, such as miR-10a and miR-451[54]. In NSCLC miR-21, miR-30d, miR-451, miR-10a, miR-30e-5p, miR-126*, miR-126 and miR-145 were differentially regulated and it was possible for some of their signatures to be picked up in the circulating blood[55]. These signatures could be used to aid prognostication in lung cancer or potentially to diagnose cancer earlier in high risk groups, although such strategies have not yet been evaluated in clinical studies.

COPD MECHANISMS WHICH INCREASE THE RISK OF CANCER

Inflammation

There is a wealth of evidence of systemic inflammation in COPD, as shown by increased levels of chemokines, cytokines and acute phase reactants[56]. Smoking can cause inflammation, but the degree seen in COPD is higher than in smokers alone and persists despite smoking cessation[57]. Inflammation in COPD also appears to be greater in severe disease[58], although its variability over time and with exacerbations has thwarted attempts to find biomarkers in the blood that relate consistently to clinical features[59]. Interleukin-6 (IL-6), C-reactive protein (CRP), IL-8 and surfactant protein D (SP-D)[60,61] levels are typically high in COPD and are important in the recruitment of inflammatory cells, although fibrinogen seems the most reliable biomarker to date[56,59]. Fibrinogen levels are associated with increased exacerbation rates and poorer outcomes[62,63], however it is a non-specific marker and as such has inherent weaknesses. More specific related markers, such as Aa-Val360 may prove more useful in the future[64].

There is an accumulation of inflammatory cells in COPD lungs, including macrophages, neutrophils, B cells and CD4+ and CD8+ T cells[65]. Macrophages release multiple inflammatory mediators including reactive oxygen species, cytokines, chemokines, extracellular matrix proteins and matrix metalloproteinases (MMPs). In COPD their function may be impaired, for instance they show impaired phagocytosis of bacteria, which may result in an increased inflammatory response to bacteria in the lower airways[66]. Neutrophils also produce reactive oxygen species, elastase and cytokines which play a role in emphysema and COPD development. Lymphocytes, including both B and T cells, are also found in high numbers in COPD lung[67] and may be involved in immune activation, leading to perpetuation of inflammation and ongoing parenchymal destruction. Such a reaction is typical of autoimmune disease, and characteristics of autoimmunity have been reported in COPD[68] although whether they are cause or effect is a matter of debate[69].

There is evidence that carcinogenesis occurs at sites of chronic inflammation[70]. For example, hepatocellular carcinoma can occur in patients with chronic hepatitis and colon cancer in the setting of colitis[71]. There is some evidence that increased inflammation may also be associated with the development of lung cancer. Epidemiologically, a cohort study of 7081 patients showed an increased risk of lung cancer in patients with a CRP of > 3 mg/dL[72]. Furthermore, a mouse model of chronic inflammation showed increased lung tumorigenesis[73]. However clinical studies of anti-inflammatory drugs, such as inhaled corticosteroids, have shown inconsistent results. A cohort study has shown lower rates of lung cancer compared to patients not taking inhaled corticosteroids[74], whilst a randomized controlled trial (RCT) did not[75]. Whether targeting pulmonary inflammation to prevent lung cancer will be beneficial therefore remains uncertain.

One way inflammation may lead to the development of lung cancer is by activation of the epithelial growth factor receptor (EGFR) cascade (Figure 1). This is activated in response to oxidative stress, neutrophil elastase and other proteases[76], thus might be expected to be overactive in COPD; recent evidence suggests this may be the case[77]. Overexpression of EGFR has been associated with a high risk of developing lung cancer and can occur years after smoking cessation[78]. The arachidonic acid metabolic pathway may also be related to COPD and lung cancer development. Inflammatory cells release ara-

Table 3 Signalling pathways common to chronic obstructive pulmonary disease and lung cancer

Signal pathway	Downstream effects	Role in COPD	Role in lung cancer	Ref.
NFκβ	↑MMPs, ↑TNFα, ↓apoptosis, ↑angiogenesis	↑inflammation	↑cell proliferation, ↓cell death, metastasis	[111]
PI3K	Activation and migration of leukocytes	↑inflammation	↑cell proliferation, ↓cell death	[112,113]
P38 MAPK	Block JNK/c-Jun, ↑TNFα	↑inflammation	Metastasis, ↓cell death	[114]
PPARγ	↓MMP9, ↓TNFα, ↓TGFβ	↓inflammation	↑cell differentiation, ↓cell proliferation	[115,116]

COPD: Chronic obstructive pulmonary disease; MMPs: Matrix-metalloproteinases; JNK: c-Jun N-terminal kinases; TNFα: Tumour necrosis factor alpha; TGFβ: Tissue growth factor beta.

chidonic acid metabolites including prostaglandins - this is mediated by cyclooxygenase enzymes (COX) including COX-2. Prostaglandin E_2, the product of COX-2, regulates the inflammatory response, but also has effects on cell proliferation, apoptosis and angiogenesis[70] and therefore may have a role in cancer development. Whilst this concept has been focused on far more in other cancers than in the lung there is some evidence it is relevant to cancer risk in COPD. Increased levels of COX-2 occur in COPD and are inversely proportional to FEV1[79]. Raised COX-2 levels relate to survival in NSCLC[80], inhibition of COX-2 reduces lung cancer in animal models[81] and patients who regularly take COX-2 inhibitors have reduced rates of lung cancer[82]. Finally, carriers of a polymorphism of the *COX-2* gene have an increased risk of lung cancer[83].

Oxidative stress

The normal metabolism of oxygen results in the development of ROS - these are usually removed from the cell by enzymes or anti-oxidants[84]. If the balance between the formation and removal of ROS is disturbed oxidative stress can occur; this may activate intracellular pathways which modulate the inflammatory response, as well as causing DNA damage (discussed above), and therefore have a role in the development of COPD and lung cancer[84]. Oxidative stress is well recognized in COPD and is particularly elevated during exacerbations[85].

Cigarette smoke is a key driver of oxidative stress. It contains noxious chemicals which are metabolized to benign and/or toxic metabolites, the latter of which can damage tissue and predispose to disease. Differences in metabolism between individuals can contribute to the risk of developing COPD or lung cancer[86,87]. An example of a metabolic enzyme involved in both diseases is Microsomal epoxide hydrolase (EPHX1)[88,89]. Many of the early studies of antioxidant genes such as this were hampered by low power; and most have not been borne out by GWAS, but there is other biological evidence (*e.g.*, *in vitro* work) delineating their role in pathogenesis, which is covered in the primary genetic papers referenced in Table 2.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is the main transcription factor that regulates phase II detoxifying antioxidant enzymes and therefore plays an important role in defence against carcinogens in smoke[93]. Nrf2 is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1); mutations in either of these genes can predispose to malignancy including NSCLC[94,95]. Defective Nrf2 occurs in COPD and this may also predispose patients with COPD to lung cancer due to increased oxidative stress[96]. Oxidative stress also increases p21 expression. p21 is acyclin-dependent kinase inhibitor whose levels are raised in alveolar epithelial cells and macrophages exposed to smoke[97], and in patients with COPD and lung cancer[98]. Elevation of p21 promotes the cell cycle to move from G_1 to G_2/M phase resulting in hyperproliferation and carcinogenesis[99].

Proteinase-antiproteinase imbalance

The balance between anti-proteinases and proteinases is an important determinant of emphysema that occurs in COPD[100]. Proteinases are also important in lung cancer development as they release growth factors TGFβ and VEGF, which can lead to tumorigenesis[19]. Proteinases common to both diseases are summarized in Table 3. A good clinical example of the importance of proteinase balance is alpha1-antitrypsin deficiency (AATD); this is a genetically determined anti-proteinase deficiency which predisposes to COPD[101]. Patients who are AATD carriers also have a 70% increased risk of developing lung cancer compared to healthy controls[102]. Drugs targeting MMPs, which are recognized in the pathogenesis of both COPD and lung cancer have been tested in early phase trials in COPD[103] and are at a more advanced stage of development in NSCLC[104].

Fibrotic pathways

Fibrotic processes are recognized in the small airway in COPD and are thought to be driven by the TGFβ1/MMP12 pathway, as TGFβ1 levels are raised in relation to the severity of airway obstruction[105]. MMP12 is normally inhibited by the binding of αvβ6 to TGFβ resulting in TGFβ activation. αvβ6 is a transmembrane receptor of the integrin family which is present on the surface of epithelial cells and is up-regulated in lung inflammation[19]. Loss of αvβ6 helps to preserve normal lung architecture and homeostasis and if it is removed in mice airspace enlargement results, suggesting it is important in the development of emphysema as well as fibrosis[106]. TGFβ also drives epithelial cell mesenchymal transition (EMT) which is a recognized pre-malignant change capable of enhancing invasion and thus predisposing to cancer development and progression[107] (Figure 1). Fibrosis due to integrins and TGFβ is regulated by galectin 3. There are raised levels of galectin 3 in COPD lung[108] and increased levels are also associated with poor prognosis in NSCLC[109].

Intracellular signaling

A number of intracellular signaling pathways, often directing processes such as inflammation, oxidative stress and protease balance, are dysregulated in both COPD and lung cancer. They are summarized in Table 3. NF-κB is particularly important due to its role in chronic inflammation. It is a transcription factor activated in inflammatory cells and in the lower airways of COPD[110] and lung cancer patients.

COPD CONSEQUENCES WHICH MAY INCREASE THE RISK OF CANCER

Hypoxia

Parenchymal destruction in COPD may ultimately result in hypoxaemia, which may activate transcription factors and result in the expression of pro-inflammatory genes[117] (Figure 1).This leads to hypoxia-inducible factor (HIF) release, VEGF expression and angiogenesis[118]. The induction of HIF is reduced in emphysema and levels of VEGF are low in emphysematous lungs which results in low levels of angiogenesis[119]. Low VEGF levels can also cause apoptosis and airspace enlargement[120]. Conversely VEGF can be increased in chronic bronchitis[118] such that the consequences in airway predominant compared to emphysema predominant COPD might differ. Hypoxia and HIF activation can also occur in lung tumors that are increasing in size and can result in progression and metastasis of lung cancer through induction of VEGF and MMPs in an animal model[121]. Circulating VEGF is associated with a poor prognosis in operated lung cancer patients as it predicts recurrence[122].

Physical inactivity

Patients with COPD often reduce their physical activity levels due to breathlessness, and have markedly reduced activity levels compared to those without airflow obstruction[123]. Physical inactivity is associated with lung cancer incidence[124] and appears to remain so even after adjustment for smoking and other lifestyle factors[125]. The mechanism behind this association is not yet clear.

CONCLUSION

Chronic inflammation and oxidative stress are the most likely mechanistic links between COPD and lung cancer. Further analysis and elucidation of the molecular mechanisms involved in the pathogenesis of COPD and lung cancer should provide us with new treatment modalities and perhaps a key to understanding how the risk of lung cancer in COPD patients may be reduced.

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REVIEW

Autotaxin and lysophosphatidic acid signalling in lung pathophysiology

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Abstract

Autotaxin (ATX or ENPP2) is a secreted glycoprotein widely present in biological fluids. ATX primarily functions as a plasma lysophospholipase D and is largely responsible for the bulk of lysophosphatidic acid (LPA) production in the plasma and at inflamed and/or malignant sites. LPA is a phospholipid mediator produced in various conditions both in cells and in biological fluids, and it evokes growth-factor-like responses, including cell growth, survival, differentiation and motility, in almost all cell types. The large variety of LPA effector functions is attributed to at least six G-protein coupled LPA receptors (LPARs) with overlapping specificities and widespread distribution. Increased ATX/LPA/LPAR levels have been detected in a large variety of cancers and transformed cell lines, as well as in non-malignant inflamed tissues, suggesting a possible involvement of ATX in chronic inflammatory disorders and cancer. In this review, we focus exclusively on the role of the ATX/LPA axis in pulmonary pathophysiology, analysing the effects of ATX/LPA on pulmonary cells and leukocytes *in vitro* and in the context of pulmonary pathophysiological situations *in vivo* and in human diseases.

Key words: Autotaxin; Lysophosphatidic acid; Lung; Acute lung injury; Pulmonary fibrosis; Asthma; Lung cancer

Core tip: In the lungs, autotaxin (ATX) is constitutively expressed in the bronchial epithelium, and all pulmonary cell types express some amount of lysophosphatidic acid (LPA) receptor. LPA affects all pulmonary cell types, mainly promoting a pro-inflammatory state. Increased ATX/LPA levels have been detected in various pathophysiological situations, both in mice and humans, including acute, allergic or chronic pulmonary inflammation; fibrosis; and lung cancer. Genetic or pharmacologic interventions targeting the ATX/LPA axis have proved beneficial for modelled disease management in animal models, establishing the ATX/LPA axis as a possible therapeutic target.

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INTRODUCTION

Autotaxin (ATX, ENPP2) is a secreted glycoprotein widely present in biological fluids, including the blood[1,2]. It is a member of the exo/ecto-nucleotide/pyrophosphatase/phosphodiesterase family of ectoenzymes (NPPs) that hydrolyse phosphodiester bonds of various nucleotides and derivatives[3]. However, ATX primarily functions as a plasma lysophospholipase D, and it is largely responsible for the bulk of lysophosphatidic acid (LPA)

production in the plasma and at inflamed and/or malignant sites[2,4,5]. LPA is a phospholipid mediator produced in various conditions both in cells and in biological fluids, and it evokes growth-factor-like responses, including cell growth, survival, differentiation and motility, in almost all cell types[2,4]. The large variety of LPA effector functions is attributed to at least six G-protein coupled LPA receptors (LPARs) with overlapping specificities and widespread distribution[6,7]. Finally, a group of transmembrane lipid-phosphate phosphatases have been suggested to act as negative regulators of LPA metabolism[8,9]. Beyond the well-established role of the ATX/LPA axis in carcinogenesis[10,11], high levels of ATX expression have been observed in non-malignant, inflamed tissues, suggesting a possible involvement of ATX in chronic inflammatory disorders[12,13]. Given the role of the ATX/LPA axis in human disease, a large number of ATX inhibitors and LPAR antagonists are being developed[14-17] in pursuit of a compound with likely therapeutic potential. The reviews cited above summarise the current knowledge on ATX, LPA and LPA receptors and their therapeutic relevance and targeting. In this review, we focus exclusively on the role of the ATX/LPA axis in pulmonary pathophysiology, analysing extensively the effects of ATX/LPA on pulmonary cells and leukocytes *in vitro,* as well as discussing these effects in the context of pulmonary pathophysiology *in vivo* and the pathogenesis of human diseases.

THE ATX/LPA AXIS IN THE HEALTHY LUNG

The gene encoding ATX consists of 27 exons and, through alternative splicing, gives rise to five protein isoforms, designated α-ε, that differ by the presence or absence of sequences encoded by exons 12, 19 and 21[18,19]. All isoforms are catalytically active, and the polybasic insertion in ATXα has been suggested to confer specific binding to cell surface heparin sulphate (HS) proteoglycans[20]. In the absence of proteomic data, ATX mRNA expression analysis indicates that ATXγ is brain specific and that ATXβ is the more abundant isoform, exhibiting a broad tissue distribution that includes the lungs[18-21]. *In situ* hybridisation localised ATX mRNA expression to the basal cells of normal human bronchial epithelium[22], and ATX can be detected in the bronchoalveolar lavage fluid (BALF) of healthy humans (unpublished data). Accordingly, immunohistochemical studies have indicated constitutive ATX expression predominantly in the mouse bronchial epithelium that could also be detected in the BALF of healthy mice[23]. Moreover, with genome-wide linkage analysis coupled with expression profiling, ATX was identified as a candidate gene involved in the control of pulmonary functions (dead space volume, V_D; total lung capacity, TLC; lung compliance, C_L; and diffusing capacity for CO, D_{CO})[24].

Because ATX is a constitutively active enzyme, the biological outcome of ATX's enzymatic action - largely LPA production and signalling - will depend on its expression levels, the local availability of its substrates, and the abundance and activity of the different LPA receptors in the microenvironment.

Lysophosphatidylcholine (LPC), the main substrate of ATX, is a highly abundant bioactive lysoglycerophospholipid present at high concentrations (100-200 μmol/L) in the circulation[25,26] and is predominantly associated with albumin and lipoproteins[12]. LPC can also be detected in the BALF of healthy mice (< 1 μmol/L, unpublished data), whereas phosphatidylcholine (PC; mostly 16:0), one of the main precursors of LPC, is a major constituent of the surfactant, which is a macromolecular complex composed primarily of lipids (90%) and surfactant proteins (SPs A-D) and is largely responsible for maintaining minimal surface tension within the alveolar surfaces[27]. It remains unknown if BALF LPC is synthesised from surfactant (or membrane) PC, if it diffuses from the circulation or if it is transported with albumin. Therefore, and given its abundance, LPC levels are not a limiting factor in ATX's enzymatic activity and LPA production, although it is unknown whether LPC's associations with other molecules (*e.g.*, carrier proteins) are masking the bioavailability of LPC as an ATX substrate.

A 50% reduction or a 100% increase in systemic (and BALF) ATX/LPA levels in genetically modified mice[28,29] does not result in any appreciable effect on gross lung pathology[23], although further pulmonary functional studies are needed. Conditional deletion of ATX from the bronchial epithelium results in significantly reduced (but not completely abrogated) levels of BALF ATX, which do not, however, affect lung development and gross pathophysiology in healthy, non-stimulated mice[23]. Therefore, fluctuations in ATX levels in the lung seem to be well tolerated under normal, healthy conditions.

LPA can also be detected in the BALF of healthy human and mouse lungs[23,30,31], the bulk of which is most likely synthesised from the enzymatic action of ATX on BALF (and most likely membrane) LPC.

LPA receptors are widely expressed throughout the body, and the lungs are no exception. All pulmonary cell types have been reported to express different LPARs, as summarised in Table 1. Compiled data analysis from multiple published works, including Northern analyses, real-time PCR and microarray data, suggest a slightly different expression profile in mouse (LPAR3 > LPAR1, LPAR2, LPAR5; no LPAR4) and human (LPAR3 > LPAR1; no LPAR2, 4, 5) lungs[6]. Therefore, different LPA receptors are expressed in the lung tissue of healthy mice, although detecting their relative abundance in different cell types will have to wait for the emergence of specific antibodies and/or conditional KO mice. Interestingly, LPAR1 has been found to be dually sequestered in caveolin-1 and clathrin subcompartments of plasmalemmal fractions in porcine cerebral microvascular endothelial cells[32], and LPAR1 has been reported to heterodimerise with CD97, an adhesion-linked GPCR[33]. LPAR1 has also been reported to cluster with CD14, the LPS co-receptor, upon treatment of MEL12 pulmonary epithelial cells with LPS,

Table 1 Expression of lysophosphatidic acid receptor in pulmonary cell types and leukocytes

Cell type	LPAR1	LPAR2	LPAR3	LPAR4	LPAR5	LPAR6	Ref.
NHBEs	+++	++	+++	-	+	+	[41]
NHBEs	+++	++	+++	-	++	+	[59]
NHBEs	++	++	++	-	NA	NA	[43]
HBE	++	++	NA	NA	NA	NA	[56]
HBE (BEAS-2B)	++	++	++	+	++	+	[59]
HBE (HBEpCs)	+++	+	++	+	+	NA	[209]
HBE (HBEpCs)	++	++	++	NA	NA	NA	[46]
HBE (HBEpCs)	++	++	++	NA	NA	NA	[49]
HBE (HBEpCs)	++	++	++	NA	NA	NA	[68]
Primary mouse tracheal EpCs	++	+++	++	++	-	NA	[57]
NHBE cells	++	++	++	-	NA	NA	[43]
A549 alveolar epithelial carcinoma	++	NA	NA	NA	NA	NA	[40]
A549 alveolar epithelial carcinoma	+++	+	-	-	NA	NA	[74]
NCI-H522 lung epithelial carcinoma	-	++	+	-	NA	NA	[74]
RLCNR rat lung adenocarcinoma	NA	NA	-	NA	++	NA	[283]
Primary mouse lung fibroblasts	+++	+	+	+	++	NA	[31]
human fetal lung fibroblasts MRC5	+++	++	++	+	+	NA	[226]
Mouse embryonic fibroblasts	+++	++	+	+++	+	NA	[101]
NHLFs CCL-151	+++	+	++	+	+	++	[81]
NHLFs	++	++	NA	NA	NA	NA	[42]
Primary mouse lung endothelial	++	+	-	+++	+	NA	[31]
HPAECs	+	++	+	+	+	+++	[106]
HMVECs	+	++	+	+	+	+++	[106]
HEV ECs	NA	NA	NA	++	NA	++	[116]
Human eosinophils	+	-	+	NA	NA	NA	[148]
Primary mouse neutrophils	-	+++	-	-	+	NA	[31]
Primary mouse alveolar macrophages	-	+	+	+	+	NA	[31]
Human alveolar macrophages	+	++	++	NA	NA	NA	[158]
Rat alveolar macrophages	++	+	-	NA	NA	NA	[158]
Human monocytes	+	-	-	NA	NA	NA	[176]
Mouse DCs	++	++	++	NA	NA	NA	[58]
Human immature DCs	+	+	+	NA	NA	NA	[172]
Human mature DCs	+	+	+	NA	NA	NA	[172]
Human immature DCs	-	+	-	NA	NA	NA	[174]
Human mature DCs	-	+	-	NA	NA	NA	[174]
Mouse immature DCs	++	+	+++	++	+++	NA	[175]
Mouse mature DCs	+++	+	+	++	++	NA	[175]
Jurkat T cells	+	+++	-	NA	NA	NA	[178]
Jurkat T cells	+	+	+	NA	NA	NA	[295]
Human CD4 T cells	+	+++	NA	NA	NA	NA	[177]
Human CD4 T cells	+	+++	-	NA	NA	NA	[176]
Human CD8 T cells	-	-	-	NA	NA	NA	[176]
Mouse CD4 T cells	+	+	+	+	+	NA	[31]
Mouse CD8 T cells	+	+++	+	+	++	NA	[31]
Human B lymphocytes	-	+	+	NA	NA	NA	[176]
Human platelets	+	NA	+	NA	NA	NA	[296]
Human platelets	+	+	+	NA	NA	NA	[295]
Human platelets	+	+	+	+	+	NA	[297]
Human mast cells	+	+	+	+	NA	NA	[199]
Human mast cells	+	+	+	-	NA	NA	[200]
Human mast cells	-	++	+	-	+++	++	[201]
Lung resident mesenchymal stem cells	+++	+	+	NA	NA	NA	[298]

+++: Strong expression; ++: Moderate expression; +: Low expression; -: No expression; NA: Not available; LPAR: Lysophosphatidic acid receptor; NHBEs: Normal human bronchial epithelial; HBE: Human bronchial epithelial; HBEpCs: Human bronchial epithelial cells; NHLFs: Normal human lung fibroblasts; ECs: Endothelial cells; HPAECs: Human pulmonary arterial ECs; HMVECs: Human pulmonary microvascular ECs; HMVECs: Human pulmonary microvascular ECs; HEVs: High endothelial venules; DCs: Dendritic cells.

an interaction abolished upon the disruption of lipid rafts[34]. Further studies are needed to explore possible homo- and hetero-dimerisation of LPARs and the effect of their possible association with other GPCRs within lipid rafts.

Complete genetic deletion of LPAR1-5 does not result in any gross pathological signs in the lungs of non-stimulated mice[6], with the exception of the development of pulmonary hypertension in aged LPAR1 and 2 double KO mice[35], which is consistent with the proven role of ATX/LPA in vascular development[6,28,36-38] and the effects of LPA on endothelial and smooth muscle cells

physiology (see below).

ATX has been shown to be necessary for embryonic development, as complete genetic deletion results in aberrant vascular and neuronal development leading to embryonic lethality[6,28,36-38]. However, preliminary studies with inducible complete genetic deletion of ATX in adult mice or long-term potent pharmacological ATX inhibition indicate no gross pathological signs (unpublished data). Moreover, and according to published reports, fluctuations in ATX/LPA levels and the abrogation of LPA receptor signalling are well tolerated in the lungs, with the exception of aged mice. Therefore, the ATX/LPA axis does not seem to have a major role in the pulmonary physiology of healthy adult mice. However, more studies are needed to determine the effect of lysophospholipid homeostasis on healthy pulmonary functions and vice versa.

LPA EFFECTS ON PULMONARY CELLS

The possible involvement of the ATX/LPA axis in pulmonary pathophysiology has been widely explored in *in vitro* studies, mainly upon LPA treatment of various cell types and lines, primary or established. The main findings, exclusively concerning cells of pulmonary origin, are summarised in Table 2, presented below together with major findings from cells of different origin, and are discussed later in the context of disease pathogenic mechanisms. Notably, all reported effects were observed at LPA concentrations much higher than the physiological LPA levels in the plasma and BALF, and thus, they address possible perturbed functions in pathophysiological situations involving the increased production of LPA at local sites. Finally, differential effects have been observed for different LPA species and in the presence of appropriate carriers (*e.g.*, albumin, gelsolin); however, the mechanisms regulating phospholipid homeostasis and LPA activity are far from being understood.

Epithelial cells

The airway epithelium, the first line of defence of the lungs against inhaled stimuli, plays a protective role through its barrier activity to inhaled insults. Increased epithelial apoptosis in response to injury is believed to play a major role in the initiation of pulmonary pathophysiological disorders, such as fibrosis. Moreover, damaged epithelial cells release a plethora of factors that contribute to repair mechanisms such as growth factors, chemokines, cytokines and prostaglandins[39]. Mouse bronchial epithelial cells have been reported to be the major ATX-producing cell type in the mouse lung[23], and transformed pulmonary epithelial cell lines (A549) have also been reported to express ATX[40]. All pulmonary cell types have been reported (with some controversy) to express at least one LPA receptor, as indicated in Table 1.

LPA signalling through LPAR1 has been reported to induce anchorage-dependent apoptosis in cultured normal human bronchial epithelial cells (NHBEs)[41], and the genetic deletion of LPAR1 or LPAR2 results in a decreased number of TUNEL$^+$ bronchial epithelial cells *in vivo* post-bleomycin (BLM)-mediated lung injury, which specifically targets epithelial cells[41,42]. ATX expression from epithelial transformed A549 cells has been reported to induce their LPA-dependent and LPA-independent migration[40], a crucial step for re-epithelisation and tissue remodelling.

The stimulation of normal human bronchial epithelial cells (HBEpCs) with LPA increases stress fibre formation, reorganises αvβ6 at their ends and leads to increased transforming growth factor-beta (TGF-β) activity *via* LPAR2/Ga_q and RhoA/Rho kinase[43]. TGF-β plays crucial roles in tissue regeneration and cell differentiation, and integrin αvβ6 has been shown previously to bind and activate TGF-β1, a mechanism that was suggested to regulate pulmonary inflammation and fibrosis[44].

LPA induces interleukin-8 (IL-8) expression from HBEpCs, the major chemoattractant of neutrophils, through nuclear factor kappa B (NF-κB)/AP1 and PKCδ/p38/extracellular regulated protein kinases (ERK)/c-Jun N-terminal kinase (JNK) pathways[45,46]. LPA levels and their effects on IL-8 expression have been reported to be regulated intracellularly by acylglycerol kinase (AGK)[47] and extracellularly by lipid phosphatase-1 (LPP1)[48] and ATX[40]. Moreover, the stimulation of IL-8 expression is mediated, at least in part, by LPA-mediated phosphorylation and transactivation of the epidermal growth factor receptor (EGFR)[49]. *In vitro* results were verified *in vivo*, where intratracheal LPA administration to mouse lungs stimulated the expression of MIP-2, the mouse homologue of IL-8, and neutrophil influx[45]. Another pro-inflammatory action of LPA in HBEpCs *in vitro* is the induction of thymic stromal lymphopoietin (TSLP) and chemokine CCL20 through CARMA3-mediated NF-κB activation[50]. TSLP stimulates dendritic cell maturation, leading to antigen presentation to T cells and the initiation of an adaptive immune response to an inhaled antigen[51], whereas CCL20 induces the chemotaxis of T cells and dendritic cells (DCs)[52]. Both cytokines are expressed in the airway of asthmatic patients and contribute to airway inflammation in mouse models of asthma[52,53].

LPA has also been reported to induce IL-13 decoy receptor α2 expression and to inhibit IL-13 signalling in HBEpCs *in vitro*[54]. IL-13 is a Th2 cytokine and a mediator of allergic inflammation and disease, the levels of which were found to be increased in the BALF of asthma patients and ovalbumin-challenged mice[55]. LPA levels were also found to be increased after segmental allergen challenge[56]. Therefore, LPA-induced stimulation of IL-13Rα2 and abrogation of IL-13 signalling would conceivably abrogate the induction of allergic asthma in mice. Heterozygous LPAR2 knockout mice exhibit reduced neutrophil infiltration in the lungs upon treatment with *Schistosoma mansoni* soluble egg antigen (SEA)[57]; however, the adoptive transfer of allergen-pulsed LPAR2$^{-/-}$DCs induce substantially more lung inflammation, pointing to

Table 2 Lysophosphatidic acid effects on different cell types

Cell type	Primary	Species	LPA effect	Receptor	Experiment	Carrier	Ref
Alveolar and bronchial epithelial	Yes	Mouse	Apoptosis	LPAR1	*In vivo*	Biological fluid	[41]
Alveolar and bronchial epithelial	Yes	Mouse	Apoptosis	LPAR2	*In vivo*	Biological fluid	[41,42]
NHBEs	Yes	Human	(anchorage dependent) Apoptosis	LPAR1	*In vitro*	FAF BSA	[41]
NHBEs	Yes	Human	TSLP, CCL20 induction		*In vitro*	No	[50]
NHBEs	Yes	Human	TGF-β activation	LPAR2	*In vitro*	No	[43]
NHBEs	Yes	Human	Induction of Soluble ST2 expression	LPAR1,3	*In vitro*	NA	[61]
Bronchial epithelial (HBEpCs)	Yes	Human	EGFR transactivation, IL-8 secretion		*In vitro*	BSA; BSA	[46,49]
Bronchial epithelial (HBEpCs)	Yes	Human	Induction of IL-13 Ralpha 2	Gαi linked	*In vitro*	No	[54]
Bronchial epithelial (HBEpCs)	Yes	Human	Epithelial barrier integrity enhancement	LPAR1,3	*In vitro*	NA; BSA	[56,209]
Bronchial epithelial (HBEpCs)	Yes	Human	Decrease of EGFR-EGF binding		*In vitro*	BSA	[66]
Bronchial epithelial (HBEpCs)	Yes	Human	COX-2 expression, PGE2 secretion	Gαi linked	*In vitro*	No	[63]
Bronchial epithelial (HBEpCs)	Yes	Human	PDGFR-β transactivation		*In vitro*	BSA	[68]
Bronchial epithelial (HBEpCs)	Yes	Human	c-Met redistribution on the membrane		*In vitro*	No	[70,299]
Bronchial epithelial (BEAS-2B)	No	Human	EGFR transactivation		*In vitro*	BSA	[66]
Bronchial epithelial (BEAS-2B)	No	Human	RANTES inhibition	LPAR1	*In vitro*	FAF BSA	[59]
R3/1 Alveolar epithelial	No	Rat	Inhibition of attachment		*In vitro*	FAF BSA	[41]
Tracheal epithelial	Yes	Mouse	COX-2 expression, PGE2 secretion	LPAR2	*In vitro/vivo*	No	[57]
H292 lung cancer epithelial	No	Human	Decrease of EGFR-EGF binding		*In vitro*	BSA	[66]
A549 alveolar epithelial carcinoma	No	Human	Decrease of EGFR-EGF binding		*In vitro*	BSA	[66]
A549 alveolar epithelial carcinoma	No	Human	p53 decrease		*In vitro*	FAF BSA	[73]
A549 alveolar epithelial carcinoma	No	Human	Cell migration	LPAR1	*In vitro*	BSA; BSA	[40,74]
NCI-H522 lung epithelial carcinoma	No	Human	Cell motility		*In vitro*	BSA	[74]
Fetal lung fibroblasts (HFL1)	No	Human	Chemotaxis		*In vitro*	Biological fluid	[31]
NLFs CCL151	No	Human	Proliferation, EGFR ectodomain shedding	Gi/o linked	*In vitro*	No	[81]
Fetal lung fibroblasts MRC5	No	Human	proliferation	LPAR1,3	*In vitro*	No	[226]
Fetal lung fibroblasts IMR-90	No	Human	Chemotaxis	LPAR1	*In vitro*		[95]
NLFs	Yes	Human	Differentiation, TGF-β expression and signaling	LPAR2	*In vitro*	No	[42]
Lung fibroblasts	Yes	Mouse	Differentiation, TGF-β expression and signaling	LPAR2	*In vitro*	No	[42]
Lung fibroblasts	Yes	Mouse	Chemotaxis	LPAR1	*In vitro*	FAF BSA	[31]
Lung fibroblasts	Yes	Mouse	Protection from apoptosis	LPAR1	*In vitro*	FAF BSA	[41]
NIH 3T3 fibroblasts	No	Mouse	Protection from apoptosis, proliferation	Gi linked	*In vitro*	FAF BSA	[99]
NIH 3T3 fibroblasts	No	Mouse	Migration, protection from apoptosis, proliferation		*In vitro*	BSA	[100]
Rat1/c-Myc fibroblasts	No	Rat	Protection from apoptosis		*In vitro*	FAF BSA	[99]
Lung endothelial	Yes	Mouse	Vascular leak/extravasation	LPAR1	*In vivo*	Biological fluid	[31]
HPAECs pulmonary endothelial	Yes	Human	Increase of the endothelial layer permeability	LPAR6	*In vitro*	FAF BSA	[106]
BPAE pulmonary artery endothelial	No	Bovine	Migration, chemotaxis		*In vitro*	FAF BSA	[127-129]
Smooth muscle cells	Yes	Rabbit, cat	Contraction		*Ex vivo*	BSA	[140]
HASM airway smooth muscle cells	Yes	Human	Proliferation, stimulation of EGFR signaling		*In vitro*	NA; BSA	[139,141]
HASM airway smooth muscle cells	Yes	Human	Activation of TGF-β		*In vitro*	NA	[143]
Dendritic cells	Yes	Mouse	Inhibition of activation	LPAR2	*In vitro, in vivo*	FAF BSA	[58]
Lung resident mesenchymal stem cells	Yes	Human	Migration	LPAR1	*In vitro*	No	[298]

LPA: Lysophosphatidic acid; NHBEs: Normal bronchial epithelial; HBEpCs: Human bronchial epithelial cells; BEAS-2B: Bronchial epithelial cell line; NLFs: Normal lung fibroblasts; HPAECs: Human pulmonary arterial endothelial cells; LPAR: Lysophosphatidic acid receptor; NA: Not available; TSLP: Thymic stromal lymphopoietin; TGF-β: Transforming growth factor beta; EGFR: Epidermal growth factor receptor; BSA: Bovine serum albumin; FAF: Fatty acid free.

an anti-inflammatory role of LPA/LPAR2[58]. Consistent with a potential anti-inflammatory role of LPA signalling, especially in the context of allergic inflammation, LPA has been found to inhibit the tumor necrosis factor (TNF)/interferon (IFN)-γ-induced production of CCL5/RANTES in an established human bronchial epithelial cell line (BEAS-2B)[59]. RANTES is a chemoattractant for eosinophils, monocytes and T-cells and seems to exacerbate asthma[60]. LPA has also been reported to induce the expression of soluble ST2 (sST2) from HBEpCs, a decoy

receptor of IL-33 that attenuates IL-33 and endotoxin-induced inflammatory responses[61]. The increased expression has also been verified *in vivo*, where the intratracheal administration of LPA increased sST2 levels in BALF[61]. However, the physiological relevance of this finding remains unknown, as the abrogation of LPA signalling *in vivo, via* the genetic deletion of LPAR1 or LPAR2, attenuates LPS-induced responses[34,62]. The controversial anti-inflammatory effects of LPA are exemplified by its stimulation of cyclo-oxygenase-2 (COX-2) expression and prostaglandin E2 (PGE2) release from HBEpCs[63]. *In vivo*, LPAR2$^{+/-}$ mice express less COX-2 and secrete lower amounts of PGE_2 compared to wild-type mice upon allergic stimulation[57]. COX-2 and PGE_2 are commonly considered potent proinflammatory mediators and are involved in several inflammatory diseases. However, in the lungs, as opposed to other parts of the body, PGE2 has a role in limiting the immune-inflammatory response and tissue repair processes[64,65]. The generation of conditional knockouts for the different LPA receptors will be instrumental in dissociating the inflammatory effects of LPA in different cell types *in vivo*. Moreover, the possible differential effects of LPA in stromal and innate immune cells, as compared to adaptive immune cells, should be addressed with appropriate bone marrow transfer experiments.

LPA stimulates PGE2 production and IL-8 secretion in HBEpCs through EGFR phosphorylation and transactivation[49,63] introducing the concept that LPA can also activate or modulate structurally distinct receptors. LPA induces a decrease in EGFR binding of EGF both in HBEpCs and established epithelial cell lines (BEAS-2B) *via* different signalling pathways, including transactivation of EGFR[66,67]. The decrease in EGF binding to its receptor is sustained in normal cells but is rapidly reversed in cancer cell lines (H292, A549)[67]. LPA has been found to transactivate receptor tyrosine kinases (RTKs) other than EGFR, such as platelet-derived growth factor receptor-β (PDGF) in lung epithelial cells. Specifically, in primary cultures of HBEpCs, LPA stimulates tyrosine phosphorylation of PDGFRβ and threonine/tyrosine phosphorylation of the downstream molecule ERK1/2, both through PDGFR kinase, suggesting that PDGFR is transactivated by LPA[68]. As PDGF promotes cell proliferation[69], PDGFR transactivation from LPA may have a proliferative role in airway epithelium.

In general, transactivation of RTKs by GPCRs induces tyrosine phosphorylation of RTKs, thereby resulting in further signal transduction. Similarly, LPA induces tyrosine phosphorylation of EGFR and PDGFRβ in HBEpCs[66,68]. By contrast, LPA has no effect on tyrosine phosphorylation of another RTK, c-Met, which is the receptor of hepatic growth factor (HGF)[70]. Rather, LPA in HBEpCs induces serine phosphorylation of c-Met and its redistribution to the plasma membrane[70]. Moreover, LPA has an inverse effect on c-Met compared to the c-Met ligand, HGF. HGF induces tyrosine phosphorylation of c-Met and its internalisation, whereas LPA reverses these effects and promotes the redistribution of the c-Met-E-cadherin complexes on the plasma membrane through PKCδ[70]. The implication of LPA on c-Met signalling, which is involved in tumour invasion and metastasis, could be of importance in lung cancer (LC) in which c-Met is overexpressed[71]. Conversely, the inhibition of HGF signalling by LPA is another link between LPA and fibrosis, in which HGF has an important protective role[72].

The ATX/LPA axis is widely known to be implicated in cancer[10,11]; however, limited studies have addressed the role of ATX/LPA in LC. A549 lung carcinoma epithelial cells express ATX, which localises to perinuclear and exocytotic vesicle-like bodies and is later secreted in the culture medium[40]. ATX has been reported to induce the migration of A549 cells, most likely through the phosphorylation of PKCδ and of the actin-binding protein cortactin, which could be inhibited by an LPAR1/LPAR3 inhibitor or knock-down of LPAR1[40]. Interestingly, mutant ATX or heat-inactivated cell supernatant were also reported to promote cell migration, proving that ATX-induced cell migration does not depend totally on ATX enzymatic activity and LPA[40]. This LPA-independent pathway of cell migration could be mediated by the binding of ATX to cell-surface receptors, such as integrin β4, which, as shown by co-immunoprecipitations, takes place even after ATX has been heat inactivated[40].

Moreover, in the context of carcinogenic epithelial cells, LPA has been shown to decrease the total cellular content of the tumour suppressor p53 in A549 lung epithelial cells, most likely through proteasomal degradation regulated by PI3K, and simultaneously to decrease the nuclear localisation of p53 and the p53-dependent transcription of cell-cycle arrest genes[73]. Overexpression of LPA receptors 1, 2 or 3 in A549 cells was found to be sufficient to cause a severe reduction in p53-dependent transcription[73]. Moreover, LPA protects A549 cells from genotoxic drugs, which normally cause nuclear accumulation of p53 and apoptosis, by reducing the total levels of p53 and preventing apoptosis[73]. This may explain the protection that LPA offers to carcinoma cells against chemotherapeutic agents. In addition, the fact that p53 inhibition regulates transcription by LPA means that LPA suppresses the G_1-S cell cycle arrest induced by p53 and favours tumour cell growth. The same A549 cell line, which predominantly expresses LPAR1, shows induction of cell motility by LPA[74], whereas its migration and invasion are inhibited *in vitro* by 1-bromo-3(*S*)-hydroxy-4-(palmitoyloxy) butyl-phosphonate (BrP-LPA), a dual-function pan-antagonist of LPA receptors and inhibitor of the lysophospholipase D activity of ATX[75,76]. Expression of LPAR1 seems to be crucial for motility, as another lung epithelial carcinoma cell line with no LPAR1 but significant LPAR2 levels is not susceptible to LPA-induced cell motility[74]. Intriguingly, when A549 cells are injected along extracellular matrix (ECM) in nude mice, the resulting tumours are inhibited, and the number of vessels is decreased by BrP-LPA, an inhibitor of ATX

and LPA signalling[76]. Therefore, LPA regulates many of the aspects of A549 cells that promote carcinogenesis, such as cell cycle promotion, migration, invasion and survival.

Taken together, these results show that LPA seems to be involved in different aspects of pulmonary epithelial pathophysiology, including migration, apoptosis, pro-(and anti-) inflammatory gene expression and transactivation of RTK receptors. However, most of the reported LPA effects in epithelial cells described above were examined *in vitro*, in the absence of cell-to-cell interactions and a functional ECM, which are defining events especially in the case of epithelial cells. Their implication on pulmonary pathophysiological situations *in vivo* is discussed below.

Fibroblasts

Fibroblasts are ubiquitous cells found in connective tissue that provide mechanical strength to tissues by providing a supporting framework of ECM[77]. Moreover, fibroblasts are important sentinel cells in the immune system, which have been suggested to play a critical role in the switch from acute inflammation to adaptive immunity and tissue repair[78]. Fibroblasts from different anatomical regions exhibit characteristic phenotypes that are maintained even after prolonged culture *in vitro*, suggesting that many fibroblasts have an imprinted phenotype. They are extremely versatile cells that display a remarkable capacity to differentiate into other components of the connective tissue, such as cartilage, bone, adipocyte and smooth muscle cells. Their differentiation to myofibroblasts, under mechanical pressure from the ECM and/or profibrotic TGF-β stimulation, regulates connective tissue remodelling by combining the ECM-synthesising features of fibroblasts with cytoskeletal characteristics of contractile smooth muscle cells. Myofibroblasts can have multiple origins, regress and disappear by apoptosis on wound epithelialisation, and may persist in fibrotic situations and cause organ dysfunction, such as pulmonary fibrosis[79]. Moreover, fibroblasts are associated with cancer cells (cancer-associated fibroblasts) at all stages of cancer progression, and their functional contribution to this process is beginning to emerge[80].

Fibroblast proliferation is required in wound healing to fill an open wound. In the lungs, LPA has been reported to promote the proliferation of established human normal lung fibroblasts (CCL151), along with ERK phosphorylation and the transcription of *c-fos*, *HB-EGF* and *amphiregulin* genes[81]. In accordance, cell migration, rounding and proliferation in response to LPA are decreased in embryonic fibroblasts from LPAR1-null mice but are not absent, consistent with redundant signalling from LPA receptors[82]. In support of a role of LPA in lung fibroblast proliferation, LPA stimulates proliferation of synovial fibroblasts mediated through the GPCR, ERK, p38 and Rho kinase signalling pathways[83]. The proliferative effects of LPA in synovial fibroblasts correlates with the development of actin stress fibres[83], in agreement with early reports on LPA effects in Swiss 3T3 fibroblasts, also indicating tyrosine phosphorylation of focal adhesion kinase (FAK), paxillin and p130[84-86]. Moreover, LPA-induced cytoskeleton reorganisation in peritoneal mesothelial cells promotes connective tissue growth factor (CTGF) expression, which in turn promotes NIH3T3 fibroblast proliferation, an effect abolished upon silencing of CTGF or LPAR1 in mesothelial cells[87].

LPA has also been shown to augment human foetal lung and human foreskin fibroblast-mediated contraction of collagen gels[88,89] and to promote the contraction of human myofibroblasts, isolated from the palmar aponeurosis of patients with Dupuytren's disease[90]. LPA-mediated contraction of myofibroblasts has been suggested to involve Rho/Rho kinase inhibition of myosin light chain (MLC) phosphatase (MLCP)[90]. In accordance, LPA has been found to increase the phosphorylation and thereby the inhibition of MLCP in Swiss 3T3 fibroblasts in a ROK-dependent manner[91]. This would therefore suggest that MLCP inhibition could play a role in LPA-induced fibroblast contraction by enhancing the effect of MLC kinase (MLCK), leading to prolonged phosphorylation of MLC and, subsequently, an increase in actin/myosin cross-bridging and contraction. Moreover, LPA has also been shown to induce an MLC-independent pathway of cell contraction through rac, p21-activated kinase 1 (PAK1) and cofilin-1-mediated membrane ruffling[92].

In addition to promoting Rho-dependent cell contraction, LPA is also a potent stimulator of Rac, leading to lamellipodia protrusion and cell migration[74,93]. This involves Gi-dependent activation of PI3K that, in turn, activates the Rac-specific guanine nucleotide-exchange factor Tiam1[93]. Moreover, LPA synergises with EGF, PDGF and β1A integrins in the stimulation of cell migration[94]. In the lung, it has been shown that LPA bound to albumin acts through LPAR1 as a chemoattractant for primary mouse lung fibroblasts. Chemotaxis induced by BALFs isolated from fibrotic mice is attenuated by more than 50% when the fibroblasts are deprived of LPAR1, suggesting that LPA is the predominant fibroblast chemoattractant in the airspaces of BLM-treated mice[31]. In addition, a selective inhibitor for LPAR1, AM966, has been found to inhibit the chemotaxis of IMR-90 human lung fibroblasts mediated by LPA[95]. The LPAR1-LPAR3 inhibitor Ki16425 inhibits chemotaxis of human foetal lung fibroblasts induced by BALF from IPF patients, showing the importance of LPAR1 in LPA-induced fibroblast chemotaxis[31]. *In vivo,* LPAR1 deletion protects against BLM-induced fibrosis in mice, most likely due to the observed decrease in fibroblast accumulation. By contrast, fibroblast collagen production and differentiation to myofibroblasts remain unaffected by LPAR1 deletion[31] but have been suggested to be regulated by LPAR2[42]. In human lung fibroblasts, LPA induces the expression of α-smooth muscle actin (αSMA), fibronectin (FN), collagen I a2 and TGF-β1 protein expression, mediated through LPAR2[42]. In support of an effect of LPA on

the differentiation of lung fibroblasts to myofibroblasts, tumour-secreted LPA promotes the differentiation of peritumor fibroblasts to myofibroblasts and accelerates hepatocellular carcinoma progression[96], as well as the expression of αSMA in human adipose tissue-derived mesenchymal stem cells[97,98].

LPA signalling, specifically through LPAR1, has been found to completely suppress the apoptosis of adherent primary mouse lung fibroblasts induced by serum deprivation[41]. Similar anti-apoptotic effects of LPA have also been reported in NIH3T3, Swiss 3T3 and Rat-1 fibroblasts[99], as well as ATX-transfected NIH3T3 fibroblasts[100], further supporting a role of ATX/LPA in mediating pathologic fibroblast accumulation.

Finally, signs of LPA-induced differential expression in fibroblasts can be extrapolated from an expression profiling study of mouse embryonic fibroblasts (MEFs)[101]. LPA induces the transcription of more than 100 immediate-early genes associated with growth and cell cycle progression, growth regulatory kinases and secreted factors such as chemokines, pro-angiogenic factors and pro-fibrotic factors. Also very prominent is the activation of genes related to cytoskeletal organisation and integrin signalling, which is in line with the role of LPA in cell motility. Simultaneously, LPA-downregulated genes are associated with adhesion[101]. Therefore, LPA seems to have a plethora of actions on fibroblasts concerning cell cycle, growth, motility and inflammation. However, when used at low concentrations, LPA enhances mostly genes associated with cell movement rather than cell growth, indicating that LPA acts predominantly as a motility factor than a growth factor at low concentrations[101]. The effects of LPA on differential gene expression in MEFs have been suggested to be mediated by beta-arrestin 2 in an independent study[102].

Endothelial cells

The endothelium is the thin layer of cells that lines the interior surface of blood vessels and lymphatic vessels forming an interface between circulating blood or lymph in the lumen and the rest of the vessel wall. Endothelial cells (ECs) are involved in many aspects of vascular biology, including barrier function, blood clotting, angiogenesis, vasoconstriction and vasodilation[103]. Although endothelial dysfunction, or the loss of proper endothelial function, is a hallmark for vascular diseases and often regarded as a key early event in the development of atherosclerosis and cardiovascular diseases, chronic lung diseases such as COPD, pulmonary hypertension and interstitial lung diseases have all been reported to have a lung vascular disease component[104,105]. Moreover, the interaction of endothelial cells with immune cells is instrumental for the extravasation of inflammatory cells at local sites.

LPA increases the permeability of an endothelial layer consisting of human pulmonary arterial ECs (HPAECs) to FITC-dextran (in transwell assays) and reduces their electrical impedance[106]. The LPA-induced loss of endothelial barrier function is associated with changes in actin stress fibre formation[106]. Similar observations have been made in human umbilical vein ECs, in which it has been shown that LPA induces endothelial hyperpermeability through the activation of RhoA and Rho kinase, master regulators of signals to the cytoskeleton[107-109]. Therefore, and despite some conflicting reports on the effects of LPA on the permeability of other endothelial systems[109-111], the increased levels of LPA in certain pulmonary pathophysiological conditions could increase endothelial permeability, thereby facilitating the influx of inflammatory cells and soluble factors. Indeed, genetic deletion of LPAR1 or LPAR2 and the resulting abrogation of LPA signalling attenuate the BLM-induced vascular leakage observed during the development of modelled pulmonary inflammation and fibrosis[31,42].

Interestingly, ECs of high endothelial venules (HEVs), largely responsible for lymphocyte extravasation into secondary lymphoid organs, have been reported to express and secrete ATX, and chemokine-activated lymphocytes express receptors with enhanced affinity for ATX[112,113]. Moreover, it has recently been shown that HEV-expressed HS plays a role in chemokine presentation and lymphocyte homing[114], while the polybasic insertion in ATXα has been suggested to confer specific binding to cell surface HS proteoglycans[20]. Impressively, intravenous injection of enzymatically inactive ATX attenuates T-cell homing to lymphoid tissues, suggesting that EC-bound-ATX is an adhesive substrate for homing lymphocytes[112]. In the same homing cascade, LPA locally produced by HEV-ATX stimulates the polarisation, motility and transendothelial migration of naïve T-cells[112,115] or the motility of the ECs[116]. Furthermore, LPA stimulates the expression of IL-1β, IL-8 and MCP-1 from human ECs[117-119] *via* the p38 and JNK pathways[119], and it has been reported that LPA activates ECs to secrete chemokines which, in the presence of LPA, might modulate interactions between the endothelium and circulating monocytes[120]. LPA increases ICAM-1 expression in HUVECs, which might also enhance interactions with leukocytes[121,122] through ROCK2[122] in an NF-κB dependent mechanism[121,122]. A similar effect has also been reported in human aortic ECs, in which LPA stimulates E-selectin and VCAM-1 expression and increases the binding of monocytes and neutrophils[123]. It has also been reported that LPA upregulates the expression of pentraxin-3 (PTX3) in a human artificial EC cell line[124]. PTX3 is an acute-phase protein produced at the sites of infection and inflammation by various tissues and cells, in particular innate immunity cells, in response to proinflammatory signals and Toll-like receptor engagement. In addition, it has recently been reported that PTX3 regulates leukocyte recruitment upon acute lung injury (ALI)[125], while genetic variation in PTX3 is associated with primary graft dysfunction after lung transplantation[126]. Although the endothelial cells of HEVs (and other endothelial systems) differ substantially from pulmonary endothelial cells, similar mechanisms may be in play in the lung, further

regulating inflammatory cell influx.

Beyond the effects of LPA in endothelial permeability and the possible regulation of the influx of inflammatory cells, LPA stimulates the migration of some (but not all) pulmonary EC types through ECM-dependent cytoskeletal rearrangements involving focal adhesions[127-129]. The migration, proliferation and differentiation of ECs are all essential steps in angiogenesis, and LPA has been reported to be involved in all processes, in different EC systems and experimental conditions (reviewed in[130]). Notably, LPA dramatically downregulates the surface expression of CD36, the receptor of thrombospondin-1 and other anti-angiogenic proteins in primary microvascular endothelial cells and promotes angiogenesis *via* a PKD-1-dependent signalling pathway[131]. LPA enhances VEGF-C expression in human endothelial cell lines through LPAR1/3, COX-2, NF-κB and EGFR transactivation-dependent mechanisms[132,133]. Therefore, the ATX/LPA axis might also stimulate angiogenesis, thereby exacerbating carcinogenesis and possibly chronic lung diseases that have been suggested to include a vascular component.

Smooth muscle cells

Smooth muscle cells (SMCs) play an important role in mediating a wide range of physiological processes, such as blood pressure regulation and airway responsiveness. Their principle function is to contract or relax in response to stimuli, and they are capable of major phenotypic changes in response to alterations in local environmental cues[134]. LPA has been suggested to be such a phenotypic modulator of SMCs, and its possible involvement in vascular diseases and atherosclerosis have been extensively reviewed elsewhere[13,135], suggesting that isolated vascular SMCs respond to LPA by proliferating and migrating. The early growth response-1 (Egr-1) transcription factor[136,137], which regulates the transcription of a large variety of genes in SMCs implicated in vascular diseases and fibrotic genes in fibroblasts[138], has been proposed to be central to LPA responses of vascular SMCs.

LPA has been reported to stimulate the proliferation of airway SMCs in marked synergism with EGF[139] and to enhance their contraction in response to serotonin and methacholine[140]. Moreover, LPA upregulates the expression of EGF receptors, increases EGF binding[141,142] and induces integrin αvβ5-mediated TGF-β activation[143], suggesting a possible involvement of LPA in asthma and obstructive lung diseases.

Remarkably, LPA has been suggested to target vascular and oncogenic pathways *via* the receptor for advanced glycation end products (RAGE)[144]. LPA has been reported to bind avidly to RAGE, which is required for LPA effects in vascular SMCs, including Akt signalling, proliferation and migration[144]. RAGE is a member of the immunoglobulin superfamily and has been shown to be a pattern recognition receptor that transduces the effects of multiple ligands, including advanced glycation end products (AGEs), advanced oxidation protein products, S100/calgranulins, high-mobility group box-1 (HMGB1) and amyloid-β peptide[145]. RAGE is highly expressed in the lungs, suggesting a potentially important role in lung homeostasis, and the disruption of RAGE levels has been implicated in the pathogenesis of a variety of pulmonary disorders, including ALI, fibrosis and cancer[145]. The discovery that it can be transactivated by LPA opens up novel research directions on the effects of LPA in the lung.

LPA EFFECTS ON LEUKOCYTES

In addition to the different immunomodulatory effects of ATX/LPA in stromal cells presented above, including the modulation of barrier functions of endothelial cells, vascular remodelling and cytokine secretion from epithelial cells, LPA has been reported to have direct effects on leukocytes. As with every cell in the body, primary alveolar leukocytes all express some LPA receptors (Table 1)[31].

Granulocytes

Eosinophils have a unique contribution in initiating inflammatory and adaptive responses due to their bidirectional interactions with DCs and T cells and to their large panel of secreted cytokines and soluble mediators[146]. They are mainly involved in parasite infections and allergic diseases; however, they have significant contributing roles in a wide range of other diseases[147]. LPA exhibits chemotactic activity towards human peripheral blood eosinophils, shown to express LPAR1,3 mRNA, both *in vitro*[148] and in the lung *in vivo*[149]. Moreover, LPA re-arranges the eosinophil actin cytoskeleton, upregulates the expression of the integrin CD11b on their surface and stimulates Ca^{++} mobilisation and the production of reactive oxygen intermediates[148]. The observed effects of LPA in eosinophils, shown pharmacologically to be mediated through LPAR1/3-$G_{i/o}$, are comparable to those obtained from other well-known chemoattractants such as C5α, PAF, CCL5, CCL11 and CCL13[148], suggesting that LPA is a potent chemoattractant and activator of eosinophils.

Like eosinophils, human peripheral blood neutrophils, the most abundant granulocytes or leukocytes in the blood and the major effectors of acute inflammation[150], respond to LPA by calcium flux and oxidative burst[151]. LPA has also been reported to stimulate neutrophil degranulation[152] and to promote neutrophil chemotaxis both *in vitro*[153] and in the lung *in vivo*[149]. Despite the limited studies and some conflicting reports[154], it seems that LPA might have a role in neutrophilic responses and therefore in acute inflammation and lung injury.

Macrophages

Macrophages (MΦs), the most plastic cells of the haematopoietic system and the predominant resident immune cells in the lungs, have well-established roles in lung homeostasis, tissue repair and immunity[155,156]. Peripheral blood monocytes and/or tissue MΦs in mice, humans and rats all express some of the receptors for LPA; how-

ever, different publications, all based on RT-PCR data, report different expression patterns[31,157-159], while bone marrow-derived MΦs were found to express all 5 major LPARs (unpublished data and[83]). All transformed monocytic cell lines (MM6, RAW, THP-1, J774A.1) have also been reported to express LPA receptors[160-162]. However, a systematic study on LPAR expression during the differentiation of monocyte to MΦs and upon inflammatory activation of primary, resident or immigrating cells is still lacking.

The same is true for ATX, as there are limited reports on ATX expression in monocytes/MΦs. LPS-stimulated transformed monocytic THP-1 cells have been reported to express ATX mRNA[163,164] that is inhibited by pharmacological inhibitors of PKR, JNK and p38 MAPK[164]. More importantly, alveolar MΦs from BLM-challenged fibrotic mice and human IPF patients have been shown with immunocytochemistry to express ATX[23]. Macrophage ATX expression has also been noted in LC patients (unpublished data). Therefore, ATX expression from inflammatory or tumour-associated MΦs would stimulate local LPA production and its plethora of effects.

As far as the effects of LPA on MΦs themselves are concerned, LPA has been shown to protect murine primary peritoneal MΦs from apoptosis induced by serum deprivation, suggested to be mediated through PI3K[165]. By contrast, LPA has no effect on macrophage proliferation[165]. In THP-1 cells, LPA significantly increases reactive oxygen intermediates (ROI) production and prostaglandin E2 release[161]. In RAW264.7 cells, LPA stimulates cell survival and induces monocyte lipid accumulation from oxidised low-density lipoprotein (ox-LDL), suggested to be mediated through PPARγ activation, and CD36 scavenger receptor uptake[166]. LPA in J774A.1 cells also induces ox-LDL uptake[162] and IL-1 expression[167]. In MM6 cells, LPA has been reported to increase cytosolic Ca^{++}, a second messenger of cellular activation that regulates diverse biological processes such as the secretion of cytokines and the expression of proinflammatory genes[160]. Therefore, the limited studies on the effects of LPA in MΦs point to a potential pro-survival and pro-inflammatory role of the ATX/LPA axis, although more studies are needed, especially in primary cells, employing flow cytometry analysis of surface expression markers.

Dendritic cells

Dendritic cells (DCs) are the most potent antigen-presenting cells specialised in the activation of naive T-lymphocytes and the initiation of the immune response and are among the major immunological cells residing in the lungs. LPA (50 μmol/L) has been shown to affect the differentiation of peripheral circulating monocytes to DCs *in vitro*, which, however, have impaired immunological functions[168]. Interestingly, LPC, the precursor of LPA and the substrate of ATX, has also been reported to promote dendritic cell maturation from monocytes, with the ability to stimulate IL-2 and IFN-γ production by allogeneic T lymphocytes[169]. Notably, LPC released from apoptotic cells has also been suggested to be a potent chemotactic signal to MΦs *via* the phagocyte receptor G2A[170,171].

Both mature and immature DCs express LPARs[1-3] but respond differently to LPA[172]. LPA induces calcium flux, actin polymerisation and chemotaxis of immature DCs, whereas LPS-exposed mature DCs are insensitive. However, LPA inhibits, in a PTX-insensitive manner, the secretion of IL-12, and TNF and enhances the secretion of IL-10 from LPS-exposed mature DCs[172]. Other groups have suggested a predominance of LPAR2 in DCs[173,174] and reported that LPA induces IL-6 and IL-8 in maturing DCs[174] but does not have these effects in mature DCs[174]. Moreover, LPA does not exert a dominant effect on the ability of DCs to stimulate Th cell polarisation but does inhibit LPS-induced responses[173]. Similarly, unsaturated LPA species (as opposed to saturated ones) are able to induce the chemotaxis of immature but not LPS-exposed mouse bone marrow-derived DCs *in vitro*, attributed to LPAR3[175]. Finally, $LPAR2^{-/-}$ DCs have been reported to induce the proliferation of co-cultured T cells and their IL-13 secretion, more so than in wild-type DCs, suggesting that LPAR2 in DCs has a suppressive role in the Th2 inflammation and airway response to allergens[58]. Indeed, adoptive transfer of $LPAR2^{-/-}$ DCs pulsed with ovalbumin (OVA) enhances lung inflammation in comparison with OVA-pulsed wild-type DCs[58]. However, a different group reported that heterozygous LPAR2 KO mice are partially protected from allergic inflammation[57].

Taken together, and despite the limited available information and observed discrepancies, LPA seems to have a pro-inflammatory role in immature DCs, promoting inflammatory cytokine secretion and their chemotaxis and maturation, whereas in mature cells, LPA has a potential anti-inflammatory role, which might depend on the allergen.

Lymphocytes

Lymphocytes, the major cellular components of the adaptive immune response, all express some LPARs, as assessed with RT-PCR and in some rare case with western blots, but the results have been somewhat conflicting[176-178]. Moreover, the presence of recently identified LPARs remains to be examined. In one such study, LPAR5 was reported to be highly expressed in gastrointestinal lymphocytes[179]. As with all cells, future FACS studies on primary cells are needed to clarify the constitutive and inducible regulation of LPA receptor expression in T- and B-cell subsets. By contrast, no ATX mRNA expression was detected in splenocytes, thymocytes and $CD8^+$ T-cells, even upon their activation with phorbol myristate acetate (PMA) (unpublished data and[83]), consistent with the expression of other NPP family members in these cells[3]. However, a human transformed pre-B-cell line (Nalm-6) was reported to express and secrete ATX, the effects of which on LPA production were suggested to be counteracted by the simultaneous expression

of LPP1[180].

LPA stimulates Jurkat leukemic T cells, leading to calcium flux and proliferation[181]. Similarly, immortalised human B lymphoblasts respond to LPA with calcium flux, MAPK activation, and immunoglobulin production[182]. However, there is a wide variety of responses to LPA regarding calcium flux depending on the cell line[183].

Jurkat cells also respond to LPA *in vitro* by migrating through a matrigel membrane, an experimental connective tissue-like barrier[184]. As mentioned above, and despite opposing findings[185], LPA produced locally by HEV-ATX has been shown to stimulate the polarisation, motility and transendothelial migration of naïve T-cells[115,116], and ATX/LPA has also been shown to affect endothelial permeability and thus the regulation of lymphocyte influx[112,113]. Similar mechanisms may also exist in B-cells, as LPA has been shown to enhance LFA-mediated adhesion of murine follicular and marginal zone B-cells to ICAM-1 *in vitro,* similar to the effects of CXCL12 and CXCL13 chemokines and PMA[186], suggesting that LPA may be involved in B-cell homing within the spleen.

LPA has also been reported to inhibit the apoptosis induced by antibodies to Fas, CD2 or CD3/CD28 of a human T lymphoblast cell line (Tsup-1), accompanied with the suppression of the apoptotic protein Bax[187]. Similarly, LPA protects B-cell lines (BJAB and I-83) and primary chronic lymphocytic leukaemia cells from apoptosis. By contrast, LPA does not protect normal B-cells from fludarabine- and etoposide-induced apoptosis[188]. LPA protects transformed pre-B cells (Nalm-1) from spontaneous or staurosporine-induced apoptosis[180]. However, indirect pro-apoptotic effects of LPA on T-cells have been reported through the upregulation of Fas in ovarian cancer cells[189,190].

LPA, surprisingly in a PTX-insensitive manner, suppresses IL-2 secretion from anti-CD3/CD28-activated $CD4^+$ T-cells, but not similarly activated $CD8^+$ cells or non-activated $CD4^+$ cells[176], although opposing results on IL-2 expression have been reported in Jurkat cells[181]. By contrast, LPA was reported to enhance PMA-induced IL-13 promoter activity and gene expression in Jurkat and human peripheral blood $CD4^+$ lymphocytes *in vitro*, but only under submaximal conditions and not by itself[178]. Therefore, it seems that LPA might co-stimulate the polarisation to Th2 responses, although both cultured human Th1 and Th2 cells responded to LPA by inducing calcium flux and chemotaxis[191].

Platelets and mast cells

Platelets are the principle effector cells in haemostasis and have additional major functions in inflammation, vascular integrity, and tissue repair. In the lungs, platelets contribute to pulmonary vascular barrier function and are required for defence against pulmonary haemorrhage[192]. Increased coagulation and depressed fibrinolysis, as a consequence of the activation of circulating quiescent platelets, result in diffuse alveolar fibrin deposition, which serves to amplify pulmonary inflammation, while the interaction of platelets with endothelial cells and leukocytes is critical in the pathogenesis of ALI[193]. Moreover, asthma is associated with a procoagulant state in the bronchoalveolar space, further aggravated by impaired local activities of the anticoagulant protein C system and fibrinolysis[194]. ATX has been reported to bind to integrins β1 and β3 on the surface of platelets[195], consistent with the integrin-mediated binding of ATX to lymphocytes[112] and insights from the crystal structure[196]. ATX was found to inhibit fibrinogen-dependent platelet aggregation and enhance their thrombin-induced LPA production[195], whereas systemic genetic overexpression of ATX in mice *in vivo* resulted in bleeding diathesis and attenuation of thrombosis[29]. On the other hand, LPA levels in serum prepared from platelet-rich plasma are 5-10-fold higher than in platelet-poor plasma[197], indicating that activated platelets are a major source of LPA in the circulation. Therefore, the recruitment of circulating ATX to the platelet surface could enhance the local LPA production during clotting, which in turn would exert its numerous effects in adjacent cells. The effects of LPA on platelets, which express the five major LPARs, include shape change, fibronectin matrix assembly, platelet-monocyte co-aggregate formation and synergism with other platelet agonists, such as epinephrine and adenosine diphosphate, and have been reviewed elsewhere[135].

Mast cells, potent effector cells of the innate immune system, are mainly implicated in pro-inflammatory responses to allergens but can also contribute to protection against pathogens[198]. LPA potently induces the proliferation and differentiation of mast cells, which also express LPARs[199-201], providing a synergistic signal with the major mast cell growth factor, stem cell factor (SCF)[199]. LPA strongly enhances the formation of secretory granules and the cell-surface expression of kit[199]. Mast cells primed with IL-4 respond to LPA by the production of chemokines, including macrophage inflammatory protein (MIP)-1b, monocyte chemotactic protein (MCP)-1, and IL-8[200]. Moreover, LPA induces histamine release from rat peritoneal mast cells and mouse skin fragments[202], and the subcutaneous administration of LPA increases plasma exudation in the skin[203]. Thus, LPA may both support reactive mastocytosis (a feature observed in several disease states) and serve as an amplifier of mucosal inflammation, in which mast cell hyperplasia is mediated by a Th2 cytokine-based mechanism.

THE ATX/LPA AXIS IN LUNG PATHOPHYSIOLOGY

Acute lung injury

Acute lung injury (ALI), or mild acute respiratory distress syndrome (ARDS), is a diffuse heterogeneous lung injury characterised by arterial hypoxemia, respiratory failure and low lung compliance, non-cardiogenic pulmonary oedema, and widespread capillary leakage leading to alveolar flooding[204]. Bacterial or viral pneumonia is the most common

cause of ALI and ARDS, but sepsis due to non-pulmonary infections, the aspiration of gastric contents, major trauma with shock and/or mechanical ventilation also commonly precipitate this type of injury[204]. Altered permeability of epithelial and endothelial barriers, inappropriate accumulation of leukocytes and uncontrolled activation of coagulation pathways are among the main pathophysiological concepts in ALI and ARDS[204], and LPA seems to affect all of them.

Elevated ATX/LPA levels have been detected in an animal model of LPS-induced ALI (unpublished data[34]), and the genetic deletion of LPAR1 or LPAR2 has been reported to moderately attenuate inflammation but not the epithelial/vascular leakage induced by LPS[62,205]. However, both pulmonary inflammation and vascular leakage in response to BLM are entirely abrogated in the absence of LPAR1 or LPAR2[31,42]. The partial protection of LPAR1- and LPAR2-null mice and attenuation of inflammation from LPS-induced lung injury are consistent with the observed LPA stimulation of IL-8 secretion from pulmonary epithelial cells *in vitro*[45,46]. IL-8 is the major chemoattractant of neutrophils[206], which in turn predominate LPS-induced inflammatory responses in ALI/ARDS[207]. LPA can also directly induce neutrophil chemotaxis *in vitro*, as well as neutrophil activation and degranulation[153,208]. However, the exogenous administration of LPA to the lungs has been reported to both increase and decrease neutrophilic accumulation and LPS-induced lung injury[149,209], highlighting the importance of assessing endogenous local control mechanisms of LPA production. Conclusive insights are expected to be obtained by the ongoing conditional genetic deletion of ATX and an examination of LPS-induced ALI severity, as well as by the creation of conditional knockouts for LPARs.

The ability of LPA to induce integrin-dependent activation of TGF-β in pulmonary epithelial cells[43] points to another pro-inflammatory role of LPA. TGF-β activation has been reported to disrupt the alveolar epithelial barrier integrity, leading to alveolar flooding[210,211]. Moreover, TGF-β is known to induce the expression of plasminogen activator inhibitor-1 (PAI1), a major inhibitor of fibrinolysis, whereas fibrin deposition is a hallmark of ALI[212]. Therefore, by promoting TGF-β activation in the pulmonary epithelium, LPA could indirectly promote epithelial barrier disruption and inhibit fibrinolysis in an environment of high TGF-β content and in this manner promote lung injury.

Moreover, LPA has been shown to increase the permeability of endothelial systems[106-108], which could facilitate the entry of inflammatory cells in the alveolar space, although there is much controversy on the issue[109-111]. *In vivo*, genetic deletion of LPAR1 or LPAR2 attenuated the BLM-induced vascular leak[31,42], indicating that LPA signalling indeed disrupts vascular endothelial barrier integrity, in turn promoting the infiltration of inflammatory cells and possibly ALI.

Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, fibrotic form of diffuse lung disease that occurs mainly in older adults and is characterised by a progressive worsening of lung functions and a poor prognosis[213,214]. Clinically, IPF is characterised by progressive, exertional dyspnoea and non-productive cough, worsening of pulmonary function and radiographically evident interstitial infiltrates (honeycombing). Histologically, IPF is associated with the appearance of Usual Interstitial Pneumonitis (UIP), which is characterised by patchy subpleural and/or paraseptal interstitial fibrosis alternating with areas of mild inflammation and normal lung. The hallmark of IPF/UIP is the presence of hyperplastic reparative epithelium overlying distinctive fibroblastic foci that deposit exuberant ECM components, leading to thickening of alveolar septa and the collapse of normal lung architecture[213]. Although the aetiology and pathogenesis of IPF remain poorly understood, a number of conditions and risk factors are weakly associated with the disease: cigarette smoking, occupational/environmental factors, gastro-oesophageal reflux, latent viral infections, and age/gender/genetic predisposition[213]. To study the pathogenetic mechanisms that govern disease activation and perpetuation, a number of animal models have been developed that reproduce the clinical features of IPF, although it remains unclear if they truly replicate the chronic and progressive forms of the disease[215]. Among them, the BLM model is the most widely used and best characterised model and is responsible, together with the site-specific and/or temporal overexpression or ablation of candidate pathogenic genes, for most of our knowledge concerning IPF pathogenesis[215,216]. In this context, current research suggests that the mechanisms driving IPF reflect abnormal, deregulated wound healing in response to repetitive pulmonary epithelial damage, involving increased vascular permeability of the endothelium, extravascular coagulation, TGF-β activation, fibroblast persistence and differentiation to myofibroblasts, leading to exaggerated collagen deposition[214,217].

Deregulated phospholipid homeostasis seems to be an integral component of pulmonary fibrosis pathogenesis. Early studies have reported the altered composition of phospholipids in IPF[218-221], whereas experiments with genetically modified mice implicate proinflammatory mediators, such as prostaglandins, thromboxanes and leukotrienes, in the pathogenesis of BLM-induced pulmonary fibrosis[222-224]. These mediators derive from arachidonic acid, which is the product of phosphatidylcholine (PC) hydrolysis by PLA2[225], with concurrent release of LPC, the substrate of ATX.

ATX shows strong staining intensity within the alveolar epithelium immediately adjacent to fibroblastic foci and lower intensity in interstitial MΦs, fibroblast-like cells and in areas of bronchiolar metaplasia in IPF lung samples[23]. A similar expression profile was also demonstrated in fibrotic non-specific interstitial pneumonia (fNSIP) samples, a histopathological pattern sharing common pathologic features with UIP. By contrast, ATX has minimal expression within the inflammatory components of

cellular NSIP lung samples and in areas of loose connective tissue, called Masson bodies, representing the pathogenetic hallmark of cryptogenic organising pneumonia (COP). The two latter pathologies represent two forms of idiopathic interstitial pneumonias (IIPs) with favourable prognoses and excellent treatment response to corticosteroids, indicating that ATX upregulation is closely associated with more progressive and irreversible forms of pulmonary fibrosis, such as IPF/UIP and fNSIP[23]. In the mouse BLM-induced fibrotic lung, high constitutive ATX expression has been noted in the bronchial epithelium, the major source of ATX in the lungs, as well as in inflammatory alveolar MΦs, resulting in increased ATX BALF levels[23,31]. However, the increase in ATX BALF closely follows BALF total protein levels, suggesting that additional ATX could be extravasated from the circulation. As a consequence of the increased ATX levels, LPA levels are also increased in the BALFs of fibrotic mouse and human lungs[23,31], even at early time points[41]. Pharmacological inhibition of ATX results in the attenuation of LPA levels, confirming that ATX is solely responsible for LPA production in the lung[23].

Conditional genetic deletion of ATX from the majority of bronchial epithelial cells or MΦs results in the attenuation of BLM-induced pulmonary inflammation and fibrosis, as indicated by the improved lung architecture, reduced inflammation and collagen production, highlighting the importance of local pulmonary ATX production and verifying ATX as a major contributor to disease pathogenesis[23]. Likewise, genetic deletion of either LPAR1 or LPAR2 also results in attenuation of the BLM-induced disease[31,42], suggesting that the ATX/LPA axis is a candidate for therapeutic interventions. Indeed, pharmacological inhibition of either ATX or LPAR1 results in attenuation of BLM-induced disease symptoms[23,95], and pharmacological inhibition of LPAR1/3 alleviates radiation-induced pulmonary fibrosis[226]. However, the relative contribution of each receptor to pulmonary inflammation and fibrosis will have to be evaluated in head-to-head studies, with animals of the same genetic background and in comparison with littermate controls.

The apoptosis of alveolar epithelial cells is found both in the lungs of IPF patients and in animal models of the disease, correlating with the increased expression of "death-inducing" TNF/TNF receptor family members and various apoptotic markers[227]. Furthermore, induction of epithelial apoptosis is sufficient to initiate a fibrotic response in animal models[228], whereas genetic or pharmacological blocking of apoptotic signals can prevent a BLM-induced fibrotic response[229]. These observations have contributed significantly to the prevailing hypothesis that the mechanisms driving IPF reflect abnormal, deregulated wound healing in response to multiple sites of on-going alveolar epithelial injury[214]. LPAR1- and LPAR2-null mice, which are both protected from the development of the BLM-induced disease, exhibit significantly reduced numbers of TUNEL$^+$ epithelial cells[31,42], suggesting that LPA promotes epithelial apoptosis upon lung injury. In agreement, LPA signalling through LPAR1 was reported to induce anchorage-dependent apoptosis in cultured normal human bronchial epithelial cells[41], although the intracellular mechanisms and the role of cell-to-cell and cell-to-ECM contacts need to be defined. Interestingly, BLM-induced, epithelial cells undergoing apoptosis *in vivo* express TNF[230], which has been suggested to stimulate ATX expression[83,231]. Therefore, stimulation of apoptosis in epithelial cells from BLM in mice or unidentified insults in humans can stimulate TNF expression, which in turn promotes ATX expression and the local production of LPA, perpetuating the damage. Moreover, the critical involvement of the cytoskeleton in epithelial apoptosis and BLM-induced disease[232], as well as the reported ability of LPA to rearrange the cytoskeleton of bronchial epithelial cells[43], argue for an additional intracellular pathway mediating the effects of LPA in epithelial cells.

Increased fibroblast accumulation, due to increased fibroblast proliferation and migration and to decreased fibroblast apoptosis, is a hallmark of IPF pathogenesis[233]. Consistent with a role for ATX/LPA in disease pathogenesis, LPA stimulates lamellipodia protrusion and fibroblast cell migration[74,93]. Moreover, LPA acts as a chemoattractant for primary mouse lung fibroblasts, and genetic deletion of LPAR1 attenuates lung fibroblast chemotaxis induced by BALF from fibrotic mice, proving that LPA is the predominant fibroblast chemoattractant in the airspaces of BLM-treated mice[31]. In humans, BALFs from IPF patients with elevated LPA levels induce fibroblast chemoattraction, in contrast with BALF from healthy individuals, an effect abrogated by an LPAR1 inhibitor[31], while LPA also induces the chemotaxis of human lung fibroblasts *in vitro*[95]. Thus, the chemotactic effect of LPA on fibroblasts could be a determining factor for the development of IPF. Moreover, the ability of LPA to promote the proliferation of lung fibroblasts *in vitro*[81,226] and its ability to completely suppress the apoptosis of adherent primary mouse lung fibroblasts[41] or non-lung fibroblasts[99] further indicate that LPA promotes pathologic fibroblast accumulation in the airspaces. Chronic fibrosis is characterised by the persistence of myofibroblasts, which promote tissue remodelling by expressing fibrogenic and extracellular mediators[234]. LPA, through LPAR2, induces the differentiation of human lung fibroblasts to myofibroblasts by inducing αSMA, FN, collagen I a2 and TGF-β1 protein expression[42], whereas LPA-mediated differentiation of peritumor fibroblasts to myofibroblasts in the liver has also been shown[96]. Therefore, the ATX/LPA axis is also implicated in pulmonary fibrosis through fibroblast recruitment, proliferation and differentiation into myofibroblasts.

It is well accepted that inflammatory mediators play a role both in the initiation and progression of pulmonary fibrosis, despite the failure of anti-inflammatory treatments[214]. A prominent effect of LPA in epithelial cells is the production of IL-8[45,46], a potent neutrophil chemoat-

tractant, suggesting that the observed increased levels of LPA in the early phases on BLM-induced lung injury[41] can promote the initiation of the inflammatory cascade. LPA stimulation of ECs also results in the upregulation of inflammatory mediators, such as IL-1β, IL-8 and MCP-1[117-119], and adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin, that might enhance interactions with leukocytes, facilitating their extravasation[119,121,123]. Moreover, EC-bound-ATX has been shown to be an adhesive substrate for homing lymphocytes[112], whereas LPA stimulates the polarisation, motility and transendothelial migration of naïve T-cells[112,115]. Genetic deletion of LPAR1 or LPAR2 results in the attenuation of vascular leakage upon BLM treatment[31,42], whereas LPA increases the permeability of an endothelial layer consisting of human pulmonary arterial ECs[106]. Therefore, LPA can also affect the inflammatory component of pulmonary fibrosis through the stimulation of cytokine production, through the modulation of the endothelial barrier and through the promotion of inflammatory cells extravasation.

TGF-β is the major pro-fibrotic factor in several organs. In the lung, it is produced from a wide variety of cells, including alveolar MΦs and neutrophils, activated epithelial and endothelial cells, fibroblasts and myofibroblasts[235]. When activated, TGF-β is a pleiotropic growth factor with chemotactic and proliferative properties, inducing macrophage and fibroblast recruitment and the secretion of a number of pro-inflammatory and pro-fibrotic cytokines[235]. TGF-β levels are increased in the BALFs of fibrotic lungs in both BLM-challenged mice and human IPF patients[236], whereas adenoviral delivery of TGF-β is sufficient to promote fibrosis in the absence of inflammation[237,238]. LPA induces TGF-β expression in pulmonary fibroblasts *in vitro*[42], and stimulation with LPA leads to increased TGF-β activity through integrin αvβ6 in bronchial epithelial cells[43] and through integrin αvβ5 in smooth muscle cells[143]. Therefore, LPA can indirectly promote pro-fibrotic responses by potentiating TGF-β activation and possibly expression.

HGF is a growth factor for epithelial and endothelial cells. It is activated only in injured tissues, the lungs included, and its expression increases post-lung injury[239]. In patients with IPF, HGF levels in BALF are increased compared to healthy subjects; however, fibroblasts from IPF patients express less HGF and have a decreased activation capability of pro-HGF[239]. In fact, the exogenous administration of HGF alleviates fibrosis and induces lung repair[72,239]. The protective effect of HGF has been suggested to be mediated through the restriction of myofibroblast recruitment, the promotion of proliferation and the survival of lung epithelial and endothelial cells[72] and the induction of myofibroblast apoptosis[239]. Compared to HGF, LPA has the opposite effects on c-Met, the basic receptor of HGF, in normal human bronchial epithelial cells: LPA induces c-Met serine phosphorylation and its redistribution to the cell membrane, and it is also capable of abrogating HGF-induced c-Met activation[70]. Therefore, LPA could indirectly have profibrotic consequences through the inhibition of HGF signalling.

Conclusively, the ATX/LPA axis may promote pulmonary fibrosis in several ways, such as the induction of vascular leakage, fibroblast migration, fibroblast differentiation, epithelial cell apoptosis, inflammatory cell influx, TGF-β signalling and HGF signalling suppression; however, anti-inflammatory effects have also been reported.

Asthma

Asthma, a common chronic inflammatory lung disease that leads to airflow obstruction[53], has an onset usually early in life in association with sensitisation to common aeroallergens. Asthma can be divided into phases, such as acute or chronic, severe or not severe, with the pathophysiology of the disease differing among the distinct phases[240]. Acute asthma, or allergic asthma, is triggered by allergens that lead to IgE reactions and the activation of mast cells located beneath the mucosa of the lower airways of the respiratory tract. Mast cells release their granules, thereby stimulating mucus production and airway smooth muscle contraction, which constricts the airway, causing the characteristic asthmatic wheezing. Furthermore, a Th2 lymphocyte response is also a predominant feature of acute asthma and, together with mast cells, lead to cytokine secretion, thus mediating inflammation in the form of eosinophil and other leukocyte recruitment[240,241]. Eosinophils, key players in asthma, further promote inflammation and enhance airway hyperresponsiveness and airflow obstruction[240]. Chronic asthma is a result of the inflammation obtained from acute asthma. The acquired chronic inflammation leads to mucosal epithelium hypersensitivity so that even simple environmental agents such as smoke can evoke asthma attacks. In persistent asthma, the lung epithelium is injured, and airway smooth muscle becomes hypertrophic[242]; both these tissues secrete inflammatory mediators[240]. Further changes in asthmatic lungs include mucus gland hypertrophy, collagen deposition and thickening of the basal lamina, increased matrix deposition and thickening throughout the airway walls[242,243], all of which contribute to airflow obstruction.

The involvement of the ATX/LPA axis in asthma was first established when it was shown that allergen exposure leads to an increase in the LPA levels in the BALF of humans[56] and a mouse model of asthma[57]. Similar results were obtained more recently: allergen challenge in asthmatic patients leads to an increase in LPA levels, accompanied by an increase in BALF ATX levels[244]. In a triple allergen asthmatic mouse model, ATX expression is localised in terminal bronchial epithelial cells and alveolar MΦs[244]. Transgenic mice overexpressing ATX in the liver, which leads to systemic 100%-200% increases in the ATX levels in the serum[29], develop increased pulmonary inflammation and higher levels of IL-4 and IL-5 in lung homogenates and BALFs upon triple allergen challenge[244]. Accordingly, heterozygous ATX full knockout mice, with a 50% reduction of systemic/serum ATX and

LPA levels[28], exhibit reduced inflammation and IL-4/5 levels upon triple allergen challenge[244], indicating a major role for ATX/LPA in asthma pathogenesis. Pharmacological treatment with an ATX inhibitor attenuates disease development[244], establishing ATX as a potential drug target in the treatment of asthma.

Allergic inflammation in LPAR2$^{-/-}$ knockout mice is also attenuated[244]. Surprisingly, heterozygous LPAR1 or LPAR2 knockout mice have also been reported to develop distinct aberrant responses upon *Schistosoma mansoni* egg sensitisation and challenge[57]. However, and in a different mouse asthma model, using systemic immunisation with ovalbumin and alum, LPAR2$^{-/-}$ knockout mice showed greater allergic sensitisation, higher eosinophilia and Th2 inflammation[58]. These observed discrepancies could be due to the different allergens utilised and/or the genetic backgrounds of the experimental and control groups of mice, urging further comparative, genetic or pharmacological studies.

As mentioned above, LPA has been reported to have mainly pro-inflammatory effects in pulmonary cell types and pulmonary inflammation, but anti-inflammatory effects have also been reported. In support of a pro-inflammatory role of the ATX/LPA axis in the development of asthma, LPA stimulates IL-8 secretion from HBEpCs *in vitro*[45,46] and IL-8 levels are elevated in mouse lungs after intratracheal LPA administration[45]. IL-8 is a major chemoattractant for neutrophils and eosinophils[245], and its levels are elevated in the BALFs of asthma patients[245]. In accordance, intratracheal administration of LPA stimulates neutrophil infiltration in mice[57] and both eosinophil and neutrophil infiltration in guinea pigs[149], although no significant association was found between LPA and eosinophil recruitment in humans[56]. However, LPA has been shown to act chemotactically on human eosinophils *in vitro*[148]. Eosinophils have a primary role in allergic inflammation, releasing upon activation cytokines and leukotrienes and their highly inflammatory granule components injuring the airway and causing persistent inflammation[240]. The mast cell is another important cell type in the initiation and perpetuation of allergic inflammation through the release of leukotrienes and cytokines[240], whereas the release of histamine from their granules activates the endothelium and increases blood vessel permeability. LPA potently induces mast cell proliferation and differentiation, formation of their secretory granules[199], chemokine production[200] and histamine release[202]. Therefore, the LPA-mediated chemoattraction of eosinophils and mast cell activation, the impairment of EC barriers[107-109] and the enhancement of EC-leukocyte interactions[120-123] can all be deteriorating factors in the pathogenesis of asthma.

LPA has also been reported to stimulate lymphocyte homing[112,116] and TSLP and CCL20 secretion from HBEpCs *in vitro*[50]. TSLP is produced from the airway epithelium upon TLR activation and acts on dendritic cell motility and activation[53,246], leading to the Th2 polarisation[53] that is crucial in asthma. CCL20 contributes to airway inflammation in mouse models of asthma[52] and is known to act on the recruitment of DCs and T cells on the airway and other mucosal surfaces[52,247,248]. Therefore, these results suggest that ATX/LPA could also regulate adaptive immune responses in asthma.

Several other findings implicate LPA in other aspects of asthma. LPA is capable of promoting the proliferation of airway smooth muscle cells[139] and enhancing the contraction of airway smooth muscle[140], which could contribute to smooth muscle mass increase and airway hypercontractility, respectively, both of which are key features in asthma. Furthermore, by activating TGF-β in airway smooth muscle cells in an integrin αvβ5-dependent way[143], LPA can again promote asthma, as TGF-β induces airway remodelling, smooth muscle thickening, ECM deposition and mucous production in an asthma model[249]. Moreover, TGF-β is required for the differentiation of the Th17 cells that are linked to asthma[250,251] and Th9[252], a Th2 subtype that participates in the inflammatory and the remodelling aspect of airway allergy[53]. TGF-β also drives the differentiation of fibroblasts to myofibroblasts, leading to the thickening of epithelial basal lamina and airway walls[241] that follows chronic and severe asthma[253]. The observed activation of TGF-β by LPA in the airway epithelium[44] and smooth muscle[143] could, thus, affect many aspects of the disease.

The reported exacerbated allergic (OVA) inflammation in LPAR2$^{-/-}$ knockout mice is correlated with an LPA-LPAR2 suppressive effect on dendritic cell activation, the subsequent T cell proliferation and Th2 allergen response[58]. In support of this proposed anti-inflammatory role of LPA/LPAR2 in asthma development, LPA was found to inhibit the TNF-α/IFN-γ stimulated CCL5/RANTES[241,254-256] production from HBEpCs *in vitro*[59], whereas RANTES was found to increase in BALFs of asthmatic patients[257], and the severity of asthma has been associated with a polymorphism in the promoter of the *RANTES* gene[258]. LPA has also been shown to induce the expression of the decoy receptors for IL-13 and IL-33, IL-13R2 and soluble ST2 in HBEpCs *in vitro*[54,61]. Notably and concerning IL-13, LPA has been shown to have an opposite effect on T cells at submaximal activation, where it actually stimulates its gene expression[178]. Airway IL-13, found at higher levels in BALF of asthma patients[55], is implicated in asthma in many ways: it promotes survival and migration of eosinophils, activation of MΦs, mast cell maturation, permeability of airway epithelial cells, airway hyperresponsiveness, mucus production and transformation of airway fibroblasts to myofibroblasts leading to collagen deposition[53,241,259,260]. In allergy, IL-13 is also necessary for the isotype switching of B cells from IgM to IgE, whereas it restricts the differentiation of Th17 cells, a subtype also implicated in asthma[261], although these processes take place in secondary lymphoid tissues[53]. IL-33 is another cytokine expressed by the airway epithelium upon PRR activation that activates lung DCs and helps sustain the Th2 response in asthma[53,262]. Therefore, LPA could attenuate asthmatic inflammation by suppressing

IL-13 and IL-33 signalling. Finally, LPA has been shown to stimulate PGE2 expression from HBEpCs *in vitro*[63], whereas epithelial cells from asthmatic patients cultured *in vitro* were shown to overproduce PGE2 compared to normal epithelium[263]. In the lung, PGE2 is bronchoprotective and suppressive of inflammation in asthma[64,262,264], although some indications that it promotes Th2 differentiation do exist[265,266]. Therefore, the induction of PGE2 by LPA could have complex consequences, mostly protective of the pathology.

Lung cancer

Lung cancer (LC) is the most prevalent form of malignancy and the major cause of cancer-related deaths worldwide. The prognosis for patients with LC remains dismal, with a five-year survival rate of 14%. Current therapeutic options are limited to classical adjuvant therapy (a combination of radiation and chemotherapy with cytotoxic drugs) following surgery[267,268]. Histopathologically, LC can be divided into two major histopathological groups: non-small-cell LC (NSCLC)[269] and small-cell LC (SCLC)[270]. Approximately 80% of LC are NSCLC, and they are subdivided into adenocarcinomas, squamous cell, bronchoalveolar, and large-cell carcinomas[271]. SCLC, which accounts for close to 18% of all lung tumours, and large-cell neuroendocrine carcinomas both have a very high proliferative and metastatic potential. SCLC and NSCLC show major differences in histopathologic characteristics that can be explained by the distinct patterns of genetic lesions found in both tumour classes[272]. The molecular origins of LC lie in complex interactions between the environment (tobacco smoke and/or inhaled carcinogens) and host genetic susceptibility. Lung tumourigenesis appears to conform to a multistep model in which 1) self sufficiency of growth signals; 2) insensitivity to anti-growth signals; 3) evasion of apoptosis; 4) increased replication potential; and 5) angiogenesis and metastasis dictate the tumorigenic process[273].

ATX was originally isolated as an autocrine motility stimulation factor from the supernatant of highly metastatic melanoma cells[274]. Since then, increased ATX expression has been detected in a large variety of cancers such as neuroblastoma, hepatocellular carcinoma, breast cancer, renal cell carcinoma, glioblastoma, thyroid carcinoma, B-cell lymphomas, and non-small cell LC (reviewed in[10]). Moreover, the plethora of actions of LPA are concordant with many of the 'hallmarks of cancer', including proliferation, the evasion of apoptosis, angiogenesis and metastasis[10,11]. LPA levels are significantly increased in malignant effusions, and its receptors are aberrantly expressed in several human cancers[10]. Notably, overexpression of ATX and/or LPARs in the mammary gland was recently reported to result in spontaneous breast cancer in aged mice[275], whereas the genetic deletion of LPAR2 attenuates tumour formation in an experimental model of colitis-associated cancer[276].

Despite the established role of the ATX/LPA axis in carcinogenesis, little is known about its involvement in LC. Meta-analysis of datasets from seven different microarray studies on NSCLC for differentially expressed genes related to survival time identified ATX as one of the 64 genes predicting potential beneficial effects of aggressive therapy of stage I LC patients[277]. ATX mRNA is overexpressed in poorly differentiated carcinomas in NSCLC patients[22], while the conditional deletion of ATX from the lung attenuates chemically induced or k-ras-driven lung carcinogenesis (unpublished data and[278]), suggesting a major contribution of ATX in lung carcinogenesis, although the related mechanisms are still not fully investigated.

In support of these data, BrP-LPA, a dual function pan-antagonist of LPA receptors and an ATX inhibitor[75,279], inhibited tumour growth and angiogenesis in a engineered three-dimensional tumour xenograft NSCLC model composed of A549 lung carcinoma epithelial cells encapsulated in 3-D ECM injected in nude mice[76]. Similarly, genetic or pharmacologic neutralisation of LPAR1 attenuates mesenchymal stem cell-dependent angiogenesis and tumour growth in a murine xenograft model of A549 human adenocarcinoma[280]. In accordance, ATX was independently reported to induce the migration of A549 cells[40], and, in the same cells, LPA was shown to decrease the nuclear localisation and cellular abundance of p53[73].

The expression of LPA receptors seems to vary in different lung tumour cells (Table 1 and related references[74,281,282]), possibly regulated by methylation[283,284], and LPAR1 mutations were reported in a rat model of lung carcinogenesis correlating with advanced staging[285]. Again, conditional knockout mice for the different LPARs are needed to examine their individual contribution to lung carcinogenesis.

Genetic deficiency of ATX and its associated effects on LPA production results in embryonic lethality due to aberrant circulation and neural tube closure[28,36,38], suggesting a major effect of ATX/LPA in angiogenesis. Supporting *in vitro* studies have suggested that LPA stimulates the expression of a large number of angiogenic genes in different endothelial and cancer cells and regulates endothelial proliferation and migration (see above; reviewed in[130]). The conditional deletion of ATX and/or LPA receptors in different endothelial systems is expected to dissect the involvement of ATX/LPA to angiogenesis, an obligatory component of carcinogenesis.

EGFR is overexpressed and functions aberrantly in various human cancers, including NSCLC in which it enhances cancer invasion and brain metastasis[286], and has been extensively used as a target of therapeutic approaches[287]. LPA has been shown to induce squamous cell carcinoma cell proliferation and motility[288], ovarian cancer cell invasion[289] and prostate cancer cell proliferation[290] through EGFR transactivation, introducing the concept that LPA can amplify carcinogenic growth signals. Likewise, LPA has been reported to affect c-Met signalling[70,291] which was found to be overexpressed and activated in NSCLC cell lines and tumour tissues[292]. In

addition, c-Met has been suggested to provide resistance to EGFR targeted therapies[293]. Therefore, suggested adjuvant therapies targeting simultaneously both EGFR and c-Met for the treatment of NSCLC[294] could be possibly enhanced by inhibitors of the ATX/LPA axis.

CONCLUSION

ATX is a secreted glycoprotein widely present in biological fluids, including BALFs, largely responsible for the bulk of LPA production in the plasma and at inflamed and/or malignant sites. In turn, LPA evokes growth-factor-like responses in almost all cell types, including pulmonary cells, through its abundant GPCR receptors. ATX/LPA have an established role in inflammation and malignant transformation, and increased ATX and/or LPA levels in the lung have been detected in both humans with pulmonary diseases such as acute lung injury, IPF, asthma, and LC and/or the corresponding animal models. Genetic or pharmacologic interventions targeting the ATX/LPA axis have proven to be beneficial for disease management in animal models, establishing the ATX/LPA axis as a possible therapeutic target.

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MINIREVIEWS

FDG-PET for predicting efficacy of EGFR-tyrosine kinase inhibitors in lung cancer

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Abstract

Non-small cell lung cancer (NSCLC) is the major cause of cancer-related deaths worldwide. Recent advances in molecular biology have resulted in the clinical use of several molecularly targeted drugs, which usually exhibit cytostatic antitumor activity, to improve the survival of NSCLC patients. The epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) gefitinib and erlotinib have been approved for the treatment of NSCLC, and several phase Ⅲ trials have demonstrated that sensitizing *EGFR* mutations are biomarkers for predicting a favorable clinical outcome of NSCLC patients treated with the EGFR-TKIs. The Response Evaluation Criteria in Solid Tumors is generally used to assess the therapeutic response to antitumor drugs based on the morphological changes in tumor size as evaluated by computed tomography or magnetic resonance imaging. However, such assessment may not always reflect the treatment efficacy of cytostatic drugs, such as EGFR-TKIs. In this regard, functional imaging methods, including ^{18}F-fluorodeoxyglucose measured by positron emission tomography (FDG-PET), are potentially beneficial. An increasing body of evidence indicates the usefulness of FDG-PET to predict treatment efficacy for NSCLC patients treated with EGFR-TKIs. In this review, we summarize the current understanding of the potential role of FDG-PET in the clinical use of EGFR-TKIs for NSCLC.

Key words: Non-small cell lung cancer; ^{18}F-fluorodeoxyglucose measured by positron emission tomography; *Epidermal growth factor receptor* mutation; Gefitinib; Erlotinib; Survival; Biomarker

Core tip: Molecularly targeted drugs including epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs), which usually exhibit cytostatic antitumor activity, have emerged for the treatment of non-small cell lung cancer. The Response Evaluation Criteria in Solid Tumors is generally used to assess the therapeutic response based on the morphological changes in tumor size as evaluated by computed tomography or magnetic resonance imaging. However, such assessment may not always reflect the clinical outcome of cytostatic drugs, such as EGFR-TKIs. In this regard, 18F-fluorodeoxyglucose measured by positron emission tomography (FDG-PET) is potentially beneficial. Here we summarize the role of FDG-PET to predict the treatment efficacy in NSCLC treated with EGFR-TKIs.

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Lung cancer is the major cause of cancer-related deaths worldwide[1]. Lung cancer comprises two major histological types: small cell lung cancer and non-small cell lung cancer (NSCLC), and the latter represents 80%-85% of

Table 1 Studies evaluating the predictive roles of early 18F-fluorodeoxyglucose measured by positron emission tomography assessment on clinical outcome in non-small cell lung cancer treated with epidermal growth factor receptor-tyrosine kinase inhibitors

Ref.	EGFR-TKIs	Patient no.	Evaluation timing	*EGFR* mutation status			Significant association	
				Mutant	Wild-type	Unknown	PFS or TTP[1]	OS
Takahashi *et al*[25]	Gefitinib	20	2 d	12	3	5	Yes	NA
Soto Parra *et al*[24]	Erlotinib	23	2 d	NA	NA	NA	Yes	No
Mileshkin *et al*[23]	Erlotinib	51	2 wk	4	30	17	Yes	Yes
Zander *et al*[26]	Erlotinib	34	1 wk	4	24	6	Yes	Yes
Benz *et al*[22]	Erlotinib	22	2 wk	4	1	17	Yes[1]	Yes
Bengtsson *et al*[21]	Erlotinib	125	2 wk	10	90	25	NA	Yes

[1]Significant association with time to progression. NA: Data not available; EGFR-TKIs: Epidermal growth factor receptor-tyrosine kinase inhibitors; PFS: Progression-free survival; OS: Overall survival; TTP: Time to progression.

all lung cancers[2]. The majority of patients with NSCLC have locally advanced or metastatic disease at the time of diagnosis, and chemotherapy with cytotoxic agents remains marginally effective[3,4]. In recent years, molecularly targeted drugs, which usually exhibit cytostatic antitumor activity, have emerged for the treatment of NSCLC. The effective use of molecularly targeted drugs requires the identification of biomarkers to predict treatment response and clinical outcomes in NSCLC patients[5]. Recent advances in basic and translational research have identified epidermal growth factor receptor (*EGFR*) mutation as the most promising biomarker for predicting the treatment efficacy of the EGFR-tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib for NSCLC patients[6-11]. However, the clinical benefit of EGFR-TKI treatment is not confined to patients whose tumors harbor *EGFR* mutations, and some *EGFR*-mutated NSCLC patients do not respond to EGFR-TKIs[12]. Furthermore, sufficient tumor samples to test *EGFR* mutation status are not always available, and alternative methods to predict the efficacy of EGFR-TKI therapy are therefore warranted.

The therapeutic response to antitumor drugs is generally evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST)[13]. On the RECIST evaluation, target lesions are measured before and after chemotherapy by morphological imaging technologies, including computed tomography (CT) and magnetic resonance imaging (MRI). However, morphological changes in tumors usually take several weeks to occur after chemotherapy, and targeted tumor lesions contain noncancerous cell components such as necrotic, cystic and fibrotic lesions. Thus, RECIST evaluation based on size-measurements of total tumor volume may not always reflect the treatment efficacy, especially when patients are treated with cytostatic drugs[14].

In a phase Ⅱ trial of gefitinib monotherapy, NSCLC patients who had achieved stable disease (SD) had a favorable prognosis compared to those with progressive disease (PD)[15]. Similarly, of the NSCLC patients treated with gefitinib, the overall survival (OS) of patients with SD was significantly longer compared to those with PD[16]. In addition, erlotinib significantly prolonged OS despite a response rate of less than 10% in a large phase Ⅲ trial, possibly due to a high proportion of patients with SD[17]. These findings highlight the limitations of the RECIST criteria regarding the assessment of treatment efficacy for cytostatic drugs such as EGFR-TKIs. In this regard, molecular imaging methods, such as ^{18}F-fluorodeoxyglucose measured by positron emission tomography (FDG-PET), are advantageous because of their ability to detect changes in glucose metabolism, proliferative activities and the vascularization of tumors, which occur earlier than morphological changes[18,19]. Currently, FDG-PET has been the most wide-spreading imaging technique used as a diagnostic tool in various cancers, including NSCLC[20].

Several lines of evidence have indicated the value of FDG-PET to predict the therapeutic response and clinical outcome of EGFR-TKI therapy in NSCLC patients as shown in Table 1[21-26]. In 2008, we reported a preliminary study that assessed the roles of FDG-PET in predicting the treatment efficacy of gefitinib in five NSCLC patients[27]. The patients underwent FDG-PET 2 d (early phase) and 4 wk (late phase) after administration of gefitinib, and FDG uptake was evaluated as the maximum standardized uptake value (SUV_{max}) of the target lesions, which were evaluable by conventional CT. Of the four patients with sensitizing *EGFR* mutations, two patients exhibited a partial response (PR), and others had SD with decreased tumor size but did not achieve PR as evaluated by RECIST. In all of these patients, FDG uptake markedly decreased at the earlier phase from baseline as assessed by a mean ± SD SUV_{max}% of 60% ± 14% (60% ± 18% and 59% ± 12% for PR and SD groups, respectively). Notably, the two patients with SD had a long-term progression-free survival (PFS) of ⩾ 12.5 mo and an OS of ⩾ 16.9 mo. While these results were obtained from a small sample size, our findings suggest that FDG-PET can potentially assess the treatment efficacy of gefitinib for NSCLC patients more accurately than morphological evaluation.

Recently, Takahashi *et al*[25] reported a similar study of 20 lung adenocarcinoma patients receiving gefitinib monotherapy. In their study, changes in tumor FDG uptake 2 d after gefitinib initiation were positively correlated with changes in tumor size assessed by CT 1 mo after the treatment. In addition, metabolic responders defined as Δ

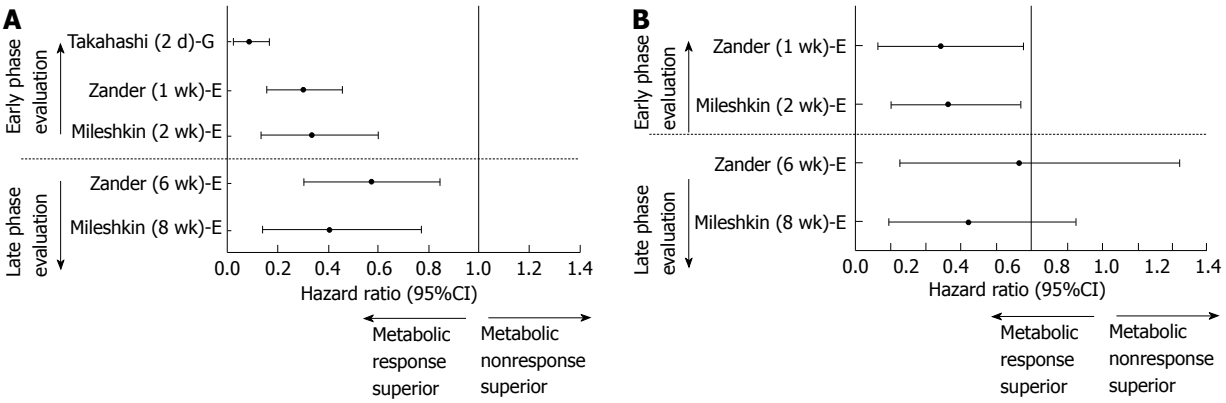

Figure 1 Progression-free survival (A) and overall survival (B) hazard ratios of 18F-fluorodeoxyglucose measured by positron emission tomography metabolic responders over the metabolic nonresponders with 95%CI based on the results from previous studies. The groups over the dotted line represent the data of early phase evaluation (2 d, 1 wk or 2 wk) and the groups under the dotted line represent the data of late phase evaluation (6 wk or 8 wk) after the initiation of epidermal growth factor receptor-tyrosine kinase inhibitor therapy. The data were available from references of [23,25,26].

SUV_{max}% < -20% had significantly longer PFS than metabolic non-responders (ΔSUV_{max}% ⩾ -20%) when SUV_{max} changes were evaluated after 2 d of treatment. This study demonstrates that the earlier metabolic response at 2 d could predict the prognosis of geftinib-treated NSCLC patients. These findings indicate that early FDG-PET assessment is useful to predict the treatment efficacy of gefitinib monotherapy compared to the conventional morphological assessment by CT or MRI.

Several studies have assessed the role of FDG-PET to predict the treatment efficacy of the EGFR-TKI erlotinib[21-24,26,28-30]. Aukema *et al*[28] assessed NSCLC patients who received neoadjuvant erlotinib by FDG-PET 1 wk after treatment. Of the resected tumors, 70% of metabolic responders defined as ΔSUV_{max}% ⩽ -25% [interquartile ranges (IQRs), 30%-91%] and 40% of non-responders (IQRs, 20%-50%) were necrotic. The metabolic and pathologic responses correlated significantly, suggesting that a change in FDG uptake is closely associated with the pathologic response to erlotinib. Other clinical studies have prospectively investigated whether an early FDG-PET assessment could predict the tumor response to erlotinib and survival in NSCLC patients. Soto Parra *et al*[24] reported that the metabolic response evaluated by FDG-PET 2 d after erlotinib initiation was significantly associated with a longer PFS in NSCLC patients. In a study by Mileshkin *et al*[23], the metabolic response (ΔSUV_{max}% ⩽ -15%) at 2 wk after erlotinib initiation was significantly associated with both improved PFS and OS in NSCLC patients receiving 2nd/3rd-line erlotinib monotherapy. Of note, in a subset of patients with wild-type *EGFR*, early metabolic responders tended to have a longer PFS compared to the metabolic non-responders[23]. Similar findings were also observed in another study, in which the metabolic response (ΔSUV_{peak}% ⩽ -30%) 1 wk after erlotinib initiation was significantly associated with both improved PFS and OS in NSCLC patients, irrespective of *EGFR* mutation status[26]. The same group subsequently investigated the predictive values of changes in FDG uptake using different SUV parameters (SUV_{max}, SUV_{2Dpeak}, SUV_{3Dpeak}, SUV_{50}, SUV_{A50}, SUV_{A41}, SUV_{70}, SUV_{A70} and SUV_{RTL}) and found that SUVmax best assesses the early metabolic response[30]. They also found that a lower residual FDG uptake measured by SUV_{max} and SUV_{2Dpeak} (but not other SUV parameters) at the early phase of treatment was associated with a significantly longer PFS[29]. Furthermore, another group reported that the patients with progressive metabolic disease (ΔSUV_{peak}% ⩾ 30%) 2 wk after erlotinib initiation had a significantly shorter time to progression and OS compared to those with stable metabolic disease or a metabolic response of ΔSUV_{peak}% ⩽ -30%[22]. In a recent study that assessed FDG-PET in 2nd/3rd-line erlotinib monotherapy for NSCLC patients, the absence of new lesions by FDG-PET 2 wk after erlotinib initiation was the most predictive marker for OS as opposed to changes in FDG uptake (ΔSUV_{max}% ⩽ -35%)[21]. However, FDG changes were a predictor of OS only when *EGFR* mutation status was not accounted for[21].

The predictive value of the late phase FDG-PET assessment on clinical outcome of EGFR-TKI-treated NSCLC patients has also been evaluated. In a recent study, of 38 NSCLC patients who underwent FDG-PET scan at 6 wk after erlotinib initiation, the metabolic responders with ΔSUV_{peak}% ⩽ -25% survived longer than the non-responders[31]. Importantly, two studies evaluated the predictive value of both early and late FDG-PET assessments on survival of NSCLC patients treated with erlotinib[23,26]. In both studies, FDG-PET response at the late phase (6 or 8 wk) was not significantly associated with improved overall survival. The hazard ratios for PFS and OS in these studies are summarized in Figure 1. Overall, these findings indicate that FDG-PET assessment at the early phases of EGFR-TKI therapy could predict the clinical outcome of NSCLC patients better than assessment at a later phase, irrespective of *EGFR* mutation status.

Previous studies have investigated the molecular mechanisms of FDG-PET to predict the tumor response to EGFR-TKIs at an early stage[32,33]. Treatment with ge-

fitinib reduced FDG uptake in the H3255 and HCC4006 NSCLC cell lines harboring sensitizing *EGFR* mutations within 2 h and reduced FDG uptake in the xenografts of these tumor cells after 2 d of treatment[33]. The gefitinib-mediated decrease in FDG uptake preceded the inhibition of cell proliferation and induction of apoptosis, and this phenomenon was accompanied with the translocation of glucose transporters from the cell membrane to the cytoplasm. This finding suggests that FDG-PET could detect the antitumor effects of EGFR-TKIs at a molecular level before phenotypic changes occur in tumors[33]. In contrast, Ullrich *et al*[32] failed to demonstrate a significant decrease in FDG uptake after 2 and 4 d of erlotinib treatment in tumor xenografts of the *EGFR*-mutated NSCLC cell lines PC9 and HCC827. These inconsistent results may be due to the differences in cellular context, such as differences in the expression levels of glucose transporters and the sensitivity to EGFR-TKIs.

Clinical studies have also revealed the diverse effects of EGFR-TKIs on the early metabolic response of FDG-PET in *EGFR*-mutant NSCLC patients[23,25,26]. For instance, in a study by Takahashi *et al*[25], 4 of 12 (33%) patients with *EGFR* mutations were metabolic non-responders. Three of these patients had stable metabolic disease and one patient had progressive metabolic disease 2 d after gefitinib initiation. Taken together, these findings suggest that FDG-PET assessment could identify the patients who benefit from EGFR-TKI therapies in a population of *EGFR*-mutated NSCLC patients. FDG metabolism is also likely closely linked to the sensitivity of NSCLC to EGFR-TKIs.

In summary, an increasing body of evidence indicates the potential of FDG-PET to predict the treatment efficacy and clinical outcome of NSCLC patients who are treated with EGFR-TKIs. The metabolic response has previously been assessed by different criteria, including EORTC[34] and PERCIST[14]. Thus, standardizing the PET response assessment is essential to establish the predictive values of FDG-PET. Moreover, further studies with larger patient numbers will be needed to evaluate the predictive values of FDG-PET to assess the clinical efficacy of EGFR-TKIs in NSCLC patients.

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BRIEF ARTICLE

Long-term survival of more than 3 years among patients with advanced non-small cell lung cancer treated with chemotherapy

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Abstract

AIM: To evaluate the prognostic factors of long-term survival of more than 3 years in patients with advanced non-small cell lung cancer (NSCLC).

METHODS: We retrospectively analyzed the records of 474 patients with advanced ⅢB/Ⅳ NSCLC who received chemotherapy as initial treatment between September 2002 and March 2007.

RESULTS: The median survival time (MST) was 12.5 mo and the 3 year and 5 year survival rates were 14.6% and 5.3%, respectively. Long-term survival of more than 3 and 5 years was observed in 65 and 16 patients, respectively. The MST for the 65 patients was 61.5 mo (range, 60.1-81.0 mo). In the 474 patients, a good performance status (PS), female sex, non-smoking status and adenocarcinoma histology were significantly associated with a favorable outcome. Furthermore, female sex, a good PS, non-smoking status and adenocarcinoma histology were significantly correlated with long-term survival of more than 3 years and most of these patients (89.2%, 58/65) received epidermal growth factor receptor-tyrosine kinase inhibitors as any line treatment. Survival analysis of long-term survivors showed that a PS of 0 was an independent prognostic factor for predicting favorable outcomes.

CONCLUSION: Our results suggest that a good PS and adenocarcinoma histology play an important role in long-term survival of more than 3 years. A PS of 0 was an independent prognostic factor for predicting favorable outcomes in patients with advanced NSCLC who survived for more than 3 years.

Key words: Non-small cell lung cancer; Long-term survivor; Chemotherapy; Performance status; Epidermal growth factor receptor-tyrosine kinase inhibitors

Core tip: The aim of this study is to evaluate the prognostic factors of long-term survival of more than 3 years in advanced non-small cell lung cancer. Female sex, good performance status (PS), non-smoker and adenocarcinoma were significantly associated with long-term survivors of more than 3 years and most patients received epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKI) at any line treatment. PS of 0 was an independent prognostic factor for predicting favorable prognosis in the long-term survivors of more than 3 years. PS of 0, adenocarcinoma and EGFR-TKI therapy play an important role in the

long-term survivors.

Kaira R, Kaira K, Shukuya T, Kenmotsu H, Ono A, Murakami H, Tsuya A, Nakamura Y, Naito T, Endo M, Yamamoto N, Takahashi T. Long-term survival of more than 3 years among patients with advanced non-small cell lung cancer treated with chemotherapy. *World J Respirol* 2013; 3(3): 110-115 Available from: URL: http://www.wjgnet.com/2218-6255/full/v3/i3/110.htm DOI: http://dx.doi.org/10.5320/wjr.v3.i3.110

INTRODUCTION

Non-small cell lung cancer (NSCLC) remains a major cause of cancer-related death worldwide. Surgery is the most common curative treatment, but for most patients with NSCLC, the tumor is often inoperable at the time of diagnosis. Platinum-based chemotherapy has resulted in a statistically significant improvement in survival compared to best supportive care[1,2]; however, the prognosis of patients with advanced NSCLC is still poor. Recent phase Ⅲ trials have reported a median survival time (MST) of 8 to 10 mo and a 1 year survival rate of 30%-35%[3].

In 2004, mutations in the epidermal growth factor receptor (*EGFR*) gene, conferring increased sensitivity to the chemotherapy drug gefitinib, were reported[4,5]. Recently, two phase Ⅲ studies of gefitinib as first-line therapy compared with platinum-based chemotherapy showed favorable outcomes in patients with advanced NSCLC harboring *EGFR* mutations[6,7]. The patients treated with gefitinib had a MST of 30.5 mo and a 2-year survival rate of 61.4%, indicating that some NSCLC patients may survive for more than 2 or 3 years.

Previous studies on the long-term survival of patients with advanced NSCLC treated with chemotherapy reported a 2-year survival rate of only 4%-6%[8-10]. However, these studies did not include NSCLC patients who received gefitinib and therefore, these data may no longer be accurate. Recently, we showed that a good performance status (PS), adenocarcinoma histology and EGFR-tyrosine kinase inhibitor (TKI) therapy are important factors associated with long-term survival of more than 5 years[11]. However, our previous study had a small sample size and was preliminary; therefore, further investigations on larger sample sizes were warranted. Moreover, there is no consensus on the definition of long-term survival for patients with advanced NSCLC (whether this should be more than 2, 3, 4 or 5 years). Previous reports have defined long-term survival in patients with advanced NSCLC as more than 2 years[8-10]. However, considering that advanced NSCLC patients responsive to gefitinib have a MST of more than 2 years, the clinical characteristics of patients with long-term survival of more than 3 years should be investigated. Against this background, we conducted a retrospective study to evaluate the prognostic factors associated with long-term survival of more than 3 years among advanced NSCLC patients who received chemotherapy as initial treatment.

MATERIALS AND METHODS

We analyzed the records of 474 patients with advanced ⅢB/Ⅳ NSCLC who received chemotherapy as initial treatment at the Department of Thoracic Oncology of Shizuoka Cancer Center between September 2002 and March 2007. NSCLC patients with recurrence after curative surgery were excluded from this study. The demographic characteristics of the 474 patients are listed in Table 1. The median patient age was 64 years (range, 23-85 years); 323 patients were male and 151 were female; 323 were smokers and 154 had never smoked; 333 had adenocarcinoma histology and 141 had non-adenocarcinoma histology; and 109 patients had clinical stage ⅢB disease and 365 had stage Ⅳ disease. The Eastern Cooperative Oncology Group (ECOG) PS was 0 in 148 patients, 1 in 240 patients, 2 in 65 patients, 3 in 20 patients, and 4 in 1 patient.

Of these 474 patients, 380 (80.2%) were treated with platinum-doublet regimens, 64 (13.5%) with single-agent regimens, and 30 (6.3%) with EGFR-TKI therapy (gefitinib or erlotinib) as first-line treatment. Two-hundred and thirty-eight patients (50.2%) received EGFR-TKI therapy as any line treatment. Staging was performed for all patients according to the Union for International Cancer Control TNM classification[12]. For TNM staging, all patients underwent computed tomography (CT) of the thorax and upper abdomen, bone scintigraphy or positron emission tomography, and brain CT or magnetic resonance imaging. Histological analysis of the tumors was based on the World Health Organization (WHO) classification of cell types[13].

Survival was recorded from the first day of treatment to the date of death or last follow-up, and the survival curves were calculated using the Kaplan-Meier method[14]. The median follow-up period was 323 d (range, 13-2069 d). Survival time was calculated at more than 3 years after the final registration. Fisher's exact test was used to examine the association between two categorical variables and probability values of < 0.05 indicated a statistically significant difference. We evaluated the efficacy of chemotherapy using the Response Evaluation Criteria in Solid Tumors, version 1.1. Survival difference was analyzed using the log-rank test. Multivariate analyses were performed using a stepwise Cox proportional hazards model to identify independent prognostic factors. Statistical analysis was performed using JMP 8 (SAS, Institute Inc., Cary, NC, United States) for Windows.

RESULTS

Survival analysis of all 474 patients

Figure 1 shows the survival curves for all 474 patients. The MST was 12.5 mo and the 1 year, 2 years, 3 years, 4 years and 5 years survival rates were 50.8%, 26.3%, 14.6%, 8.2% and 5.3%, respectively. Long-term sur-

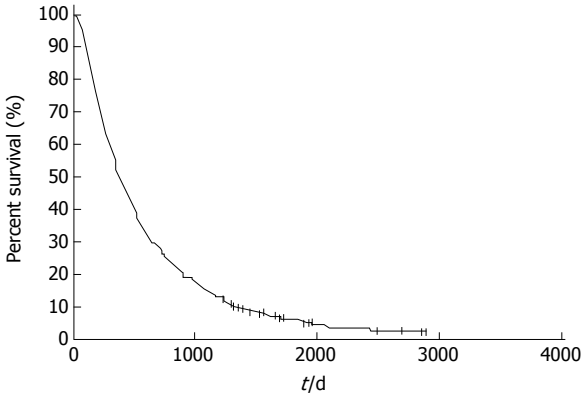

Figure 1 For the entire cohort (n = 474), the median survival time was 12.5 mo and the 1 year, 2 years, 3 years, 4 years and 5 years survival rates were 50.8%, 26.3%, 14.6%, 8.2% and 5.3%, respectively.

Table 1 Patient's characteristics (all patients) n (%)

Variables		No. of patients (n = 474)
Age (yr)		
(median 64)	< 65	264 (55.7)
(range 23-85)	≥ 65	210 (44.3)
Gender		
	Male	323 (68.1)
	Female	151 (31.9)
Performance status		
(ECOG)	0	148 (31.2)
	1	240 (50.6)
	2	65 (13.7)
	3	20 (0.04)
	4	1 (0.002)
Smoking		
	Yes	320 (67.5)
	No	154 (32.5)
Histology		
	Adenocarcinoma	333 (70.3)
	Non-adenocarcinoma	141 (29.7)
Clinical stage		
	ⅢB	109 (23.0)
	Ⅳ	365 (77.0)

ECOG: Eastern Cooperative Oncology Group.

vival of more than 3 years was observed in 65 patients and long-term survival of more than 5 years was observed in 16 patients. Univariate analysis showed that a good PS, female sex, non-smoking status and adenocarcinoma histology were significantly associated with a favorable outcome. Multivariate analysis demonstrated that a good PS and adenocarcinoma histology were independent prognostic factors for predicting a favorable prognosis (Table 2).

Demographic characteristics of patients who achieved long-term survival of more than 3 years

Of the 474 patients, 65 (33 men and 32 women) with a median age of 65 years (range, 35-78 years) survived for more than 3 years, with a MST of 61.5 mo (range, 60.1-81.0 mo). The PS, clinical stage and histology of the patients were as follows: 35 patients had a PS of 0, 25 had a PS of 1, and 5 had a PS of 2-3; 18 patients had stage ⅢB disease and 47 had stage Ⅳ disease; 65 patients had adenocarcinoma histology. With regard to metastatic sites, 23 patients had bone metastases, 18 had brain metastases, and 9 had synchronous brain and bone metastases. With regard to the efficacy of first-line treatment, a partial response was noted in 29% of cases (19/65). The median treatment-free interval between first and second-line therapy was 518 d (range, 26-1901 d) and the median total number of therapeutic lines was 4 (range, 1-13 lines).

Table 2 Univariate and multivariate analysis in overall survival in all patients

Variables		Univariate analysis (log-rank test) P value	Multivariate analysis (Cox's proportional hazard models) P value
Age (yr)	< 65/≥ 65	0.4158	0.5172
Gender	Male/Female	< 0.0001	0.0982
PS	0-1/2-4	< 0.0001	< 0.0001
Smoking	Yes/No	< 0.0001	0.1363
Histology	AC/Non-AC	< 0.0001	< 0.0001
Clinical stage	ⅢB/Ⅳ	0.2151	0.5216

PS: Performance status; AC: Adenocarcinoma.

We then compared the demographic characteristics of patients who did (n = 65) and did not (n = 409) achieve survival of more than 3 years (Table 3). Female sex, a good PS, non-smoking status and adenocarcinoma histology were significantly correlated with long-term survival of more than 3 years. The treatment regimens of patients who did and did not achieve long-term survival were also compared. Among patients who did not achieve long-term survival, 325 (79.4%) received platinum-doublet regimens, 59 (14.4%) received single-agent therapy, and 25 (6.1%) received EGFR-TKI therapy as first-line treatment. In contrast, among long-term survivors, 55 (84.6%) received platinum-doublet regimens, 5 (7.7%) received single-agent therapy, and 5 (7.7%) received gefitinib as first-line treatment. Demographic characteristics did not differ significantly between patients who did and did not achieve long-term survival, according to the first-line regimen. As any line treatment, 180 patients (44.0%) who did not achieve long-term survival and 58 patients (89.2%) who did achieve long-term survival received EFGR-TKI; this difference was statistically significant (P < 0.0001).

With regard to response to first-line chemotherapy among the 65 patients who achieved long-term survival, 17 patients were responders and 48 patients were non-responders, resulting in a response rate of 26%.

Survival analysis in patients who achieved long-term survival of more than 3 years

Univariate analysis did not identify any statistically significantly prognostic factors (Table 4). We excluded 5 patients with a PS of 2-3 from these 65 patients, thus evaluating 60 patients with a PS of 0-1 by univariate and multivariate analyses (Table 5). A PS of 0 was found to

Table 3 Comparison of patient's demographics between survivors (n = 65) of more than 3 yr and those (n = 409) of less than 3 yr

Variables		< 3 yr (n = 409)	≥ 3 yr (n = 65)	*P* value
Age	< 65/≥ 65	231/178	21/34	0.2267
Gender	Male/Female	290/119	33/32	0.0024
PS	0-1/2-4	329/80	60/5	0.0225
Smoking	Yes/No	291/118	35/30	0.0090
Histology	AC/Non-AC	268/141	65/0	< 0.0001
Clinical stage	ⅢB/Ⅳ	91/318	18/47	0.3428

PS: Performance status; AC: Adenocarcinoma.

Table 4 Univariate analysis in overall survival in 65 long-term survivors

Variables		No. of patients	Univariate analysis (log-rank test) *P* value
Age	< 65/≥ 65	31/34	0.9448
Gender	Male/Female	33/32	0.3467
PS	0-1/2-3	60/5	0.7468
Smoking	Yes/No	35/30	0.9835
Clinical stage	ⅢB/Ⅳ	18/47	0.7627

PS: Performance status.

Table 5 Univariate and multivariate analysis in overall survival in 60 long-term survivors

Variables		No. of patients	Univariate analysis (log-rank test) *P* value	Multivariate analysis (Cox's proportional hazard models) *P* value
Age	< 65/≥ 65	29/31	0.8099	0.9421
Gender	Male/Female	31/29	0.4133	0.3676
PS	0/1	35/25	0.0158	0.0244
Smoking	Yes/No	33/27	0.9062	0.5170
Clinical stage	ⅢB/Ⅳ	18/42	0.8139	0.6781

PS: Performance status.

be an independent prognostic factor for predicting a favorable outcome.

DISCUSSION

The present study showed that advanced NSCLC patients who survived for more than 3 years had a good PS and adenocarcinoma histology. Most patients who survived for more than 3 years received platinum-containing chemotherapy as initial treatment and EGFR-TKI as any line chemotherapy. Multivariate analysis of long-term survivors showed that a PS of 0 was an independent prognostic factor for predicting a favorable outcome.

A previous study reported that the best prognostic factors for long-term survivors were non-metastatic disease status and response to chemotherapy[14]. In this previous study, 1052 patients treated with platinum-based chemotherapy were analyzed and the 2 years and 5 years survival rates were 7.4% and 1.8%, respectively. All patients who survived for more than 5 years had limited disease and were treated by complementary thoracic radiation and/or surgery. Other recent studies also reported that a good PS, adenocarcinoma histology and EGFR-TKI therapy contributed to long-term survival of more than 2 years[9,11]. As EGFR-TKI therapy contributes to prolonged survival, previous reports on long-term survivors who did not receive this treatment may not be useful. Therefore, retrospective studies that include a treatment history of EGFR-TKI therapy are needed to identify the prognostic factors for a favorable outcome.

Our study suggested that a PS of 0, but not 1, was an important factor for predicting long-term survival of more than 3 years. In the ECOG experience, the rate of survival for more than 2 years in patients with metastatic NSCLC was 4.0% and pretreatment characteristics associated with long-term survival were an initial PS of 0, no bone metastases, female sex, no subcutaneous metastases, no larger cell histology, a prior weight loss of less than 5%, and no liver metastases[15]. The experience of the South West Oncology Group also documented that a good PS, female sex and an age of more than 70 years were significant independent survival predictors[16]. A good PS is known to be closely associated with a favorable outcome after chemotherapy in patients with advanced NSCLC. Although adenocarcinoma histology, use of EGFR-TKI, and an initial good PS are essential in order to achieve survival for more than 3 years in cases of advanced NSCLC, the outcome of patients with a PS of 0 may be different from that of patients with a PS of 1.

In July 2002, gefitinib was approved for pretreated NSCLC patients in Japan in clinical practice. Recently, Satouchi *et al*[17] reported on the predictive factors associated with the prognostic benefits of gefitinib, showing that survival was significantly better for female sex, adenocarcinoma histology, never-smoked status, a favorable PS and *EGFR* mutation positivity. Recent clinical studies demonstrated that the use of gefitinib or erlotinib resulted in significantly longer survival than platinum-based chemotherapy in patients with advanced NSCLC harboring *EGFR* mutation[18,19] and the MST of patients treated with gefitinib was approximately 3 years (27.7 mo)[18]. In multivariate analysis, *EGFR* mutation positivity and a PS of 0-1 have been described as independent predictors of a favorable prognosis.

It is currently unclear whether *EGFR* mutation is a prognostic factor in NSCLC patients not treated with EGFR-TKI. Therefore, Kosaka *et al*[20] examined the prognostic significance of *EGFR* mutation in a large cohort of patients with surgically treated lung adenocarcinoma. In their study, univariate analysis demonstrated that patients with *EGFR* mutations have favorable survival compared to those without *EGFR* mutations (P = 0.0046). However, *EGFR* mutation positivity was not

independently associated with poor outcome in cases of resectable lung adenocarcinoma not treated with EGFR-TKI and was a predictive factor for cases treated with gefitinib, but not for pulmonary adenocarcinoma not treated with gefitinib. In the present study, one of the limitations was that the *EGFR* mutation status had not been analyzed in all patients. Therefore, whether a PS of 0 is a useful factor for predicting favorable prognosis compared with *EGFR* mutation positivity remains unknown. Accordingly, further study is warranted for the confirmation of our results.

While PS is an important factor in determining outcomes in cases of NSCLC, there is limited available data on the distribution of PS among NSCLC patients. Recently, Kawaguchi *et al*[21] showed that PS is an independent favorable prognostic factor in a large-scale retrospective study of 26957 patients with NSCLC. In their study, most patients with a PS of 0 presented with stage I disease and were never-smokers and overall survival differed significantly between patients with a PS of 0 and those with a PS of 1. Moreover, outcomes differed significantly in patients with advanced NSCLC between those with a PS of 0 and those with a PS of 1. Qi *et al*[22] also reported that pretreatment quality of life was an independent prognostic factor for overall survival in patients with advanced NSCLC. These reports suggest that PS before treatment may be closely associated with long-term survival in patients with advanced NSCLC. Furthermore, outcomes are thought to differ between treated NSCLC patients with a PS of 0 and those with a PS of 1, but no data are available in the literature on the long-term survival of chemotherapy-treated patients with advanced NSCLC. At present, three major scales are used to measure PS in oncology, Karnofsky PS, ECOG PS and WHO PS, although the ECOG PS scale has been shown to be more effective than the Karnofsky PS scale in discriminating between patients with different prognoses[23]. Our results indicate a strong relationship between a PS of 0 and long-term survival in advanced NSCLC patients treated with chemotherapy, indicating that the ECOG PS scale may be an appropriate measure for predicting long-term survival.

In conclusion, our results suggest that a good PS and adenocarcinoma histology play an important role in long-term survival of more than 3 years. A PS of 0 was an independent prognostic factor for predicting favorable outcomes in patients with advanced NSCLC who survived for more than 3 years. In the future, a large-scale study including *EGFR* mutation analysis might be considered for determining the prognostic factors of patients with advanced NSCLC who are treated with chemotherapy and achieve long-term survival of more than 3 years.

COMMENTS

Background

Surgery is the most common curative treatment but for most patients with non-small cell lung cancer (NSCLC), the tumor is often inoperable at the time of diagnosis. Recent phase Ⅲ trials have reported a median survival time of 8 to 10 mo and a 1 year survival rate of 30%-35%.

Innovations and breakthroughs

The authors' results suggest that a good performance status (PS) and adenocarcinoma histology play an important role in long-term survival of more than 3 years. A PS of 0 was an independent prognostic factor for predicting favorable outcomes in patients with advanced NSCLC who survived for more than 3 years.

Applications

PS of 0, adenocarcinoma and epidermal growth factor receptor-tyrosine kinase inhibitors therapy play an important role in the long-term survivors.

Peer review

This is a flawless manuscript, very well written, which can be useful for the readers and investigators in lung cancer.

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