Table 2
Percentage of perceived physical health and perceived mental health by gender and age.

	Age (year)	N	Perceived Physical Health**					Perceived mental health **				
						Poor perceived physical health					Poor perceived mental health	
			Very sufficient	Sufficient	Normal	Insufficient	Very insufficient	Very sufficient	Sufficient	Normal	Insufficient	Very insufficient
Men	20–29	164	32.9	31.1	28.7	6.7	0.6	32.3	25.0	34.1	6.7	1.8
	30–39	218	31.7	24.3	38.5	5.5	0.0	23.4	22.9	44.5	8.7	0.5
	40–49	205	22.4	25.4	39.5	11.2	1.5	19.0	21.0	44.4	12.7	2.9
	50–59	186	14.0	26.3	42.5	15.6	1.6	15.6	22.6	50.0	8.1	3.8
	60–69	205	17.1	21.5	37.6	18.5	5.4	17.1	21.0	50.2	10.7	0.5
	70+	185	14.1	18.4	34.6	22.7	10.3	15.7	20.5	49.2	7.6	5.4
	Total	1163	22.0	24.3	37.1	13.3	3.2	20.3	22.1	45.7	9.2	2.4
Women	20–29	153	31.4	20.9	39.9	7.8	0.0	28.1	22.2	41.2	7.8	0.7
	30–39	234	32.1	24.4	34.6	8.1	0.9	28.6	29.5	31.2	9.4	1.3
	40–49	219	24.2	26.5	39.7	8.2	0.9	19.2	28.3	43.8	6.8	1.8
	50–59	251	20.3	21.1	43.0	13.1	2.4	16.3	27.9	43.4	9.6	2.8
	60–69	266	16.9	29.3	32.7	17.7	3.4	18.4	23.7	46.6	9.8	1.5
	70+	273	11.4	16.8	37.7	24.5	9.5	16.1	16.5	52.0	11.7	2.6
	Total	1396	21.7	23.2	37.8	14.0	3.2	20.5	24.6	43.5	9.4	1.9

** Significant difference among age groups (χ^2 test, $P < 0.01$).

Table 3
Percentage of sleep problems and their associations by gender and age.

Sleep Problems	Total	Men ($n = 1163$)				Women ($n = 1396$)			
		Total	20–39 years	40–59 years	⩾60 years	Total	20–39 years	40–59 years	⩾60 years
DIS (%)	[a]								
Not at all	75.5	78.7	80.6	78.8	76.7	72.9	74.9	75.3	69.4
Less than once a week	9.3	7.7	6.0	8.7	8.5	10.6	10.6	10.6	10.6
Once or twice a week	7.7	7.5	7.9	7.4	7.2	7.8	8.0	5.3	9.8
Three or more times a week	7.2	5.5	4.7	4.9	6.9	8.5	6.2	8.5	10.2
DMS (%)	[a]	[b]				[b]			
Not at all	62.1	66.2	77.0	67.8	54.1	58.7	66.1	60.9	51.4
Less than once a week	11.3	9.8	9.4	9.2	10.8	12.5	10.3	11.9	14.5
Once or twice a week	11.4	10.5	7.1	12.5	11.8	12.2	8.8	12.6	14.3
Three or more times a week	15.2	13.4	6.5	10.5	23.1	16.6	14.7	14.5	19.9
EMA (%)		[b]				[b]			
Not at all	80.6	81.7	86.9	82.9	75.4	79.7	87.3	81.9	72.2
Less than once a week	7.6	6.5	5.8	4.9	9.0	8.5	5.9	7.9	10.9
Once or twice a week	6.5	6.4	4.7	6.6	7.7	6.7	4.1	6.0	9.1
Three or more times a week	5.2	5.3	2.6	5.6	7.7	5.0	2.3	4.0	7.8
EDS (%)	[a]								
Not at all	96.2	95.4	96.6	94.6	95.1	96.9	96.6	97.0	97.0
Less than once a week	1.8	2.8	1.6	3.8	2.8	1.1	1.0	1.7	0.6
Once or twice a week	0.8	0.6	0.5	0.8	0.5	0.9	1.0	1.1	0.7
Three or more times a week	0.6	0.8	1.0	0.5	0.8	0.4	0.5	0.2	0.6
PSQ (%)		[b]				[b]			
Very good	13.6	13.3	13.4	12.0	14.6	13.9	15.2	9.1	17.1
Fairly good	64.3	65.1	63.1	63.4	68.7	63.6	59.2	64.7	65.9
Fairly bad	20.0	19.5	22.0	22.3	14.4	20.4	24.0	23.2	15.4
Bad	1.7	1.9	1.6	2.3	1.8	1.5	1.3	2.1	1.1
Sleep duration (%)	[a]	[b]				[b]			
SSD (<5 h)	4.0	3.6	3.7	4.6	2.6	4.4	3.9	5.3	3.9
5–9 h	92.8	92.2	94.0	93.3	89.2	93.3	94.8	94.5	91.1
LSD (⩾9)	3.2	4.2	2.4	2.1	8.2	2.4	1.3	0.2	5.0

DIS, difficulty initiating sleep; DMS, difficulty maintaining sleep; EMA, early morning awakening; EDS, excessive daytime sleepiness; PSQ, poor sleep quality; SSD, short sleep duration; LSD, long sleep duration.
[a] Significant difference between gender (χ^2 test, $P < 0.05$).
[b] Significant difference among age groups (χ^2 test, $P < 0.05$).

status. Moreover, it was revealed that the dose effect of insomnia symptoms was more remarkable on perceived mental health status than on perceived physical health status. Schubert et al. [16] found a similar tendency in the elderly, i.e., that the number of insomnia symptoms was positively associated with poor perception of mental and physical function. It was postulated from these results that more severe insomnia associated with a greater number of insomnia symptoms resulted in poorer perceptions of mental and physical health, although it was not possible to identify any causal relationship on the basis of this cross-sectional study.

4.4. Sleep problems and poor perceived health status

In the present study DMS, PSQ, SSD, and LSD were independently associated with an increased risk for poor perceived

Table 4
Association of number of insomnia symptoms with poor perceived physical health and poor perceived mental health.

Number of insomnia symptoms	Total	Poor perceived physical health			Poor perceived mental health		
			Adjusted[a]			Adjusted[a]	
	(%)	(%)	OR	95%CI	(%)	OR	95%CI
0	67.2	11.5	1.00		7.4	1.00	
1	18.5	22.8	2.03	1.55–2.67**	13.1	1.89	1.37–2.62**
2	8.4	31.9	3.11	2.22–4.36**	22.9	3.64	2.51–5.28**
3	5.9	39.1	4.28	2.94–6.22**	36.0	7.06	4.80–10.41**

[a] Adjusted for age group, gender, size of community, educational achievement, and marital status.
** $P < 0.01$.

physical health status, while DIS, EDS, and PSQ were independently associated with an increased risk for poor perceived mental health status, suggesting differential effects of individual sleep problems on perceived health status.

Several epidemiological studies have suggested that, among various symptoms of insomnia, DMS showed the strongest association with poor physical health conditions such as cardiovascular disease, diabetes, gastroenterological disorders, respiratory disease, and chronic pain [26,45–49]. It is likely that physical conditions that cause pain, impair breathing, or produce gastric irritation would disrupt sleep and, thus, lead to DMS. In this respect, in the present study, DMS was considered more likely to be a consequence of physical distress during sleep.

Previous epidemiological studies have suggested that both SSD and LSD are associated with various underlying physical conditions such as cardiovascular disease, arterial hypertension, diabetes, and obesity, as well as all-cause mortality [50–52]. Studies conducted in a laboratory setting have also indicated the wide ranging adverse effects of SSD on physical function (e.g., metabolism and cardiovascular health) [53–56], while little is known about the adverse effects of LSD on physical function. The association in the present study between both SSD and LSD and poor physical health status, independent of insomnia symptoms, may be comparable to previous findings obtained in epidemiological, and, in part, human experimental studies.

Sleep problems are reportedly common among individuals with poor mental health. Epidemiological studies have revealed that more than one third of patients with mental disorders have sleep complaints [57–59]. It is also widely recognized that sleep problems can be a risk factor for poor mental health [60–65]. Recent prospective longitudinal studies have clearly indicated that the relationship between sleep problems and mental disorders is bidirectional, each being potentially the cause or result of the other [66,67]. More recently, a longitudinal study has shown that DIS is specifically associated with poor mental health [25], suggesting a differential effect of insomnia subtypes on mental condition. Although our investigation was cross-sectional in design, the results may be comparable with those of recent studies.

EDS is a symptom of sleep apnea syndrome, circadian rhythm sleep disorders, insomnia, sleep-related movement disorders, or hypersomnia of central origin, including narcolepsy [68]. Most of these conditions are frequently associated with poor mental health [69–73]. The significant association between EDS and poor mental health status found in the present study may be due to the well-acknowledged high comorbidity between EDS and poor mental health status.

In the present study, only PSQ was independently associated with an increased risk for both poor perceived physical or mental health status. Previous studies have suggested that PSQ is associated with poor physical condition, such as the presence of hypertension or diabetes [74,75], and with poor mental health conditions such as anxiety disorders or mood disorders [76]. The associations between PSQ and other sleep variables are still unclear. However, since the positive association between PSQ and poor physical or mental perceived health status remained significant after adjustment for other sleep problems, PSQ was likely to contribute independently to poor perceived health statuses. A prospective study is warranted to examine the complex interactions among PSQ, other sleep variables, and perceived health status. The findings obtained in our study and previous ones suggest that improvement of sleep problems may lead to better perceived physical and mental health status.

There were some limitations to our study. First, since this was a cross-sectional survey, any causal relationship could not be determined. A future prospective study will be required to clarify the nature of the actual relationships we found. Second, in the present study, sleep problems were assessed using a retrospective, self-reported approach. Although some studies have reported that self-reported data on sleep status concur with physiologic data

Table 5
Association of poor perceived physical health with sleep problems.

	Poor perceived physical health					
	Crude		Adjusted[a]		Adjusted[b]	
	OR	95%CI	OR	95%CI	OR	95%CI
Gender						
Men	1.00		1.00		1.00	
Women	1.06	0.86–1.30	0.91	0.73–1.13	0.90	0.71–1.14
Age (year)						
20–39	1.00		1.00		1.00	
40–59	1.97	1.41–2.74**	2.10	1.49–2.97**	2.15	1.50–3.10**
60+	4.83	3.56–6.55**	3.76	2.70–5.22**	3.83	2.68–5.48**
Sleep problems						
DIS						
No	1.00		1.00		1.00	
Yes	3.02	2.36–3.86**	2.93	2.26–3.79**	1.32	0.94–1.84
DMS						
No	1.00		1.00		1.00	
Yes	2.73	2.21–3.39**	2.35	1.87–2.94**	1.50	1.14–1.98**
EMA						
No	1.00		1.00		1.00	
Yes	3.13	2.40–4.09**	2.70	2.04–3.57**	1.08	0.75–1.54
EDS						
No	1.00		1.00		1.00	
Yes	1.73	0.81–3.72	1.90	0.85–4.27	0.93	0.40–2.13
PSQ						
No	1.00		1.00		1.00	
Yes	2.39	1.91–2.99**	3.23	2.53–4.13**	1.93	1.43–2.62**
Sleep duration						
SSD (<5 h)	3.73	2.48–5.62**	4.42	2.85–6.84**	2.53	1.56–4.10**
5–9 h	1.00		1.00		1.00	
LSD (⩾9)	3.80	2.41–5.99**	2.11	1.29–3.47**	2.10	1.24–3.57**

DIS, difficulty initiating sleep; DMS, difficulty maintaining sleep; EMA, early morning awakening; EDS, excessive daytime sleepiness; PSQ, poor sleep quality; SSD, short sleep duration; LSD, long sleep duration.
[a] Adjusted for age group, gender, size of community, educational achievement, and marital status.
[b] Adjusted for age group, gender, size of community, educational achievement, marital status, hypnotic medication use, and other sleep problems.
** $P < 0.01$.

Table 6
Association of poor perceived mental health with sleep problems.

	Poor perceived mental health					
	Crude		Adjusted[a]		Adjusted[b]	
	OR	95%CI	OR	95%CI	OR	95%CI
Gender						
Men	1.00		1.00		1.00	
Women	0.96	0.75–1.23	0.93	0.73–1.19	0.87	0.66–1.13
Age (year)						
20–39	1.00		1.00		1.00	
40–59	1.33	0.97–1.83	1.46	1.05–2.03 *	1.42	1.00–2.02
60+	1.39	1.02–1.90 *	1.19	0.84–1.69	1.10	0.75–1.62
Sleep problems						
DIS						
No	1.00		1.00		1.00	
Yes	4.18	3.18–5.48**	4.15	3.15–5.47**	1.59	1.11–2.29*
DMS						
No	1.00		1.00		1.00	
Yes	2.70	2.11–3.47**	2.68	2.07–3.46**	1.29	0.93–1.80
EMA						
No	1.00		1.00		1.00	
Yes	3.79	2.84–5.07**	3.69	2.74–4.96**	1.31	0.89–1.95
EDS						
No	1.00		1.00		1.00	
Yes	6.18	3.13–12.21**	6.27	3.15–12.48**	3.12	1.47–6.65**
PSQ						
No	1.00		1.00		1.00	
Yes	3.94	3.06–5.07**	4.20	3.24–5.45**	2.25	1.63–3.10**
Sleep duration						
SSD (<5 h)	4.08	2.64–6.30**	4.05	2.61–6.29**	1.65	0.98–2.76
5–9 h	1.00		1.00		1.00	
LSD (⩾9)	1.51	0.80–2.82	1.09	0.56–2.12	0.96	0.44–2.08

DIS, difficulty initiating sleep; DMS, difficulty maintaining sleep; EMA, early morning awakening; EDS, excessive daytime sleepiness; PSQ, poor sleep quality; SSD, short sleep duration; LSD, long sleep duration.

[a] Adjusted for age group, gender, size of community, educational achievement, and marital status.

[b] Adjusted for age group, gender, size of community, educational achievement, marital status, hypnotic medication use, and other sleep problems.

* $P < 0.05$.

** $P < 0.01$.

[77,78], objective data (i.e., physiologic measurements such as electroencephalography) are desirable. It would be difficult to collect physiologic data in such a large epidemiologic study of the general population. The use of other subjective measures (e.g., sleep diary) in future studies would be helpful. Third, our dichotomized assessment of perceived health status based on a five-level rating system using self-reported questionnaires might have resulted in classification bias, although similar dichotomizations were conducted in previous studies [22,79]. Fourth, we asked the subjects about their perception of physical or mental health status. Further investigations should try to evaluate in more detail the background of perceived physical and mental health statuses by examining physical and mental morbidity together with the multi-item questionnaires on mental and physical health status. Fifth, non-response bias due to the sampling method should be considered. The response ratio obtained in the present study (54%) should be considered carefully in the light of non-response bias, although distribution of gender and age did not differ between population estimated data and the present study sample. Factors that could specifically influence the associations between perceived health statuses and sleep problems were not fully excluded by using multiple logistic regression analyses. However, the questions on perceived health status and sleep problems were unlikely to be related to forensic problems, religious belief, ethical attitude, or specific sense of value, which could have substantially distorted the associations between perceived health status and sleep problems.

In conclusion, the present study has shown that sleep problems are important indicators of perceived physical and mental health status, and that each individual sleep problem contributes differentially to perceived physical or mental health status in the general adult population. A further longitudinal epidemiological study will be required to examine the causal relationship between sleep problems and perceived health status.

Conflict of interest

Dr. Uchiyama has received research support from Astellas Pharma, Meiji Seika Pharma, Nippon Boehringer Ingelheim, Pfizer Japan, Sanofi-Aventis, MSD, Taisho Pharmaceutical, Kao Corporation, and Takeda Pharmaceutical and has consulted for Pfizer Japan, Sanofi-Aventis, Kao Corporation, and Takeda Pharmaceutical. All other authors declare that they have no conflicts of interest.

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: doi:10.1016/j.sleep.2012.03.011.

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Tumor necrosis factor superfamily member LIGHT induces epithelial–mesenchymal transition in A549 human alveolar epithelial cells

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ABSTRACT

Fibrosis is an abnormal response to organ injury, characterized by accumulation of activated fibroblasts at the sites of injury. Fibroblasts arise from several sources, including resident fibroblasts and circulating fibrocytes that infiltrate organ tissue. Recently, epithelial–mesenchymal transition (EMT) has been recognized as a source of mesenchymal cells. EMT is induced by various growth factors, such as transforming growth factor (TGF)-β1, and enhanced by inflammatory cytokines. Recently the tumor necrosis factor superfamily member LIGHT has been implicated in the pathogenesis of inflammatory disease and airway remodeling in severe asthma. We hypothesized that LIGHT might contribute to the pathogenesis of airway fibrosis via enhancement of EMT. Therefore, we investigated LIGHT's ability to induce EMT. A549 cells were stimulated with LIGHT, TGF-β1 or both for 48 h. To estimate EMT, we evaluated the expression of epithelial and mesenchymal markers using immunocytochemistry, Western blotting and quantitative RT-PCR. Signaling pathways for EMT were characterized by Western analysis to detect phosphorylation of Erk1/2 and smad2. LIGHT enhanced TGF-β1-induced EMT both morphologically, by suppressing E-cadherin and enhancing vimentin, and functionally, by enhancing cell contractility. Additionally, LIGHT induced EMT without TGF-β1. Evaluation of the mechanism showed that LIGHT did not induce TGF-β1 production or affect the smad-*snai1* pathway. Inhibition of Erk1/2 phosphorylation reduced LIGHT-induced EMT, indicating the Erk1/2 pathway to be a key pathway in LIGHT-induced EMT. In summary, LIGHT enhanced TGF-β1-induced EMT but also induced EMT via the Erk1/2 pathway by itself, without TGF-β1 signaling. LIGHT may contribute to the pathogenesis of airway fibrosis through enhancement of EMT.

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1. Introduction

Fibrosis, that is, fibroblast activation with generation of provisional extracellular matrix (ECM), is an abnormal response of organs to injury, inflammation or stress [18]. Successful tissue repair relies on a balance between ECM synthesis and degradation, as well as re-epithelization of damaged epithelial surfaces. Abnormal tissue repair and fibrosis are often suggested to be associated with a variety of chronic progressive diseases, including pulmonary fibrosis, cardiovascular fibrosis, liver cirrhosis, end stage kidney disease, systemic sclerosis and autoimmune disease [26]. Idiopathic pulmonary fibrosis (IPF), which is the most common interstitial lung disease, is a devastating, progressive respiratory disease, with a mean survival of 2–3 years from initial diagnosis. To date, there is no effective treatment that shows reversal of disease progression and survival benefit [20]. A key histological feature of IPF is formation of fibroblastic foci that reflect sites of active, ongoing fibrogenesis. Increased numbers of fibroblastic foci have been associated with disease activity and a more rapid disease progression of IPF [13,19]. Lung fibroblasts arise from several sources, including resident pulmonary fibroblasts and circulating fibrocytes that infiltrate the lung. Recently, epithelial–mesenchymal transition (EMT), a process whereby epithelial cells undergo transition to a mesenchymal phenotype that gives rise to fibroblasts, has been recognized as a source of mesenchymal cells [6].

Abbreviations: EMT, epithelial–mesenchymal transition; LIGHT, homologous to lymphotoxins, exhibits inducible expression and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes; TNFSF, tumor necrosis factor superfamily; TGF, transforming growth factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; α-SMA, α-smooth muscle actin.

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EMT is an important process during fetal development and progression of cancer, such as tumor invasion and tumor metastasis. EMT permits epithelial cells to acquire the capacity to migrate by down-regulating epithelial markers, such as E-cadherin, and gaining expression of mesenchymal markers, such as vimentin and α-smooth muscle actin (SMA) [11,23]. EMT has been increasingly implicated in the pathogenesis of tissue fibrosis, such as in the kidney [3] and lung [29]. EMT can be induced by such growth factors as transforming growth factor (TGF)-β, fibroblast growth factor-2, epidermal growth factor and hepatocyte growth factor. These growth factors are associated with the tissue repair process and reported to be upregulated in chronic inflammatory disease. Additionally, chronic inflammation has been shown to promote fibrotic disease [21]. Several inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin-1β, were reported to enhance EMT [1,12,28]. Thus, chronic inflammation can contribute to tissue fibrosis through TGF-β-induced EMT enhanced by inflammatory cytokines.

The tumor necrosis factor superfamily member ligand LIGHT (TNFSF14; homologous to lymphotoxins, exhibits inducible expression and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes [17]) has been implicated in the pathogenesis of such inflammatory diseases as rheumatoid arthritis and inflammatory bowel disease [8,25]. Moreover, LIGHT is recognized as a key mediator in chronic airway inflammatory diseases, such as bronchial asthma, and it is associated with the disease severity of asthma [2,7]. Furthermore, LIGHT plays an important role in the pathogenesis of airway remodeling that is associated with TGF-β and IL-13 produced by inflammatory cells [2]. However, there are no data regarding a possible effect of LIGHT on EMT.

Taken together, EMT may contribute to tissue fibrosis and be increased in chronic inflammation. LIGHT is a key cytokine in airway inflammation and fibrosis. Thus, we hypothesized that LIGHT might contribute to the pathogenesis of airway fibrosis through enhancement of EMT. We first investigated whether LIGHT affected TGF-β1-induced EMT, causing morphological changes and acquisition of contractility, which is a property of mesenchymal cells. We then investigated whether LIGHT itself induced EMT. Finally, we examined the mechanisms of LIGHT-induced EMT, elucidating the intracellular signaling.

2. Materials and methods

2.1. Reagents

The details of the reagents are presented in the online supplement.

2.2. Cells and cell cultures

A549 cells were purchased from the American Type Culture Collection (Manassas, VA). The details of cell culture are provided in the online supplement.

2.3. Gel contraction assay

Cells were cast into collagen gels using previously reported methods. The details are provided in the online supplement.

2.4. Quantitative reverse transcriptional PCR (RT-PCR) analysis of messenger RNA

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). The details of RT-PCR are provided in the online supplement. Individual data were normalized against the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Untreated control samples were set to 1.0, and the values of the fold change in expression following treatment were presented as bar graphs ± standard error of the mean.

2.5. Immunostaining of cultured cells

A549 cells were seeded into chamber slides (IWAKI), and immunocytochemical staining was performed using the streptavidin–biotin–peroxidase method (LSAB2 Kit/HRP; DAKO, Kyoto, Japan). The details are provided in the online supplement.

2.6. Western blot analysis

The details of the methods and antibodies are provided in the online supplement.

2.7. Antibodies

The antibodies (all purchased from Cell Signaling Technology, Beverly, MA) used were rabbit anti-human E-cadherin antibody #3195 1:3000, rabbit anti-vimentin antibody #5741 1:5000, rabbit anti-smad2 antibody #3122 1:2000, rabbit anti-phospho-smad2 antibody #3104 1:3000, rabbit anti-p44/42 mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (Erk1/2) antibody #9102 1:3000, rabbit anti-phospho-Erk1/2 antibody #9101 1:3000 and anti-rabbit IgG, HRP-linked antibody #7074 1:15,000.

Equal protein loading was confirmed by probing the blot with antibody against α-tubulin (Sigma–Aldrich) at a 1:5000 dilution and anti-mouse HRP-linked antibody at a 1:15,000 dilution.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The concentration of TGF-β1 was estimated by using Quantikine ELISA human TGF-β1 immunoassay (R&D Systems #DB100B) according to the manufacturer's instructions. The details are provided in the online supplement.

2.9. Statistics

Results were confirmed by repeating experiments on at least three separate occasions. Data shown in the figures are pooled data for each experiment and expressed as the mean ± SEM. Analyses were performed using JMP (Version 9; SAS Institute Inc., Tokyo, Japan). Samples with multiple comparisons were analyzed for significance by analysis of variance (ANOVA). When ANOVA indicated significant differences between groups, Tukey–Kramer's HSD was applied. P values of <0.05 were considered to be significant.

3. Results

3.1. LIGHT enhances TGF-β1-induced EMT in A549 cells

A549 cells, a human alveolar epithelial cell line, were incubated with TGF-β1 (5 ng/ml) with/without LIGHT (10 ng/ml) for 48 h. Phase contrast images showed that the treated cells changed in morphology from a cobblestone appearance—a characteristic of epithelial cells—to a spindle-shaped appearance that is characteristic of mesenchymal cells. Immunocytochemical staining confirmed that the morphological changes were associated with loss of expression of an epithelial marker, E-cadherin, and acquisition of expression of a mesenchymal marker, vimentin (Supplemental Fig. 1). In addition, costimulation with TGF-β1 and LIGHT led to a

more spindle-like, elongated shape compared with stimulation with TGF-β1 alone, with less immunoreactivity for E-cadherin and more for vimentin. Immunoblots (Fig. 1A) showed that A549 cells underwent EMT, characterized by loss of E-cadherin and acquisition of vimentin. We used quantitative (q)-RT-PCR to confirm whether the morphological and immunoreactivity changes correlated with expression of epithelial and mesenchymal marker genes (Fig. 1B). A549 cells costimulated with TGF-β1 and LIGHT for 48 h showed significantly decreased expression of mRNA for E-cadherin and significantly increased expression of mRNA for vimentin compared with stimulation with TGF-β1 alone. Thus, LIGHT enhanced TGF-β1-induced EMT morphologically.

Next, we performed gel contraction assay to determine whether EMT-induced cells acquired contractility, a key property of mesen-

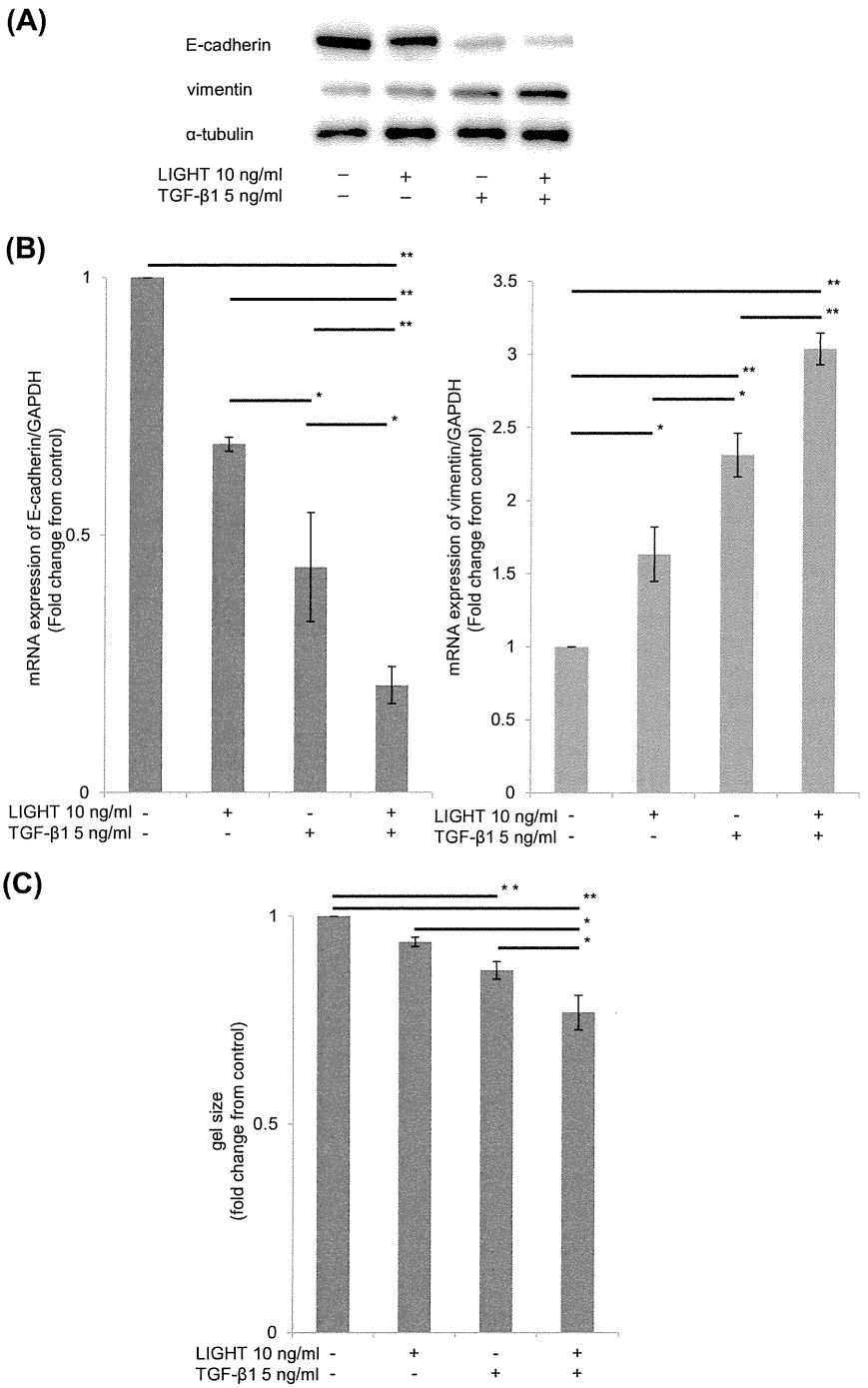

Fig. 1. LIGHT enhances TGF-β1-induced EMT in A549 cells. (A) Western blotting was performed for E-cadherin (upper images) and vimentin (lower images) in A549 cells treated with LIGHT (10 ng/ml) and/or TGF-β1 (5 ng/ml) for 48 h. (B) We determined the expression of mRNA for E-cadherin and vimentin in A549 cells stimulated with LIGHT (10 ng/ml) and/or TGF-β1 (5 ng/ml) for 48 h. E-cadherin mRNA expression was significantly decreased by the combination of LIGHT and TGF-β1 compared with TGF-β1 alone. $n = 5$ separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$. (C) We investigated the effects of LIGHT and/or TGF-β1 on collagen gel contraction mediated by A549 cells. Costimulation with LIGHT and TGF-β1 significantly decreased the gel size compared with TGF-β1 alone. $n = 6$ separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

chymal cells. The gel contraction assay—in which fibroblasts are cultured in three-dimensional gels of type I collagen—has been used as an ideal *in vitro* model of the contraction that characterizes both normal wound repair and fibrosis [4]. In the assay, mesenchymal cells are thought to attach to collagen fibers through integrin-dependent mechanisms and generate mechanical tension, which leads to tissue contraction and reduced size of collagen gels. Fig. 1C shows that stimulation with TGF-β1 significantly decreased the gel size compared with no stimulation. Costimulation with LIGHT and TGF-β1 significantly decreased the gel size compared with TGF-β1 alone. Thus, LIGHT enhanced the contractility seen in TGF-β1-induced EMT.

These results indicate that LIGHT enhanced TGF-β1-induced EMT and that cells that underwent EMT gained cell contractility, a mesenchymal cell property.

3.2. LIGHT induces EMT in A549 cells

Because we found that LIGHT enhanced TGF-β1-induced EMT, we further evaluated whether LIGHT itself could induce EMT. We examined for a concentration-dependent effect of LIGHT on the expression of cell surface markers associated with EMT. Stimulation with various concentrations (0.1–50 ng/ml) of LIGHT for 48 h induced EMT in a concentration-dependent manner, as evidenced by suppression of E-cadherin and expression of vimentin, N-cadherin and α-SMA (Fig. 2A). The expressions of E-cadherin and vimentin mRNA were quantified by q-RT-PCR. LIGHT significantly reduced the E-cadherin mRNA level while simultaneously increasing expression of vimentin, concentration-dependently (Fig. 2B).

3.3. Mechanisms of LIGHT-induced EMT in A549 cells

Since we found that LIGHT enhanced TGF-β1-induced EMT and itself induced EMT of A549 cells, we tried to elucidate the mechanisms of LIGHT-induced EMT.

3.3.1. LIGHT does not induce TGF-β1 production

First, we analyzed the effect of LIGHT on TGF-β1 production to determine whether LIGHT-induced EMT was due to production of TGF-β1, as an autocrine mechanism. We stimulated A549 cells

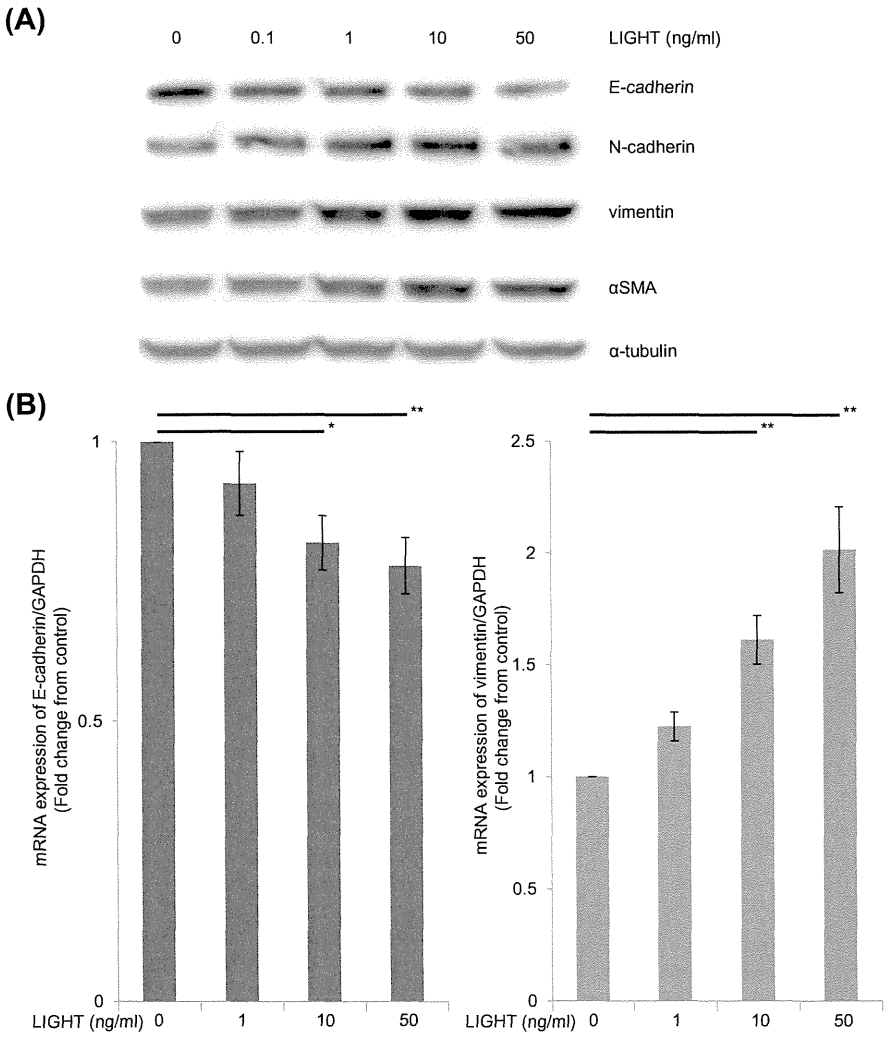

Fig. 2. LIGHT induces EMT in A549 cells. (A) We determined the expression of epithelial and mesenchymal markers in A549 cells. The cells were stimulated with various concentrations of LIGHT (0, 0.1, 1, 10, 50 ng/ml) for 48 h. LIGHT reduced expression E-cadherin (as simultaneously increasing expression of N-cadherin, vimentin and α-SMA (as mesenchymal markers), concentration-dependently. (B) We investigated the expression of mRNA for E-cadherin and vimentin in A549 cells stimulated with various concentrations of LIGHT (0, 1, 10, 50 ng/ml) for 48 h. LIGHT significantly reduced E-cadherin mRNA levels while simultaneously increasing expression of vimentin, concentrationdependently. $n = 6$ separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

with various concentrations of LIGHT (0, 10, 50 ng/ml) for 24 h, measured the concentration of TGF-β1 in the cell supernatant using an ELISA kit (Supplemental Fig. 2A) and examined the cells' expression of TGF-β1 mRNA by q-RT-PCR (Supplemental Fig. 2B). Neither the TGF-β1 concentration nor the TGF-β1 mRNA expression level differed significantly between the control and LIGHT-stimulated groups. Thus, LIGHT did not induce production of TGF-β1, which is the most potent known inducer of EMT, or enhance TGF-β1-induced EMT by an autocrine mechanism.

3.3.2. LIGHT does not affect the smad-snai1pathway

Next, since LIGHT enhanced TGF-β1-induced EMT, we examined the effect of LIGHT on the smad-*snai1* signaling pathway, which is one of the major pathways in TGF-β1-induced EMT [24]. We first evaluated phosphorylation of smad2 by immunoblot assay.

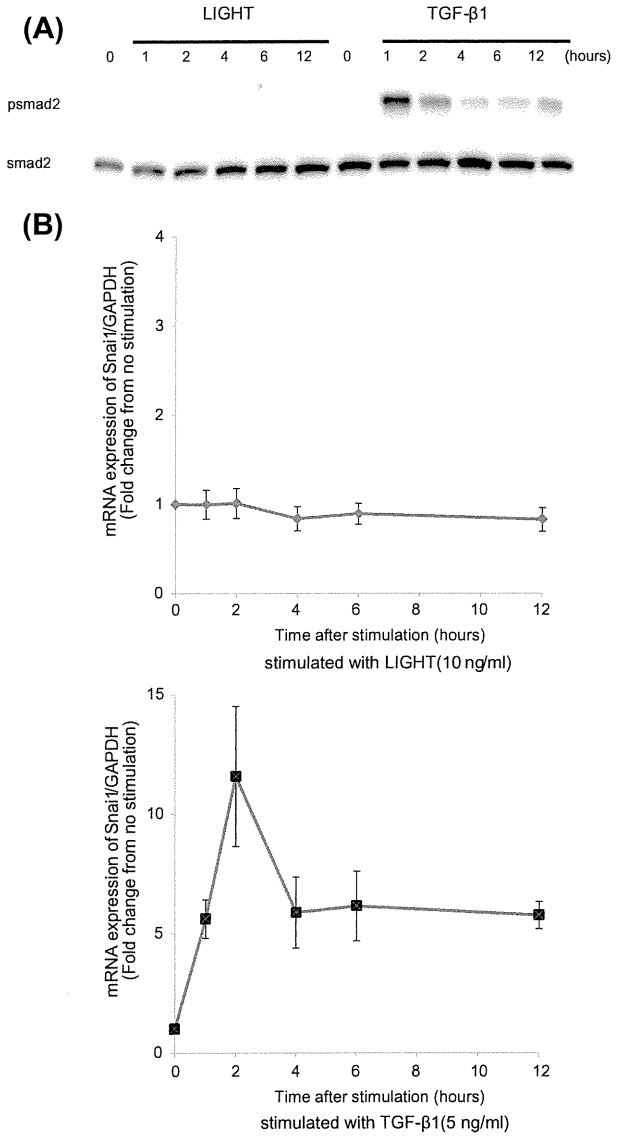

Fig. 3. LIGHT does not affect TGF-β1 production or the smad-*snai1* pathway. (A) We demonstrated time-course of effect of LIGHT (10 ng/ml) on the smad2 pathway, which is one of the major pathways associated with the TGF-β1 receptor. TGF-β1 induced phosphorylation of smad2, but LIGHT did not. (B) We showed time-course of effect of LIGHT on the *snai1* pathway, which is one of the major repressors of E-cadherin. TGF-β1 induced expression of *snai1* mRNA, but LIGHT did not.

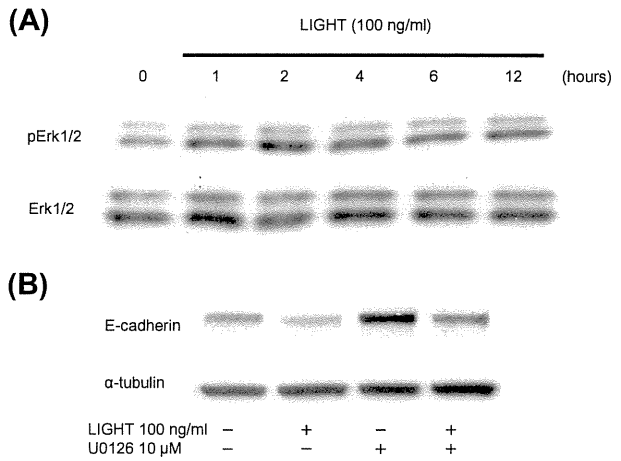

Fig. 4. Erk1/2 signaling is essential for LIGHT-induced EMT. (A) We demonstrated timecourse of effect of LIGHT on Erk1/2 phosphorylation. Erk1/2 phosphorylation was induced by LIGHT after 1 h of stimulation; the level of phosphorylation was at its peak from 2 to 4 h and then decreased by 12 h. (B) We investigated effect of Erk 1/2 inhibitor on E-cadherin expression. A549 cells were pre-treated for 1 h with U0126, a potent and specific inhibitor of Erk1/2 phosphorylation. U0126 prevented loss of E-cadherin.

Fig. 3A shows that LIGHT did not induce smad2 phosphorylation, whereas TGF-β1 did.

We then used q-RT-PCR to evaluate the expression of *snai1* mRNA, which works downstream of smad-signaling (Fig. 3B). *Snai1* mRNA expression was induced by TGF-β1 and was strongest at 2 h after stimulation, but LIGHT did not affect *snai1* mRNA expression. Therefore, LIGHT did not affect either TGF-β1 production or the smad-*snai1* pathway.

3.3.3. Erk1/2 signaling is essential for LIGHT-induced EMT

We next evaluated the effect of LIGHT on phosphorylation of Erk1/2. Fig. 4A shows that LIGHT induced Erk1/2 phosphorylation after 1 h of stumulation; the level of phosphorylation was at its peak from 2 to 4 h and then decreased by 12 h. To determine whether the Erk1/2 pathway was involved in LIGHT-induced EMT, we treated A549 cells with pharmacological inhibitors of the pathway prior to induction of EMT with LIGHT. We used U0126, a potent and specific inhibitor of Erk1/2 phosphorylation, to inhibit the Erk1/2 pathway. Fig. 4B shows that pre-treatment of A549 cells with U0126 prevented loss of E-cadherin. Taken together, these results show that Erk1/2 signaling is essential for LIGHT-induced EMT.

4. Discussion

This study demonstrated that LIGHT enhanced TGF-β1-induced EMT as well as the contractility of cells that underwent EMT. It also generated the first evidence that LIGHT itself induces EMT, without TGF-β1. LIGHT-induced EMT was shown to be an Erk1/2-dependent process, characterized by morphological transition from a typically epithelial cobblestone appearance to spindle-shaped, elongated cells expressing mesenchymal markers—vimentin, N-cadherin and α-SMA—with simultaneous loss of an epithelial protein—E-cadherin—that is an important caretaker of the epithelial phenotype and function [5].

Recently, LIGHT was reported to be involved in various inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease [8,25]. In severe asthma, which is a chronic airway inflammatory disease, LIGHT levels in the sputum of asthma

patients were negatively associated with lung function [7], suggesting that LIGHT is associated with airway remodeling. LIGHT induced airway fibrosis in severe asthma by enhancing secretion of TGF-β from macrophages and IL-13 from eosinophils [2]. Moreover, the levels of LIGHT were increased in bronchoalveolar lavage (BAL) fluid from patients with scleroderma—a cause of pulmonary fibrosis—and lung inflammation [16]. Therefore, LIGHT is an important mediator not only in inflammatory lung disease, but also in fibrotic lung disease. However, there have been few reports concerning the mechanisms of LIGHT-associated fibrosis, leading us to examine the effect of LIGHT on EMT, which is one of the mechanisms involved in tissue fibrosis. We demonstrated that LIGHT enhanced TGF-β1-induced EMT and also induced EMT by itself, in the absence of TGF-β1. Thus, LIGHT's role in lung fibrosis and the pathogenesis of fibrotic lung disease may be played out through EMT. TGF-β1 is a key mediator in the pathogenesis of pulmonary fibrosis. Enhanced TGF-β1 signaling was reported to contribute to induction of EMT in tissue fibrosis [9,10]. Moreover, inflammatory mediators, such as pro-inflammatory cytokines, reportedly enhanced TGF-β1-induced EMT [1,12,28]. That suggests that tissue inflammation might accelerate tissue fibrosis through EMT. However, there were few reports that EMT occurred without TGF-β1 [14], so we examined the mechanism of LIGHT-induced EMT without TGF-β1. First, we assumed that the mechanism of LIGHT-induced EMT involved enhanced production of TGF-β1, since TNF-α induces TGF-β1 expression [22]. However, LIGHT did not induce either TGF-β1 production or mRNA expression. This means that the mechanism of LIGHT-induced EMT is not autocrine TGF-β1 signaling. The smad pathway and Erk phosphorylation are major pathways in TGF-β1-induced EMT [27]. Here, we found that LIGHT induced Erk phosphorylation without involving the smad pathway. Inhibition of Erk1/2 by a specific inhibitor, U0126, abrogated the decrease of E-cadherin in A549 cells. These data indicate that Erk1/2 signaling is required in LIGHT-induced EMT.

A limitation of our study is that we demonstrated EMT induction in A549 cells, which is a cancer cell line, not in normal human epithelial cells. Thus, it is difficult to extrapolate our results directly to the pathogenesis of pulmonary fibrosis. However, A549 cells are widely used to study the functions of airway epithelial cells, and several studies yielded evidence of EMT in the lung by using A549 cells as a model of airway fibrosis [15,30]. Further experiments need to be carried out using normal human airway epithelial cells.

In summary, TGF-β1 suppressed E-cadherin expression and induced vimentin expression, thereby inducing EMT in A549 human alveolar epithelial cells. LIGHT enhanced the TGF-β1-induced EMT. The cells that underwent EMT acquired contractility, which was enhanced by costimulation with LIGHT and TGF-β1. Moreover, LIGHT itself induced EMT via the Erk1/2 pathway, without TGF-β1 signaling. LIGHT may contribute to the pathogenesis of airway fibrosis through enhancement of EMT.

Author contributions

Author contributions: Y.M., Y.Y., M.M, M.K., T.J., H.T, T.N., and K.T. conception and design of research; Y.M., and Y.Y. performed experiments; Y.M., and M.K. analyzed data; Y.M., Y.Y., M.M., and T.J. interpreted results of experiments; Y.M., and Y.Y. prepared figures; Y.M. Drafted manuscript; Y.M. Edited and revised manuscript; Y.M., Y.Y., and T.K. Approved final version of manuscript; Y.M., Y.Y., H.T., T.N., and T.K.

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Disclosures

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.097.

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RESEARCH Open Access

Comparison of gene expression profiling between lung fibrotic and emphysematous tissues sampled from patients with combined pulmonary fibrosis and emphysema

Masayuki Hanaoka*, Michiko Ito, Yunden Droma, Atsuhito Ushiki, Yoshiaki Kitaguchi, Masanori Yasuo and Keishi Kubo

Abstract

Background: Combined pulmonary fibrosis and emphysema (CPFE) is characterized by both emphysema of the upper zone and diffuse parenchymal lung disease with fibrosis of the lower zone of the lung on chest computed tomography. The aim of this study was to investigate the mechanism of CPFE regarding gene expressions by comparing the results of microarray sequences between fibrotic and emphysematous lesions in the lungs of CPFE patients.

Results: The expression profiles of the fibrotic and emphysematous lesions were remarkably different in terms of function. Genes related to the immune system, structural constituents of the cytoskeleton, and cellular adhesion were overexpressed in fibrotic lesions, while genes associated with the cellular fraction, cell membrane structures, vascular growth and biology, second-messenger-mediated signaling, and lung development (all processes that contribute to the destruction and repair of cells, vessels, and the lung) were overexpressed in emphysematous lesions.

Conclusions: The differences in gene expression were detected in fibrotic and emphysematous lesions in CPFE patients. We propose that the development of coexisting fibrotic and emphysematous lesions in CPFE is implemented by these different patterns of gene expressions.

Keywords: Emphysematous lesion, Cellular fraction, Fibrotic lesion, Gene expression profiles, Immune system, Lung

Background

One of the most demonstrably clinical features of combined pulmonary fibrosis and emphysema is that emphysema of the upper zones and diffuse parenchymal lung disease with fibrosis of the lower zones of the lungs are both presented on chest computed tomography [1]. The distinctive features of CPFE include cigarette smoking, severe dyspnea, hypoxemia at exercise, subnormal spirometry findings, and severely impaired lung diffusion capacity [1,2]. Severe pulmonary hypertension is frequently observed in CPFE, and is thought to determine its prognosis [1,3]. The substantial pathogenesis of CPFE is still unresolved because CPFE is not just one identical phenotype of either idiopathic pulmonary fibrosis (IPF) or emphysema [1,2]. Genetic factors have been shown to play significant roles in the development of IPF. Fibrotic lung specimens from patients with IPF exhibited misexpressions of genes encoding proteins probably involved in the metabolism of the extracellular matrix (ECM), chemokines, and tissue remodeling [4]. Genetic factors are also believed to be associated with the pathogenesis of emphysema. Lung tissues from patients with severe emphysema displayed irregular expression of genes involved in inflammation, the ECM, cytokines, chemokines, apoptosis, and stress responses [5].

We hypothesized that coexistent fibrosis and emphysema are programmed by differential gene expressions

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in the corresponding lesions in the lungs of smokers susceptible to CPFE. Given the importance of genetic susceptibility in understanding the etiology and pathogenesis of CPFE, we performed the whole-genome microarray to sequence the gene expression profiling on fibrotic and emphysematous tissues sampled from three patients with CPFE to identify genes distinguishably expressed in fibrotic lesions and emphysematous lesions in lung tissues of patients with CPFE.

Results

Data deposition

The data reported in this paper have been deposited in the Gene Expression Omnibus database [GEO:GSE38934].

Among all the specimens of lung tissue from the resected lobes of the six patients (see Methods), six lung specimens from three patients with CPFE (three specimens from fibrotic lesions and three specimens from emphysematous lesions) were selected to apply to the current gene expression profiling study after confirming the pathology in fibrotic lesions and emphysematous lesions by H & E staining. Figures 1,2,3 show the chest computed tomography and H & E staining microscopic images for each case, representatively showing the upper zone with emphysema, the lower zone with fibrosis, the cancer shadow, and the position of sampling without cancerous lesions. All three patients were male ex-smokers (smoking from 38 to 72 pack-years) aged from 60 to 78 years. The specimens of fibrotic lesions showed usual interstitial pneumonia in histology, characterized by fibroblastic foci and excessive deposition of the ECM (Figures 1f,2f,3f).

Summary of *t* test results, volcano plot and hierarchical clustering analysis

Figure 4 presents the paired *t* test results summary as a comparison between fibrotic and emphysematous lesions on the microarray data (Figure 4a) and the volcano plot as a representation of the point of intersection of the fold-change (emphysema vs. fibrosis) relative to the *P* value (emphysema vs. fibrosis) (Figure 4b). Hierarchical clustering analysis was performed to build a heat map (Figure 4c), which beautifully separated fibrotic and emphysematous lesions into two clusters.

Gene functional classification for fibrotic lesions in CPFE

One hundred and forty genes with a signal log ratio (SLR) cutoff of at least 1 were overexpressed in tissues with fibrotic lesions versus emphysematous lesions (Additional file 1: Table S1). When enrichment analysis was applied to this set of genes, five functional annotation clusters emerged (Figure 5); the most highly

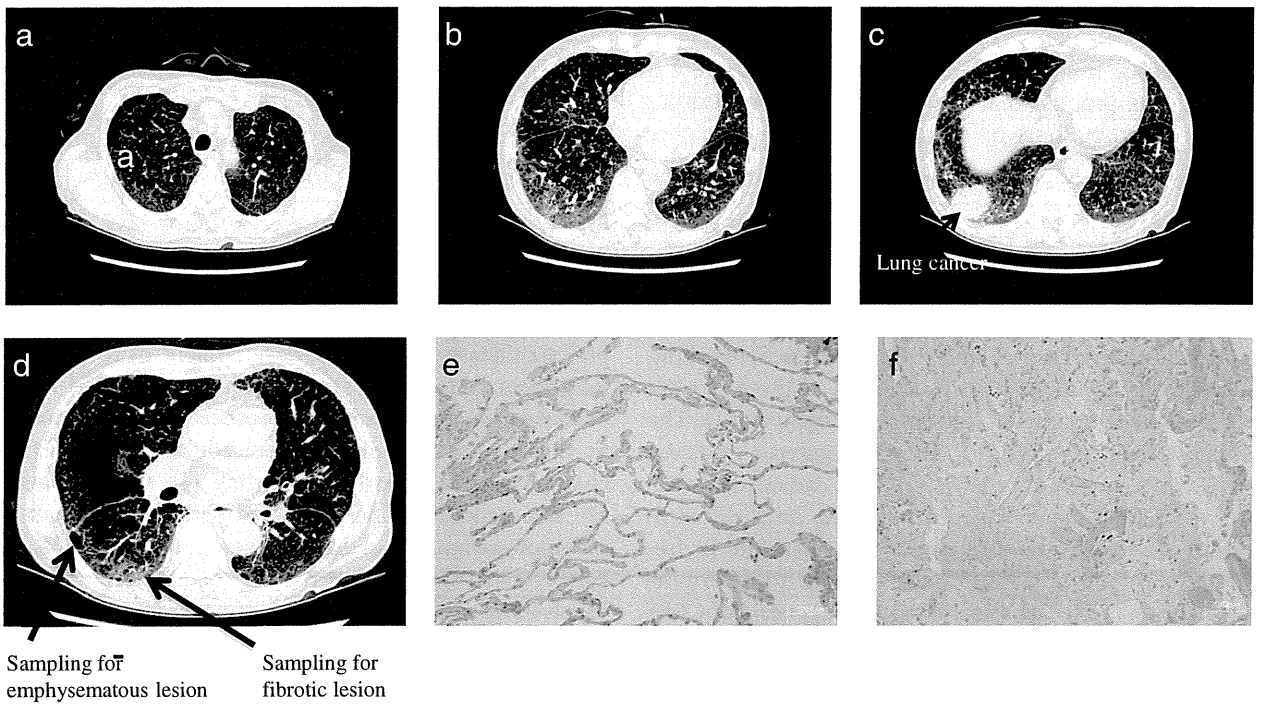

Figure 1 Chest computed tomography and H & E staining microscopic images for case 1. Upper panel: emphysematous change in the upper zone of the lung (**a**), fibrotic lesion at the lung base (**b**), and the cancer shadow (arrow) at the right inferior lobe (**c**). Lower panel: positions (arrows) of sampling in the emphysematous lesion and the fibrotic lesion in the right inferior lobe without cancerous lesions (**d**), and the H & E staining microscopic images of specimens of the emphysematous lesion (**e**) and the fibrotic lesion (**f**).

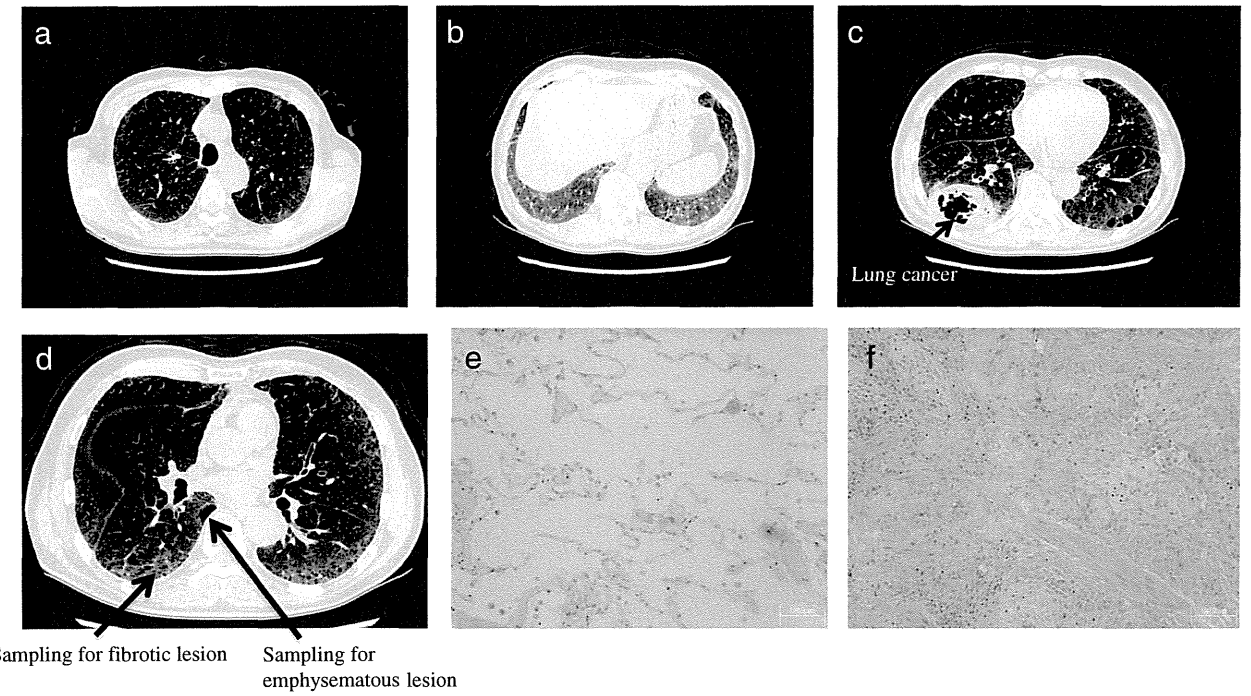

Figure 2 Chest computed tomography and H & E staining microscopic images for case 2. Upper panel: emphysematous change in the upper zone of the lung (**a**), fibrotic lesion at the lung base (**b**), and the cancer shadow at the right inferior lobe (**c**). Lower panel: positions (arrows) of sampling in the emphysematous lesion and the fibrotic lesion in the right inferior lobe without cancerous lesions (**d**), and the H & E staining microscopic images of specimens of the emphysematous lesion (**e**) and the fibrotic lesion (**f**).

enriched was a cluster of immunoglobulin-like molecules with an enrichment score of 13.13. The other clusters were based on annotations of immunoglobulin heavy constant region (score of 5.57), immunoglobulin variable region (score of 3.55), structural constituent of cytoskeleton (score of 3.55), and cell adhesion (score of 2.94).

Gene functional classification for emphysematous lesions in CPFE

After application of an analogous analysis process, 148 genes were identified as overexpressed in emphysematous tissues relative to fibrotic tissues (Additional file 2: Table S2). Of the six functional annotation clusters in the emphysematous lesion dataset (Figure 6), the cellular fraction cluster was the most enriched, with a score of 3.86. The other functional clusters were proteins in structure of membrane (score of 2.47), regulation of blood vessel (score of 2.36), angiogenesis and blood vessel development (score of 1.85), second-messenger-mediated signaling (score of 1.7), and lung development (score of 1.65).

Discussion

The present study demonstrates in patients with CPFE in which pulmonary fibrosis and emphysema coexisted that the genes overexpressed in the fibrotic lesions were functionally different from those overexpressed in the emphysematous lesions. The genes overexpressed in the fibrotic lesions were annotated as contributing to functions involving immunoglobulin, the structural constituents of the cytoskeleton, and cellular adhesion – biological functions that are related to the pathophysiology of fibrosis. The set of genes overexpressed in the emphysematous lesions were annotated as associated with cell membrane structures, vascular growth and biology, second-messenger-mediated signaling, and lung development – all processes that contribute to the destruction and repair of cells, vessels, and the lung. These annotation-based clusters suggest that functionally different genes expressed in susceptible fields of the lungs eventually contribute to the formation of either fibrosis or emphysema, depending on the pattern of gene expression in that field.

The major finding of the present investigation is that genes belonging to the clusters enriched for immunoglobulin-like molecules, the immunoglobulin constant region, and the immunoglobulin variable region were markedly expressed in the fibrotic lesions. The roles of autoimmunity in interstitial lung diseases (fibrosis in pathology) associated with connective tissue disorders such as systemic sclerosis, systemic lupus erythematosus, and rheumatoid arthritis are well established [6]. Autoimmunity has been demonstrated to be

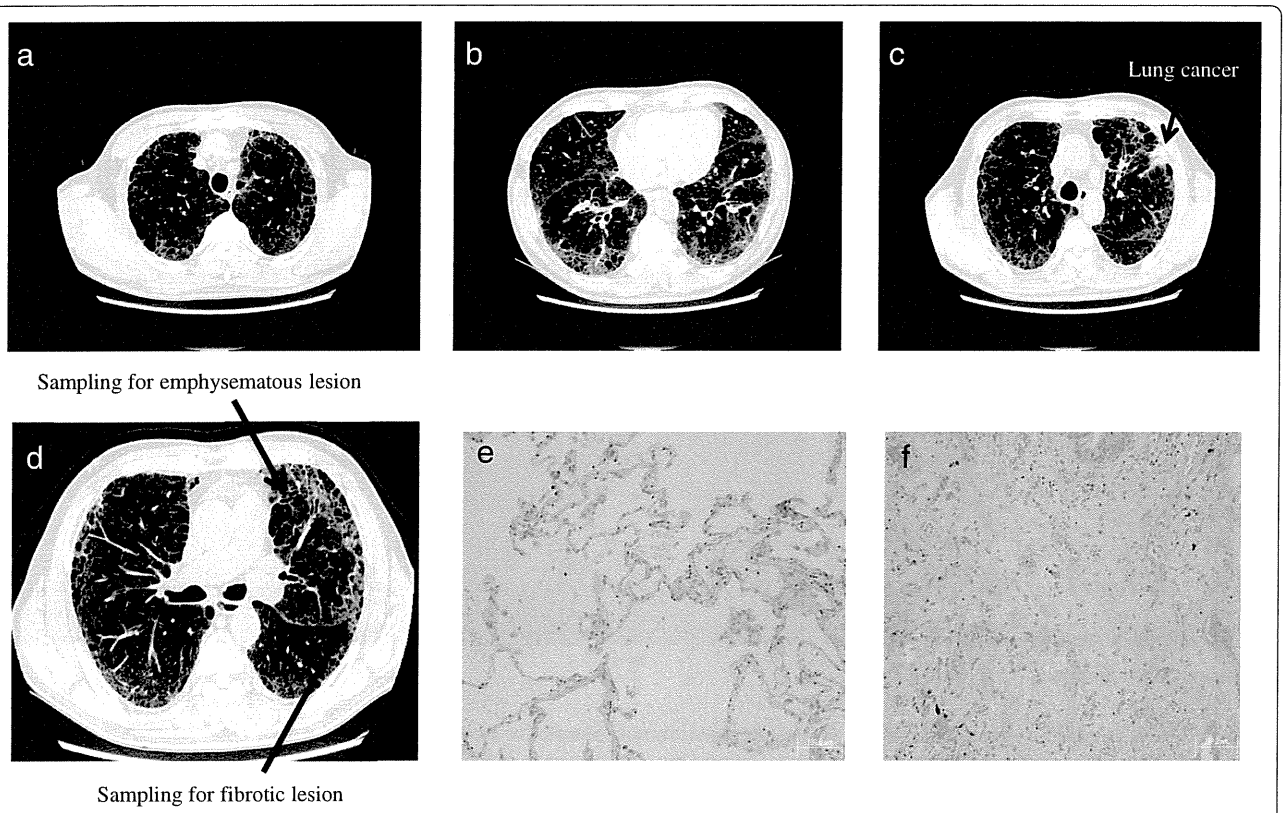

Figure 3 Chest computed tomography and H & E staining microscopic images for case 3. Upper panel: emphysematous change in the upper zone of the lung (**a**), fibrotic lesion at the lung base (**b**), and the cancer shadow at the left superior lobe (**c**). Lower panel: positions (arrows) of sampling in the emphysematous lesion and the fibrotic lesion in the left superior lobe without cancerous lesions (**d**), and the H & E staining microscopic images of specimens of the emphysematous lesion (**e**) and the fibrotic lesion (**f**).

one of the mechanisms of alveolar injury responsible for IPF [6,7]; the presence of autoantibodies in the sera of patients with IPF has been demonstrated elsewhere [8,9]. Additionally, the initial hypothesis for the mechanism of pulmonary fibrosis was that pulmonary inflammation was a prominent and necessary feature of the fibrotic process [10]. Those genes involved with immunoglobulin were probably expressed by the inflammatory cells in the infiltrates that were clearly presented in the fibrotic tissues. However, to date no candidate genes related to immunoglobulin-like molecules or members of the immunity system annotation cluster have been reported to be associated with lung fibrosis either in case–control association studies [11,12] or in studies based on human genome oligonucleotide microarrays [13]. Our observations provide the first evidence of a positive association of the immune system and pulmonary fibrosis at the genetic level. This major finding further supports the inflammation hypothesis of pulmonary fibrosis [10,14].

Different from other findings in which the results of gene expression in pulmonary fibrosis were resulted from the comparisons of the gene expression datasets between fibrous tissues and healthy lung tissues [13] or hypersensitivity pneumonitis lung tissues [15], we found that gene members of the structural constituent of cytoskeleton (keratin, claudin, dystonin) annotation cluster were overexpressed in CPFE fibrotic lesions versus emphysematous lesions. This observation provides genetic evidence in support of the latest mechanism of pulmonary fibrosis [16], which postulates that the losses of epithelial cells, endothelial cells, and alveolar–capillary barrier basement membrane integrity contribute to the pathogenesis of pulmonary fibrosis. Furthermore, we identified overexpression of the gene members of the cell adhesion annotation cluster in the fibrosis lesions of CPFE. Previously, cell adhesion molecules were shown to be expressed in the lungs of patients with IPF [17]. Cell–cell and cell–ECM interactions are critical for the pathogenesis of pulmonary fibrosis [18]. One of the essential mechanisms by which cells interact with the microenvironment is through the expression of cell adhesion molecules [18]. Inflammatory cells may adhere to and injure lung parenchymal cells through binding to intercellular adhesion molecule 1. After initial injury, the abnormalities in interepithelial adhesion interactions, the

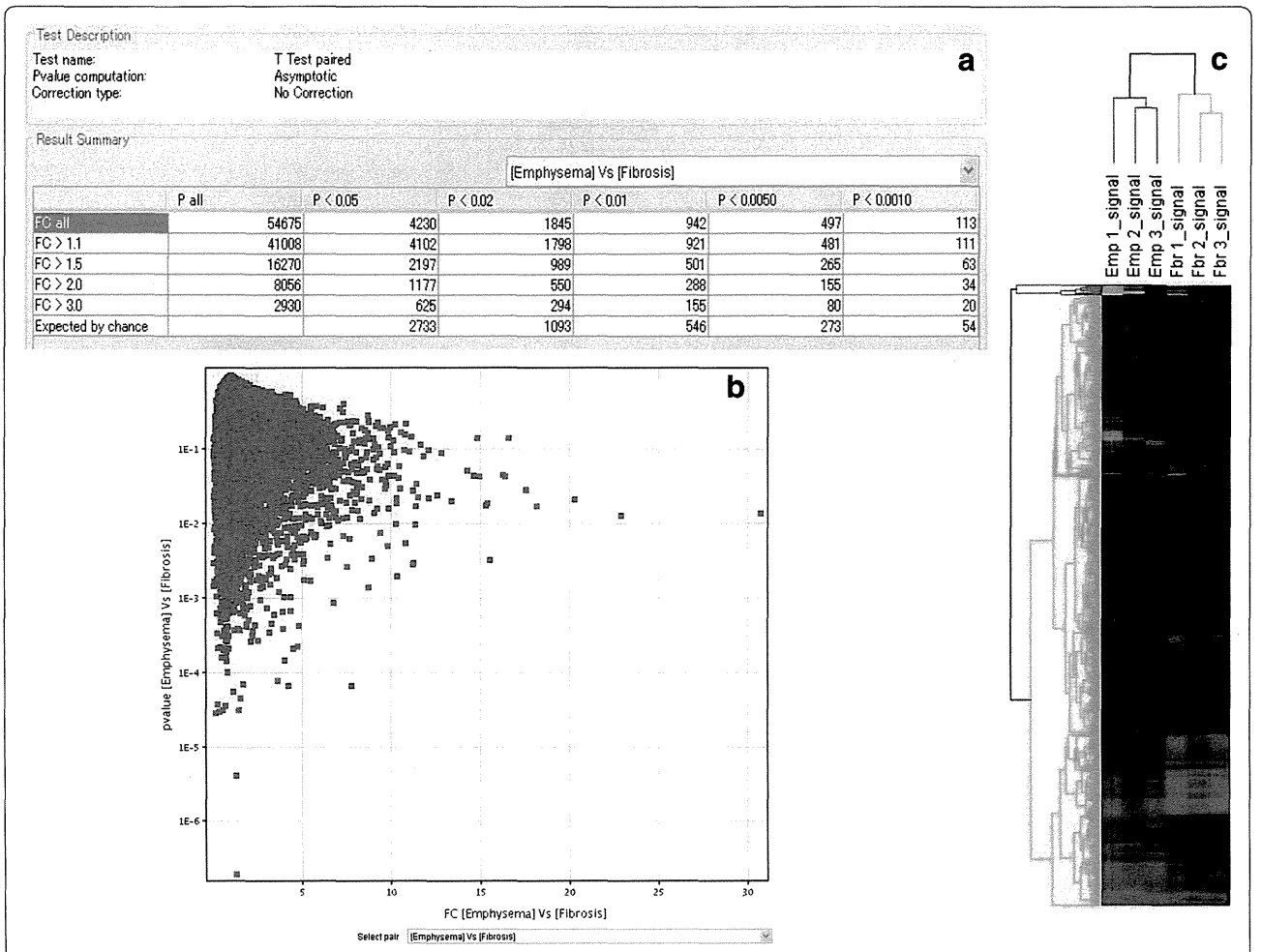

Figure 4 Comparison of the signals between emphysematous and fibrotic lesions, fold-change and *P* value intersection, and hierarchical clustering analysis. Paired *t* test results summary comparing between emphysematous and fibrotic lesions on the microarray data (**a**), volcano plot representing the point of intersection of fold-change (FC) (emphysema vs. fibrosis) relative to *P* value (emphysema vs. fibrosis) (**b**), and heat map built as the result of hierarchical clustering analysis (**c**). Emp 1_singal, Emp 2_singal, and Emp 3_singal indicate the signals detected in the specimens of emphysematous lesions in case 1, case 2, and case 3, respectively. Fbr 1_singal, Fbr 2_singal, and Fbr 3_singal indicate the signals detected in the specimens of fibrotic lesions in case 1, case 2, and case 3, respectively.

interactions of ECM molecules with both epithelial cells and fibroblasts, and the disordered healing process eventually result in fibrosis [18].

Regarding the genes expressed in emphysematous lesions in CPFE, our results showed that the genes involved in cellular and membrane fractions, genes encoding proteins in membranes, genes related to blood vessel size, development, and angiogenesis, and genes related to second-messenger-mediated signaling were overexpressed versus fibrotic lesions of CPFE. We propose that the overexpression of genes with functions contributing to the cellular fraction and membrane structure may lead to the injury of pulmonary alveoli to cause emphysema. In addition, our observation of the overexpressed genes regulating blood vessel size, development, and angiogenesis in the emphysematous lesions in CPFE may explain the genetic susceptibility to high prevalence (up to 47%) of pulmonary hypertension [1] in CPFE. We suggest that the overexpression of these vascular biology-related genes orchestrates the development and progression of pulmonary hypertension in CPFE. This hypertension may be a clinical characteristic that distinguishes CPFE from severe emphysema without fibrosis; the latter has a lower frequency of pulmonary hypertension than CPFE [1] and expresses downregulated endothelium-related genes [5].

The main limitation of the present study is the very small sample size, with only three patients with CPFE, which occurred because lung tissue specimens were only occasionally available following invasive operations for other diseases; for example, lung cancer. Further study with a larger sample size is necessary to confirm our

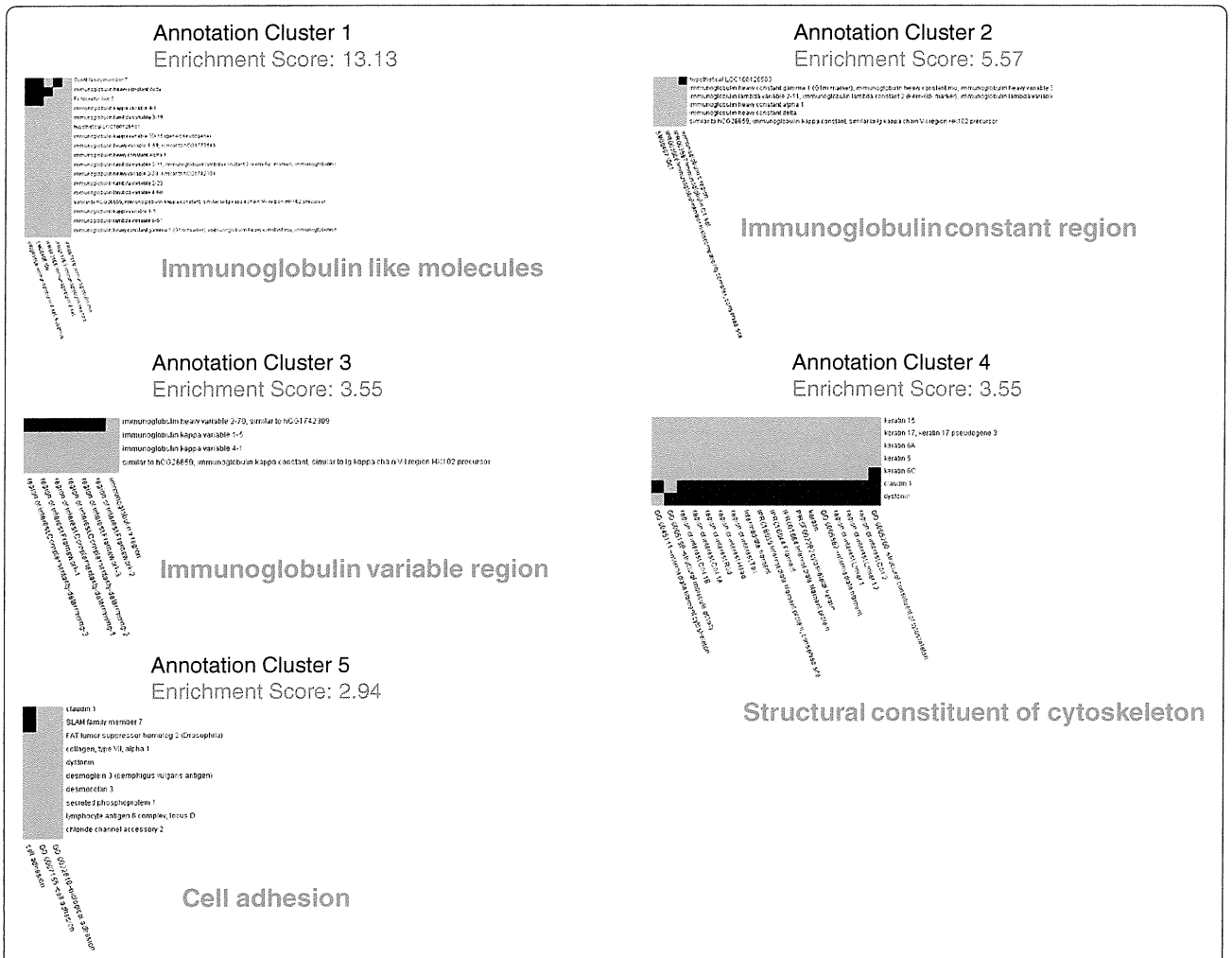

Figure 5 Highly related genes and corresponding annotations in fibrotic lesions of combined pulmonary fibrosis and emphysema. Two-dimensional view maps of the relationships between highly related genes and their corresponding annotations in fibrotic lesions of combined pulmonary fibrosis and emphysema (CPFE) using the Gene Functional Classification Tool (http://david.abcc.ncifcrf.gov/gene2gene.jsp) from the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/home.jsp). Individual gene names in the gene group are listed on the right-hand side of the map and functional annotation terms are presented on the lower side of the map. Annotation cluster 1 for immunoglobulin-like molecules, annotation cluster 2 for immunoglobulin heavy constant region, annotation cluster 3 for immunoglobulin variable region, annotation cluster 4 for structural constituent of cytoskeleton, and annotation cluster 5 for cell adhesion. Green rectangle, corresponding gene-annotation association positively reported in DAVID; black rectangle, corresponding gene-annotation association not yet reported in DAVID.

observations. Nevertheless, we believe that the present results motivate a new direction for understanding the coexistence of fibrosis and emphysema in CPFE.

Conclusions

In summary, we have demonstrated that gene expression differs between fibrotic and emphysematous lesions in CPFE. In the fibrotic lesions, genes associated with the immune system are highly expressed, while genes related to the cellular fraction, membrane biology, and vascular biology are highly expressed in emphysematous lesions. We propose that the development of coexistent fibrotic and emphysematous lesions in CPFE is implemented by these different patterns of gene expression.

Methods

Patients and specimens

According to the characteristics of CPFE addressed by Cottin and colleagues [1], six patients were selected for the current study. All six patients were originally diagnosed with lung cancer and high-resolution computed tomography showed not only the cancerous lesions but also emphysematous lesions in the upper zones and fibrotic lesions in the lower zones. The fibrotic and emphysematous lesions were located within the resected

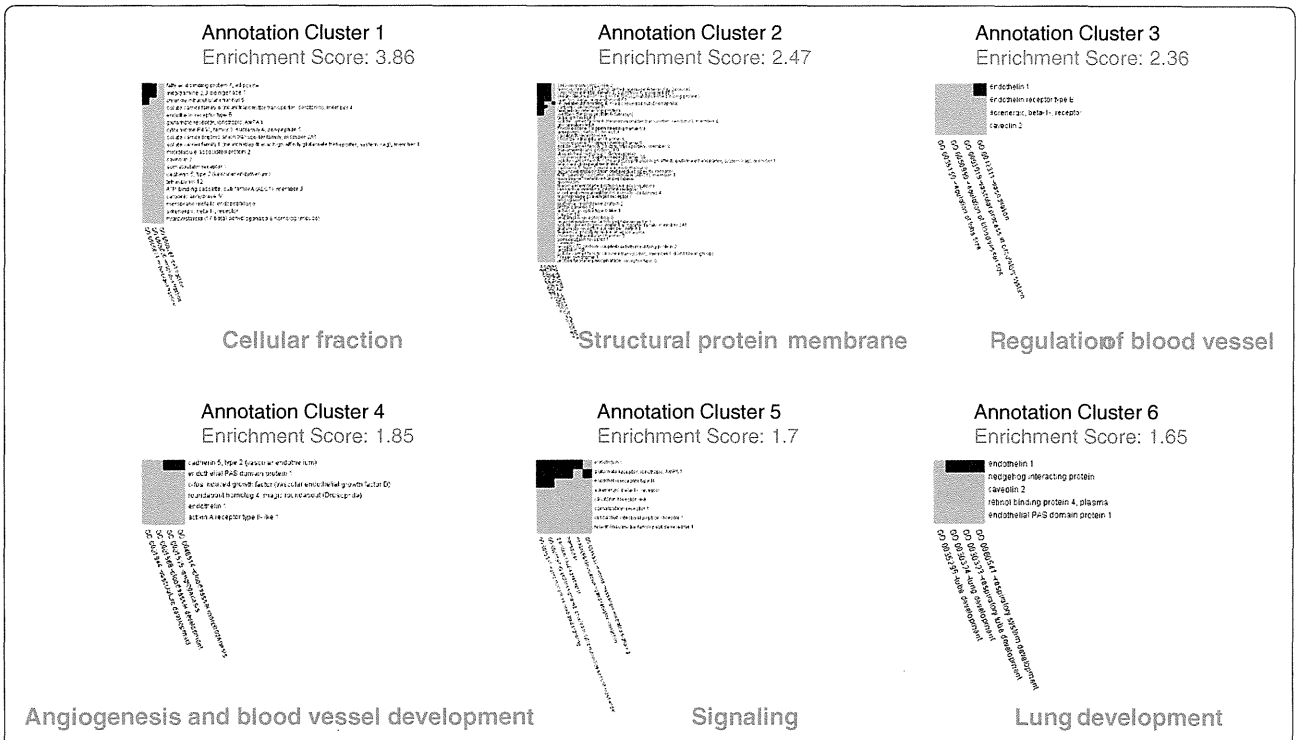

Figure 6 Highly related genes and corresponding annotations in emphysematous lesions of combined pulmonary fibrosis and emphysema. Two-dimensional view maps of the relationships between highly related genes and their corresponding annotations in emphysematous lesions of combined pulmonary fibrosis and emphysema (CPFE) using the Gene Functional Classification Tool (http://david.abcc.ncifcrf.gov/gene2gene.jsp) from the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/home.jsp). Annotation cluster 1 for cellular fraction, annotation cluster 2 for proteins in structure of membrane, annotation cluster 3 for regulation of blood vessel, annotation cluster 4 for angiogenesis and blood vessel development, annotation cluster 5 for signaling, and annotation cluster 6 for lung development. Green rectangle, corresponding gene-annotation association positively reported in DAVID; black rectangle, corresponding gene-annotation association not yet reported in DAVID.

lobes. Further pulmonary function tests also indicated that impairment of carbon monoxide diffusing capacity and subnormal spirometry were present in these patients. CPFE was also diagnosed for these patients. Lung lobectomy was thought to be the optimal treatment for the lung cancer after consulting with chest surgeons. Specimens of fibrotic lesions and emphysematous lesions in lung tissues were sampled from the resected lobes of the six patients. Immediately after the lobectomy, the lung tissues with fibrosis and emphysema were separated from the resected lobes, and the lung tissue adjacent to each specimen was cut for histological confirmation of the absence of cancerous cells. The pathological types of fibrosis and emphysema were examined and confirmed after H & E staining by a pathologist who was blinded to the purpose of the study. This study was approved by the Ethics Committee of Shinshu University School of Medicine, and written informed consent was obtained from all patients.

RNA isolation

Lung tissue specimens were immediately frozen in dry ice and stored at −80°C. RNA was extracted from the lung tissues using TRIzol Reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's protocol. RNA for microarray analysis was purified using the RNeasy MinElute Cleanup Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

GeneChip expression preprocessing

In accordance with the standard Affymetrix protocol from the GeneChip Expression Analysis Technical Manual Revision 5 [19], 2 μg total RNA was processed, biotinylated, fragmented, and hybridized to the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA). The Human Genome U133 Plus 2.0 chips consist of 54,765 probe sets and provide comprehensive coverage of the transcribed human genome on a single array, allowing the analysis of more than 47,000 transcripts and variants, including 38,500 well-characterized human genes plus approximately 6,500 new genes [20]. Immediately following hybridization, the probe array underwent an automated washing and staining protocol on the Affymetrix Fluidics Station 450. The

prepared GeneChips were then scanned using the Affymetrix GeneChip Scanner 3000. Each probe array was scanned twice and the software calculated an average of the two images, defined the probe cells, and computed the intensity for each cell. The double scan improved assay sensitivity and reduced background noise. Each complete probe array image was stored in a separate data file.

Data processing

The scanned images were analysed with GeneChip Operating Software version 1.4 (Affymetrix 690036) and Microarray Suite version 5.0 (Affymetrix). The advantages of the Microarray Suite include the associated *P* values indicating statistical significance for detection and change calls, the confidence limits being associated with expression change values, and the negative expression values being eliminated [21]. The profiling dataset from the emphysematous lesions was used as background when extracting the differentially expressed genes in the fibrotic lesions, and the data from the fibrotic lesions were used as background when extracting the differentially expressed genes in the emphysematous lesions. The trimmed mean target intensity of each array was set to 500. Differentially expressed genes were extracted using DNA Microarray Viewer (Kurabo, Osaka, Japan).

All microarray data of the individual lung tissue of the six specimens are freely available through the Gene Expression Omnibus repository [GEO:GSE38934].

Data analysis

The paired *t* test was performed for comparison of the detected signals between fibrotic and emphysematous lesions on the microarray data. The volcano plot was derived from the summary results of the paired *t* test to represent the point of intersection of fold-change (emphysema vs. fibrosis) relative to *P* value (emphysema vs. fibrosis). Clustering analysis was then carried out with log-transformed average signals of both fibrotic and emphysematous lesions on the microarray data and the heat map was built (Avadis 4.3; Strand Scientific Intelligence, San Francisco, CA, USA), according to the result of the hierarchical clustering analysis.

The SLR algorithm measures the magnitude and direction of the change between transcript levels of the experimental and background chips. A SLR of 1 represents a twofold increase in the abundance of an mRNA, and a value of −1 represents a twofold reduction in transcript expression. In the present analysis, we extracted genes with SLR >1 and SLR <1 for the fibrotic/emphysematous and emphysematous/fibrotic ratios, respectively. We then systematically annotated the large list of genes according to their biological functions using bioinformatics resources from the Database for Annotation, Visualization and Integrated Discovery [22], which provides the ability to explore and view functionally related genes together, as a unit, to concentrate on the large biological network rather than at the level of an individual gene [9]. Condensing large gene lists into biologically meaningful modules greatly improves the ability to assimilate large amounts of information and thus switches functional annotation analysis from a gene-centric analysis to a biological module-centric analysis [9]. Since an enrichment score of 1.3 is equivalent to 0.05 on the non-log scale, we focused on gene clusters with scores ≥1.3 to address genes hypothetically involved in the phenotypes of the examined tissues [9].

Additional file

Additional file 1: Table S1. One hundred and forty genes with signal log ratio over 1 were overexpressed in lung tissues with fibrotic lesions versus tissues with emphysematous lesions.

Additional file 2: Table S2. One hundred and forty eight genes with signal log ratio less than 1 were overexpressed in lung tissues with emphysematous lesions versus tissues with fibrotic lesions.

Abbreviations

CPFE: Combined pulmonary fibrosis and emphysema; ECM: Extracellular matrix; H & E: Hematoxylin and eosin; IPF: Idiopathic pulmonary fibrosis; SLR: Signal log ratio.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MH conceived of the study, participated in its design and coordination, and drafted the manuscript. MI collected samples, performed the experiments and statistical analysis, and analyzed the data. YD analyzed the data and drafted the manuscript. AU, YK, and MY were responsible for the clinical data of the patients for diagnosis. KK conceived and designed the study. All authors read and approved the final manuscript.

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