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Immunohistochemical Evidence for the Association between Attenuated mTOR Signaling and Diffuse Alveolar Damage, A Fatal Lung Complication

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Targeted anticancer therapies have been developed to interfere with specific target molecules including those of downstream pathways required for tumor growth and progression. Mammalian target of rapamycin (mTOR) has been considered as one of the target molecules of cancer growth, and its inhibitors have been reported to exert an anticancer effect in various malignant tumors. The pulmonary disorder is one of the major side effects of anticancer drugs including mTOR inhibitor (mTORi), and the diagnosis of lung injury induced by medication is difficult because of non-specific nature of the radiological findings. In this study, we present the detailed autopsy findings of a patient who developed diffuse alveolar damage (DAD) following mTORi treatment for metastatic renal cell carcinoma. We also studied 19 cases of DAD derived from other diseases and 9 cases with non-pathological lung. Of interest, pneumocytes of the patients with DAD, who received other anticancer drugs or contacted bacteria, demonstrated significantly lower mTOR activities than pneumocytes of those with non-pathological lung tissue, as judged by the immunohistochemical analysis. In contrast, both pneumocytes and T cells in DAD tissues of the patient treated with mTORi showed higher mTOR activities than those of patients with DAD of other causes, suggesting that the enhanced mTOR signaling may be involved in the development of DAD after mTORi treatment. This unexpected finding needs to be confirmed in other patients treated with mTORi. In conclusion, the attenuated mTOR signaling in pneumocytes may contribute to the pathogenesis of DAD in patients without mTORi treatment.

Keywords: diffuse alveolar damage; immunohistochemistry; lung injury; mammalian target of rapamycin; mammalian target of rapamycin inhibitor
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Introduction

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that acts as a central regulator of various biological processes, including cell proliferation and cell metabolism. In addition, mTOR is regulated by the upstream phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway (Manning and Cantley 2003). Recently, mTOR inhibitors (mTORi) such as everolimus and temsirolimus have been used to treat metastatic renal cell carcinoma in Japan (Pirrotta et al. 2011). In the future, mTORi is expected to be used for other malignancies, such as breast cancer (Saini et al. 2013).

A previous study has reported a 15-31% incidence of lung injury after treatment with mTORi (Dabydeen et al. 2012). The vast majority of these cases are clinically mild; however, fatal cases, such as those associated with diffuse alveolar damage (DAD), are rare (Pham et al. 2004; Dabydeen et al. 2012). The mTORi-induced lung injuries are most often diagnosed by clinical findings and/or imaging without pathological examination (Dabydeen et al. 2012). To the best of our knowledge, only one study has reported detailed histopathological findings associated with mTORi-induced lung injury (Pham et al. 2004), in which they have described the pathological findings associated with mTORi-induced lung injury, including organizing pneumonia, focal fibrosis, and non-specific pneumonia. Moreover, the detailed function of mTOR in the lung and

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the mechanism of mTORi-induced lung injury remain largely unknown despite the frequency of lung injury associated with mTORi. It is important to investigate the role of mTOR activity in lung injury to identify risk factors of fatal lung injury associated with mTORi therapy. Because the number of cancer patients treated with mTORi in the future is expected to increase, the incidence of these lung injuries may also increase (Saini et al. 2013).

We recently performed an autopsy of a patient who developed DAD after treatment with everolimus for renal cell carcinoma with pulmonary metastases. This is the first reported autopsy of a patient with fatal lung injury associated with mTORi. First, we measured mTOR activity in human lung tissue by immunohistochemical analysis of factors in the mTOR pathway. These assays were performed on specimens from the patient with DAD resulting from mTORi treatment as well as 19 patients with DAD without mTORi treatment and nine patients without lung disease. We particularly focused on mTOR activities in pneumocytes and T cells. The mechanisms underlying DAD are complicated and poorly understood. Several previous studies have reported that drug-induced lung injury, including DAD, is associated with direct injury to type 1 pneumocytes, activation of T cells, and/or apoptosis of type 2 pneumocytes (Guinee et al. 1996; Pirmohamed et al. 2002; Parra et al. 2007). In addition, the mTOR pathway has been associated with apoptosis of various cells, including pneumocytes (Fielhaber et al. 2012), and regulation of CD8-positive T cells (Araki et al. 2009; Waickman and Powell 2012).

Therefore, in the present study, we evaluated mTOR activity in pneumocytes, T cells, and T-cell subpopulations in lungs with DAD and fatal lung injury associated with mTORi treatment to describe mTORi-induced lung pathology.

Materials and Methods

Patient selection

The Ethics Committee at the Tohoku University School of Medicine approved the research protocol (2011-595).

Case history of mTORi-induced lung injury: This is an autopsy case of 63-year-old man. He was a life-long never-smoker without past or family history of interstitial pneumonia. The patient underwent unilateral nephrectomy for renal failure due to autosomal dominant polycystic kidney disease (ADPKD) 5 years prior to his death. Subsequent pathological examination identified incidental clear cell renal cell carcinoma. There was no recurrence for two years following nephrectomy; therefore, unilateral renal transplantation was performed. However, one year after the transplantation, the renal cell carcinoma recurred followed by systemic metastases, including lung metastases. The patient underwent chemoradiation and arterial embolization without clinical improvement. Therefore, everolimus (10 mg/day) was started.

42 days after initiation of everolimus treatment, there was a sharp increase in the patient's serum levels of KL-6 (888 U/mL; normal, < 500 U/mL) and surfactant protein D (SP-D) (219 ng/mL; normal, < 110 ng/mL). At that time, the patient did not have any clinical symptoms, and his plain chest radiograph was unremarkable. However, 52 days after initiation of everolimus treatment, he developed dyspnea. Computed tomography (CT) demonstrated bilateral ground-glass attenuation and patchy airspace consolidation (Fig. 1A), which was not consistent with an infectious process. Based on the clinical course and CT findings, he was diagnosed with drug-induced interstitial pneumonia due to everolimus. Everolimus was discontinued, and prednisolone (50-60 mg/day) was administered. His repeat CT and respiratory condition worsened (Fig. 1B), and despite methylprednisolone pulse therapy, the patient died of respiratory failure 119 days after initiation of everolimus therapy.

The autopsy examination revealed marked chest-wall adhesion of the right lung and multiple foci of metastatic clear cell renal cell carcinoma in both lungs (Fig. 2A). Histologically, both lungs exhibited DAD associated with hyaline-membrane formation. In the right superior lobe (Fig. 2B), there was organization with fibroblastic foci (Fig. 2C) and fibrin exudation (Fig. 2D), consistent with acute-on-

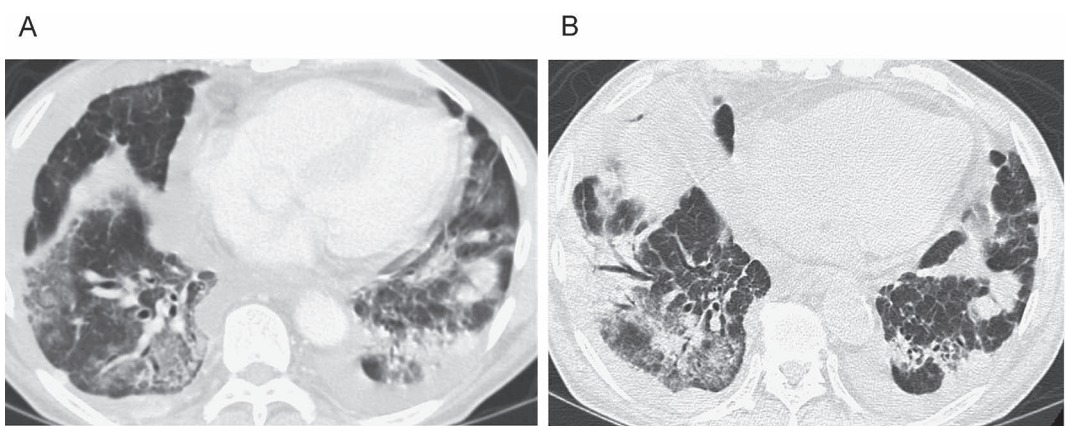

Fig. 1. Radiological findings in a patient with lung injury after treatment with mammalian target of rapamycin inhibitor (mTORi).
(A) Fifty-two days after initiation of everolimus treatment, computed tomography (CT) demonstrated bilateral ground-glass attenuation and patchy airspace consolidation. (B) Seventy-six days after initiation of everolimus treatment, the consolidation spread, and the CT findings worsened.

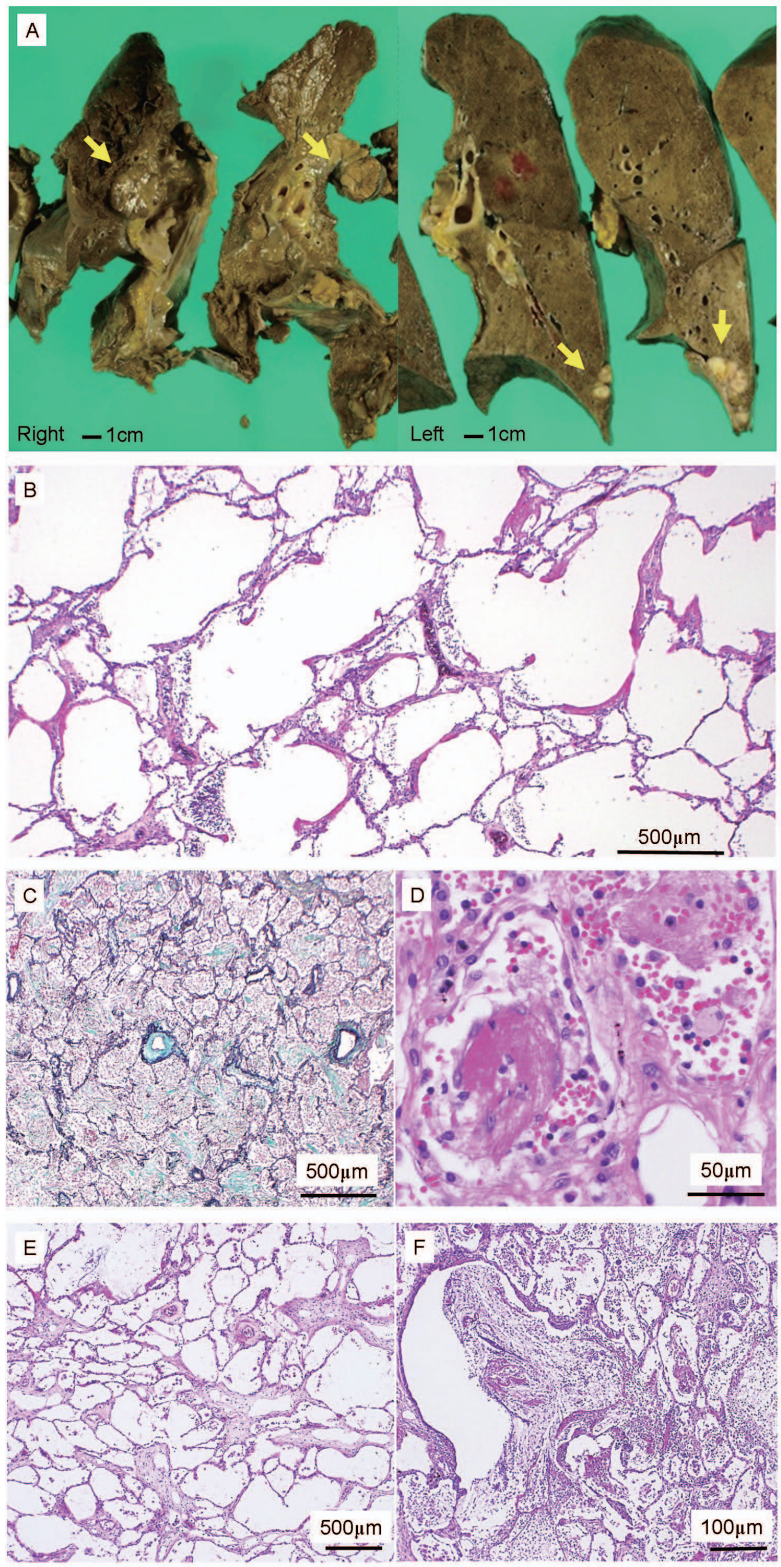

Fig. 2. Autopsy findings in a patient with lung injury after treatment with a mammalian target of rapamycin inhibitor (mTORi). (A) Macroscopic appearance. The lungs exhibited chest-wall adhesion and multiple foci of metastatic clear cell renal cell carcinoma (arrow). (B) Hematoxylin and eosin (H & E) staining. On histological examination, both lungs exhibited diffuse alveolar damage (DAD) with hyaline-membrane formation. (C) Elastica-Masson staining. Organization with fibroblastic foci in the right superior lobe. (D) H & E staining. Fibrin exudation in the right superior lobe. (E) H & E staining. Slight, focal fibrosis in the alveolar septum was observed. (F) H & E staining. Focal neutrophil infiltration was observed in some areas.

chronic lung injury. Focally, there was slight fibrosis in the alveolar septum without apparent changes of acute lung injury (Fig. 2E). There were small foci of neutrophil infiltration suggestive of bacterial infection (Fig. 2F).

Autopsies of patients with DAD without mTORi treatment: First, we screened 24 cases (22 cases diagnosed with DAD in 2008-2012 and two cases diagnosed with gefitinib-induced DAD in 2003) from the autopsy files of Tohoku University Hospital. We excluded five cases diagnosed with neutrophilic inflammation or other interstitial pneumonia following histopathological evaluation. The remaining 19 cases were selected for this study. The characterization and cause of DAD in the selected patients are summarized in Table 1.

Autopsies of patients without lung injury or apparent lung disease: First, we screened 16 cases of sudden death in 2008-2012 due to acute myocardial infarction, aortic-aneurysm rupture, stroke, and other causes from the autopsy files of Tohoku University Hospital. We excluded seven cases that showed lung diseases. The remaining nine cases without apparent lung disease were selected for this study. The characterization and clinical diagnoses of the selected patients are summarized in Table 1.

Immunohistochemistry

The present study utilized a Histofine Kit (Nichirei, Tokyo, Japan), which employs the streptavidin-biotin amplification method. The characteristics of the antibodies used in this study are summarized in Table 2. The antigen-antibody complex was visualized using 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCL buffer [pH 7.6], and 0.006% H_2O_2), and sections were counterstained with hematoxylin.

We used immunoreactivity against phospho-mTOR (p-mTOR, Ser 2448) and phospho-eukaryotic initiation factor 4-binding protein 1 (p-4EBP1, Thr 70) in order to evaluate activity of the Akt/mTOR

Table 1. The summary of clinicopathological findings of the cases examined in the present study.

Case	age	sex	Cause of DAD (clinical diagnoses)	Pneumocyte		T cell	
				p-mTOR score	p-4EBP1 score	p-mTOR score	p-4EBP1 score
DAD-1	54	M	Bacterial bronchopneumonia	0	0	0	0
DAD-2	84	F	Lung cancer	0	0	0	0
DAD-3	76	M	Bacterial bronchopneumonia	0	0	0	0
DAD-4	62	M	Lung cancer	0	0	0	0
DAD-5	73	M	Lung abscess	0	0	0	0
DAD-6	60	M	Lung cancer	0	0	0	0
DAD-7	47	F	Bacterial bronchopneumonia	1	1	0	0
DAD-8	61	M	Unknown	0	0	0	0
DAD-9	57	M	Gastric cancer	0	0	0	0
DAD-10	76	M	Lung cancer	0	0	0	0
DAD-11	69	M	Malignant mesothelioma	0	0	0	0
DAD-12	58	M	Lung cancer	0	0	0	0
DAD-13	81	M	Colon cancer	1	1	0	0
DAD-14	89	M	Unidentified fever	0	1	0	0
DAD-15	71	M	Lung cancer	1	0	0	0
DAD-16	61	M	Acute myocardial infarction	0	0	0	0
DAD-17	75	M	Lung cancer	0	0	0	0
DAD-18	78	M	Gefitinib-induced lung injury	0	0	0	0
DAD-19	60	M	Gefitinib-induced lung injury	0	1	0	0
			Cause of death (clinical diagnoses)				
NP-1	70	M	Ruptured aortic aneurysm	2	1		
NP-2	64	F	Ruptured aortic aneurysm	2	2		
NP-3	48	F	Amyotrophic lateral sclerosis	0	1		
NP-4	87	M	Ruptured aortic aneurysm	2	2		
NP-5	70	M	Acute myocardial infarction	0	1		
NP-6	80	M	Subarachnoid hemorrhage	0	0		
NP-7	79	F	Hepatic failure	0	2		
NP-8	76	M	Amyotrophic lateral sclerosis	2	2		
NP-9	79	F	Cardiac perforation	2	2		

19 DAD cases and nine non-pathological lung cases. M, male; F, female; DAD, diffuse alveolar damage; NP, non-pathological; p-mTOR, phospho-mammalian target of rapamycin; p-4EBP1, phospho-eukaryotic initiation factor 4-binding protein 1.

Table 2. Antibodies and their conditions of immunostaining used in the present study.

primary antibody	dilution	source	antigen retrieval	positive control
CD3	1/500	DAKO (Glostrup, Denmark)	AC	Tonsil
CD4	1/30	Leica Biosystems (Newcastle, UK)	AC	Tonsil
CD8	1/50	DAKO (Glostrup, Denmark)	AC	Tonsil
Foxp3	1/100	Abcam (Cambridge, MA)	AC	Tonsil
p-mTOR (Ser 2448)	1/50	Cell Signaling Technology (Beverly, MA, USA)	MW	Breast carcinoma
p-4EBP1 (Thr 70)	1/50	Cell Signaling Technology (Beverly, MA, USA)	MW	Breast carcinoma

AC, autoclave treatment; MW, microwave treatment; p-mTOR, phospho-mammalian target of rapamycin; p-4EBP1, phospho-eukaryotic initiation factor 4-binding protein 1.

pathway. Immunopositive pneumocytes and T cells were classified into three groups according to the percentage of positive cells as follows: score 0 (negative), < 10%; score 1, 10-50%; score 2, ≥ 50%.

All cases of DAD, including one with mTORi treatment and 19 cases without, exhibited relatively homogenous lymphocyte infiltration into the alveolar walls. Next, we evaluated the immunoreactivity of lymphocytes in areas with the most numerous CD3-positive T cells and the fewest neutrophils in order to exclude the influence of lung infection. We counted the number of T cells positive for CD3, CD4, CD8, Foxp3, p-mTOR, and p-4EBP1 per 0.0625 mm^2 using a 100-point grid with a known area (0.0625 mm^2 at 400 × magnification) attached to the ocular of the microscope. In the case of mTORi-induced lung injury, we utilized double immunostaining with DAB for p-mTOR and Vector-blue for CD3 to evaluate further the p-mTOR immunopositivity in T cells. We also calculated the CD4/CD8 ratio.

Statistical analysis

Statistical analysis was performed using the StatView 5.0 J software (SAS Institute Inc., Cary, NC). The CD4/CD8 ratio was evaluated using ANOVA and the Bonferroni test. p-mTOR and p-4EBP1 immunoreactivity was evaluated using the Mann-Whitney *U* test.

Results

mTOR activity in pneumocytes

Results are summarized in Table 1 and Table 3. In the case of lung injury after mTORi treatment, pneumocytes were diffusely immunopositive for p-mTOR and p-4EBP1 (Fig. 3A, B). In almost all cases of DAD without mTORi treatment, pneumocytes were negative for p-mTOR and p-4EBP1 (Fig. 3C-E). Only three cases (DAD-7, 13, 15) exhibited focal immunopositivity for p-mTOR, and four cases (DAD-7, 13, 14, 19) exhibited focal immunopositivity for p-4EBP1. Among the nine non-pathological cases, five cases (NP-1, 2, 4, 8, 9) exhibited diffuse immunopositivity for p-mTOR in pneumocytes, while the pneumocytes were immunonegative in the other four cases. Eight cases (NP-1, 2, 3, 4, 5, 7, 8, 9) exhibited p-4EBP1 immunoreactivity, while one case (NP-6) was immunonegative. There were significant differences in p-mTOR immunopositivity between a case of lung injury with mTORi treatment and cases of DAD without mTORi treatment ($p = 0.0181$) and between cases of DAD without mTORi treatment and cases with non-pathological lungs ($p = 0.0101$). Finally, there were significant differences in p-4EBP1 immunopositivity between a case of lung injury with mTORi treatment and cases of DAD without mTORi treatment ($p = 0.0293$) and between cases of DAD without mTORi treatment and cases with non-pathological lungs ($p = 0.0002$).

mTOR activity in T cells of lungs with lung injury with mTORi treatment and DAD without mTORi treatment

T cells were identified by CD3 immunopositivity (Fig. 4A, D). In the case of lung injury with mTORi treatment, no T cells were immunonegative for p-mTOR (Fig. 4B), but many were positive for p-4EBP1, consistent with mTOR activation (Fig. 4C). On the other hand, none of the 19 cases of DAD without mTORi treatment exhibited T cells that were immunopositive for p-mTOR or p-4EBP1 (Table 1, Fig. 4E, F). Accordingly, the number of p-4EBP1 immunopositive T cells significantly differed between these two groups ($p < 0.0001$) (Table 3).

T-cell subpopulations in lungs with DAD

We examined the distribution of three types of T cells, including CD4-positive, CD8-positive, and Foxp3-positive T cells. In the case of lung injury with mTORi treatment and all 19 cases of DAD without mTORi treatment, there was a predominance of CD8-positive T cells (Fig. 5A, B, D, E) with a CD4/CD8 ratio of 0.47 ± 0.338 (mean ± standard deviation [S.D.]). In the case of lung injury with mTORi treatment and two cases of DAD without mTORi treatment (Case DAD-6 and -19), CD4-positive T cells were few. Foxp3-positive T cells were not detected in the case of lung injury with mTORi treatment or any of the 19 cases of DAD without mTORi treatment (Fig. 5C, F).

Discussion

In the present study, we have shown that the mTOR activity was significantly lower in the pneumocytes in cases of DAD without mTORi treatment than in those of cases with non-pathological lungs. In addition, the mTOR activities in both pneumocytes and T cells were significantly higher in the case of DAD with mTORi treatment than

Table 3. Summary of scoring of immunohistochemistry.

p-mTOR score in pneumocytes				
	score 0	score 1	score 2	*p* value
NP (*n* = 9)	4 (44.4%)	0 (0%)	5 (55.6%)	
DAD (*n* = 19)	16 (84.2%)	3 (15.8%)	0 (0%)	0.0101 (vs. NP)
Lung injury with mTORi treatment (*n* = 1)	0 (0%)	0 (0%)	1 (100%)	0.0181 (vs. DAD)
p-4EBP1 score in pneumocytes				
	score 0	score 1	score 2	*p* value
NP (*n* = 9)	1 (11.1%)	3 (33.3%)	5 (55.6%)	
DAD (*n* = 19)	15 (79.0%)	4 (21.0%)	0 (0%)	0.0002 (vs. NP)
Lung injury with mTORi treatment (*n* = 1)	0 (0%)	0 (0%)	1 (100%)	0.0293 (vs. DAD)
p-mTOR score in T cells				
	score 0	score 1	score 2	*p* value
DAD (*n* = 19)	19 (100%)	0 (0%)	0 (0%)	not significant
Lung injury with mTORi treatment (*n* = 1)	1 (100%)	0 (0%)	0 (0%)	
p-4EBP1 score in T cells				
	score 0	score 1	score 2	*p* value
DAD (*n* = 19)	19 (100%)	0 (0%)	0 (0%)	< 0.0001
Lung injury with mTORi treatment (*n* = 1)	0 (0%)	0 (0%)	1 (100%)	

NP, non-pathological lung; DAD, diffuse alveolar damage without mTORi treatment; mTORi, mammalian target of rapamycin inhibitor; p-mTOR, phospho-mammalian target of rapamycin; p-4EBP1, phospho-eukaryotic initiation factor 4-binding protein 1.

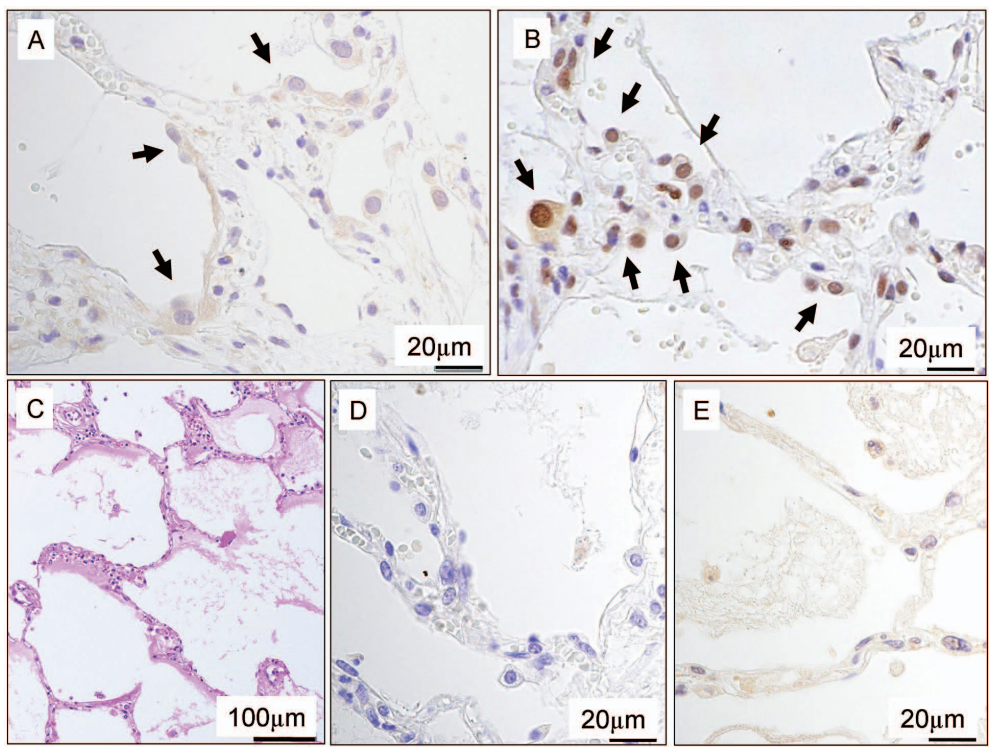

Fig. 3. Mammalian target of rapamycin (mTOR) activity in pneumocytes.
In the case of lung injury with mTOR inhibitor (mTORi) treatment, pneumocytes were diffusely immunopositive for (A) phospho-mTOR (p-mTOR) and (B) phospho-eukaryotic initiation factor 4-binding protein 1 (p-4EBP1) (arrow). (C) Hematoxylin and eosin staining of diffuse alveolar damage (DAD) (Case DAD-10). In case of DAD, pneumocytes were immunonegative for (D) p-mTOR and (E) p-4EBP1.

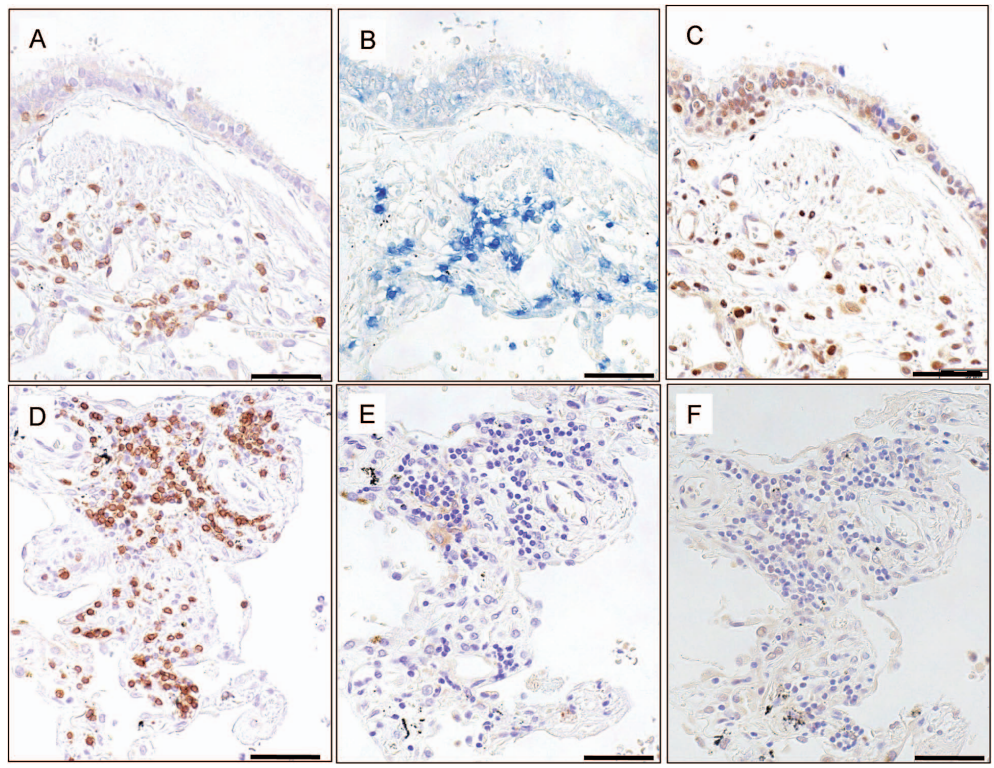

Fig. 4. Mammalian target of rapamycin (mTOR) activity in T cells.
(A, B, C): Case of lung injury with mTOR inhibitor (mTORi) treatment. (D, E, F): Case of diffuse alveolar damage (DAD) without mTORi treatment (Case DAD-13).
(A) CD3-immunopositive T cells infiltrating the alveoli and around the bronchi. (B) T cells were immunonegative for phospho-mTOR (p-mTOR) in double-immunostaining with 3,3′-diaminobenzidine for p-mTOR and Vector-blue for CD3. (C) Numerous T cells were immunopositive for phospho-eukaryotic initiation factor 4-binding protein 1 (p-4EBP1). (D) CD3-immunopositive T cells infiltrating the alveoli. The DAD case exhibited T cells that were negative for (E) p-mTOR or (F) p-4EBP1. Likewise, none of the remaining 18 cases of DAD exhibited T cells immunopositive for p-mTOR or p-4EBP1. Scale bar, 50 μm.

those in cases of DAD without mTORi treatment. Thus, the attenuated or enhanced mTOR signaling in pneumocytes and T cells may contribute to the pathogenesis of DAD.

To the best of our knowledge, there are no previous reports on mTOR activity in fatal lung diseases or the mechanisms of mTOR-induced lung injury. Therefore, the present study assessed mTOR activity in pneumocytes and T cells in cases of DAD with or without mTORi treatment. We employed antibodies against mTOR phosphorylated at Ser 2448 and 4EBP1 phosphorylated at Thr 70. mTOR possesses two adjacent phosphorylation sites for Akt (Thr 2446 and Ser 2448). Ser 2448 is phosphorylated by Akt both *in vitro* and *in vivo* (Sekulic et al. 2000; Reynolds et al. 2002; Hay and Sonenberg 2004). In addition, mTOR directly phosphorylates Thr 37 and Thr 46 and subsequently phosphorylates Thr 70 and Ser 65 on 4EBP1 (Hay and Sonenberg 2004). Therefore, the phosphorylation of mTOR at Ser 2448 and 4EBP1 at Thr 70 indicates the activation of the mTOR pathway. Nevertheless, future studies should assess the relationship between other phosphorylated sites and mTOR and 4EBP1 activities.

The cases with non-pathological lungs displayed different patterns of p-mTOR and p-4EBP1 immunoreactivity in the pneumocytes. This variation between the non-pathological lungs may reflect differences in known regulators of the mTOR pathway, such as amino acids, oxygen, growth factors, and cell stress (Laplante and Sabatini 2012); however, the correlation between these factors and mTOR activity in non-pathological lungs remains unknown. Interestingly, the pneumocytes in cases of DAD without mTORi treatment displayed significantly less mTOR activity than those in non-pathological lungs. Several studies have reported that mTOR promotes cell growth by regulating various processes, including anabolism, apoptosis, and cell cycle, through the mTOR complex 1 (mTORC1) and mTORC2 pathways. Accordingly, these studies have shown that inhibiting mTOR activity reduces cell growth (Inoki et al. 2003, 2006; Laplante and Sabatini 2012). Results from the present study suggest that low mTOR activity may be associated with pneumocyte damage in cases of DAD without mTORi treatment.

In addition, results from the present study demonstrated significantly higher mTOR activity in both pneumocytes and T cells in the case of lung injury with mTORi treatment than in cases of DAD without mTORi treatment.

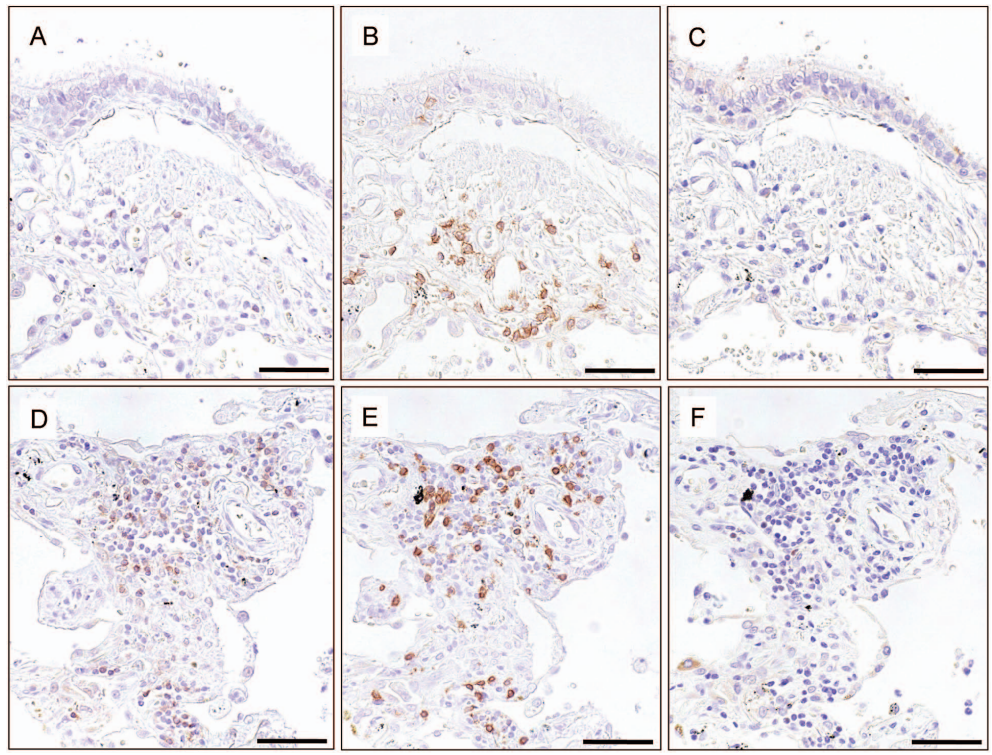

Fig. 5. T-cell subpopulations in the lungs.
(A, B, C): Case of lung injury with mammalian target of rapamycin inhibitor (mTORi) treatment. (D, E, F): Case of diffuse alveolar damage (DAD) without mTORi treatment (Case DAD-13).
Lung injury with mTORi treatment exhibited (A) scant CD4-positive T cells, (B) numerous CD8-positive T cells, and (C) no Foxp3-positive T cells. The DAD case exhibited (D) CD4-immunopositive T cells, (E) CD8-immunopositive T cells, and (F) no Foxp3-positive T cells. Scale bar, 50 μm.

This suggested that mTORi exerted an effect on pneumocytes and T cells during lung injury. The relative elevation in mTOR activity despite mTORi treatment could be due to the following factors. First, the patient died 119 days after initiation of mTORi treatment and 67 days after its discontinuation upon clinical diagnosis of mTORi-induced lung injury. Therefore, the suppressive effects of mTORi on the mTOR pathway had likely resolved by the time of autopsy. Second, mTORi treatment could have resulted in feedback regulation of the mTOR pathway that was more pronounced at the time of the patient's death. There is no evidence that mTOR inhibition changes the number of mTOR phosphorylation sites; nevertheless, previous studies have reported feedback mechanisms in mTOR signaling (Watanabe et al. 2011). Specifically, mTOR-stimulated ribosomal protein S6 kinase (S6K) inhibits insulin receptor substrate (IRS) through multiple phosphorylation sites on IRS and phosphorylated Rictor, resulting in an inhibition of mTOR signaling. Moreover, mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt/mTOR pathway (O'Reilly et al. 2006; Wan et al. 2007). However, the mechanisms of mTOR inhibition or activation during mTORi-induced pneumocyte injury remain unknown.

Another possibility is that infiltrating lymphocytes mediate lung damage. Therefore, in the present study, we evaluated subpopulations of T cells in the lungs of patients with fatal lung injury. In cases of DAD without mTORi treatment and lung injury with mTORi treatment, CD8-positive T lymphocytes were predominant, and there were no apparent regulatory T cells (Tregs) positive for Foxp3. In non-pathological lungs, there were too few lymphocytes infiltrating the alveolar walls to be evaluated. However, there is a correlation between the CD4/CD8 ratio in lung tissue and that in the bronchoalveolar lavage fluid (BALF) (Yamadori et al. 2000). In a previous report of healthy never-smokers, the CD4/CD8 ratio in the BALF was 2.80 ± 1.68 (Nagai et al. 1992). In the present study, the CD4/CD8 ratio in cases of lung injury with mTORi treatment and DAD without mTORi treatment was 0.47 ± 0.338. Therefore, the CD4/CD8 ratio in lung injury with or without mTORi treatment was exceedingly low. Araki et al. (2009) reported differentiation of CD8-positive memory T cells resulting from mTORi. The results of that study are consistent with our hypothesis that in DAD, low mTOR activity is correlated with a predominance of CD8-positive T cells. Additionally, it has been reported that mTORi upregulates Tregs (Thomson et al. 2009). However, the effects of the mTOR pathway on Tregs *in vivo* remain unknown. Therefore, future studies must investigate the correlation between mTOR activity and T-cell subpopulations.

It is important to assess the activity and function of

mTOR in the lungs. Results from the present study suggest that mTOR activation participates in mTORi-induced lung injury. In addition, the activated mTOR pathway has been considered as a biomarker of mTORi sensitivity (Nishi et al. 2013). However, the present study only assessed one case of lung injury with mTORi treatment at one time point. Therefore, the present results should be validated using animal models, cell culture, flow cytometry, and other methods.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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