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Original Article

Implications of thyroid transcription factor-1 gene methylation in carcinogenesis of interstitial pneumonia-related non-terminal respiratory unit lung adenocarcinoma

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Abstract: The present study aimed to elucidate the mechanisms underlying the histogenesis of interstitial pneumonia (IP)-related lung adenocarcinoma (LADC). We focused on the methylation of thyroid transcription factor 1 (*TTF-1*). The *TTF-1* locus was highly methylated in IP-LADCs compared to non-IP-LADCs. Among the IP-LADCs, the non-terminal respiratory unit (TRU) LADCs showed marked hypermethylation in CpG sites in a particular intragenic region. This region was also found to be highly methylated in the IP lungs. The hierarchical dendrogram based on methylation levels divided the IP lungs into three different clusters. One of them showed a methylation profile similar to that of non-TRU LADCs. The non-TRU LADCs developed from this cluster with a significantly higher frequency. Moreover, bronchiolar metaplasia lining honeycomb/cystic lesions in IP lungs, IP-related non-TRU LADCs, and bronchiolar epithelia in healthy lungs were separately collected by microdissection and examined for methylation. Bronchiolar metaplasia showed hypermethylation, but bronchiolar epithelia did not. The methylation patterns in bronchiolar metaplasia were similar to those in non-TRU LADCs. In summary, a particular region of *TTF-1* was highly methylated in IP-related non-TRU LADCs and bronchiolar metaplasia, supporting the theory that IP-related non-TRU LADCs may develop from bronchiolar metaplasia lining honeycomb/cystic lesions.

Keywords: Non-TRU lung adenocarcinoma, bronchiolar metaplasia, *TTF-1* methylation, interstitial pneumonia

Introduction

Lung cancer is the leading cause of cancer-related death in developed countries [1]. Various factors play a role in the development of lung cancer [2, 3]. Diffuse interstitial lung disease or interstitial pneumonia (IP) is one of the factors proven to increase the risk of lung cancer [4]. The incidence of lung cancer in patients with IP is reportedly 4-15% [3, 5, 6].

IP is a rare and incurable disease. The disease prevalence is 3.4 per million people in Japan [3, 7]. IP affects the alveolar septa, causing air spaces to collapse, and causes tissue remodeling, progressing to honeycomb lesions [8]. Our recent study demonstrated that IP-related lung cancer develops at a significantly higher frequency in honeycomb lesions or alveolar cystic lesions lined with bronchiolar metaplasia (honeycomb/cystic lesions) [9].

Among lung cancers, adenocarcinoma (LADC) is the most frequent histologic subtype [10]. Large variations have been reported in the histologic appearance of LADC [10]. These variations may result from differences in cancer progenitor cells and/or the wide spectrum of molecular genetic alterations. In the recent classification, LADC is divided into two groups: the

terminal respiratory unit (TRU) type and the non-TRU type, based on their morphologic features and immunohistochemical profiles [10-12]. TRU LADC consists of club-shaped neoplastic cells that are immunohistochemically positive for thyroid transcription factor-1 (TTF-1), and it accounts for up to 90% of LADCs [10, 11]. Non-TRU LADC shows an appearance similar to bronchiolar surface epithelial cells (ciliated columnar epithelial cells) and is immunohistochemically negative for TTF-1 [10, 12]. A recent study demonstrated that *TTF-1* gene hypermethylation frequently occurs in non-TRU LADC, suggesting that methylation could play an essential role in TTF-1 inactivation and histogenesis of this type of LADC [13]. Most IP-related LADCs that develop in honeycomb/cystic lesions are of the non-TRU type [9], and they are often associated with bronchiolar metaplasia lining honeycomb/cystic lesions [9, 14, 15].

All of these findings prompted us to examine *TTF-1* gene methylation in IP-related LADCs and IP lungs (non-tumor parts) and analyze the relationship between them to elucidate the potential molecular mechanism of IP-related LADC development.

Materials and methods

Patients and lung tissues

A total of 123 patients with IP who underwent surgical lung biopsy and 35 patients without IP who underwent a surgical operation (lower lobe lobectomy) for lung cancer (31 TRU LADCs, 1 non-TRU LADC, 2 squamous cell carcinomas, and 1 large cell carcinoma) at the Kanagawa Prefectural Cardiovascular and Respiratory Center (Yokohama, Japan) between 2011 and 2018 were included. Among the patients with IP, 13 patients who had lung cancer (3 TRU LADC, 6 non-TRU LADC, 2 squamous cell carcinoma, and 2 large cell carcinoma) underwent surgical operations (piratical resection). Samples from the lower lobes of the lungs were subjected to the analyses. Three to five millimeter square tissue fragments were snap-frozen for bisulfite conversion-based DNA sequencing. In addition, 10 mm square tissue sections were embedded in OCT compound and snap-frozen for laser-capture microdissection, followed by bisulfite conversion-based DNA sequencing. Informed consent for research use of the resected materials was obtained from all subjects. The ethics committees of Yokohama City University (A180726009/A180726008, 2018.7.26) and Kanagawa Prefectural Cardiovascular and Respiratory Center (KCRC-17-0015/KCRC-17-0016, 2017.10.4) approved this research plan.

Conventional histopathologic examination

Lung tissues were fixed with buffered 10% formaldehyde solution for approximately 24 h and embedded in paraffin. Sections were cut from tissues and stained with hematoxylin and eosin (H&E), Elastica Van Gieson (EVG), and Alcian Blue Periodic Acid Schiff (ALB-PAS). We classified the IPs according to the ATS/ERS/JRS/ALAT 2018 criteria [8] through multidisciplinary discussions on pathologic, clinical, and radiologic findings, involving three pathologists, two radiologists, and three pulmonologists. The cases examined here (123 in total) included 25 idiopathic pulmonary fibrosis, 25 non-specific IP, 2 cryptogenic organizing pneumonia, 2 desquamative IP, 4 respiratory bronchiolitis-interstitial lung disease, 2 pleuroparenchymal fibroelastosis, 20 chronic hypersensitivity pneumonia, 16 collagen-vascular disease-associated interstitial lung disease, and 27 unclassifiable lesions.

Immunohistochemistry

Tissue sections were incubated with a blocking solution to block endogenous peroxidases and non-immune-specific protein binding. They were then boiled in antigen retrieval buffer. Then, the sections were incubated with the primary antibody against TTF-1 (mouse monoclonal antibody clone 8G7G3/1, DAKO, Ely, UK), followed by incubation with a horseradish peroxidase-labeled anti-mouse immunoglobulin antibody. Immunoreactivity was visualized using diaminobenzidine as a substrate, and the nuclei were lightly counterstained with hematoxylin.

Laser-capture microdissection

Bronchial epithelial cells, metaplastic cells, and LADC cells were separately collected using a

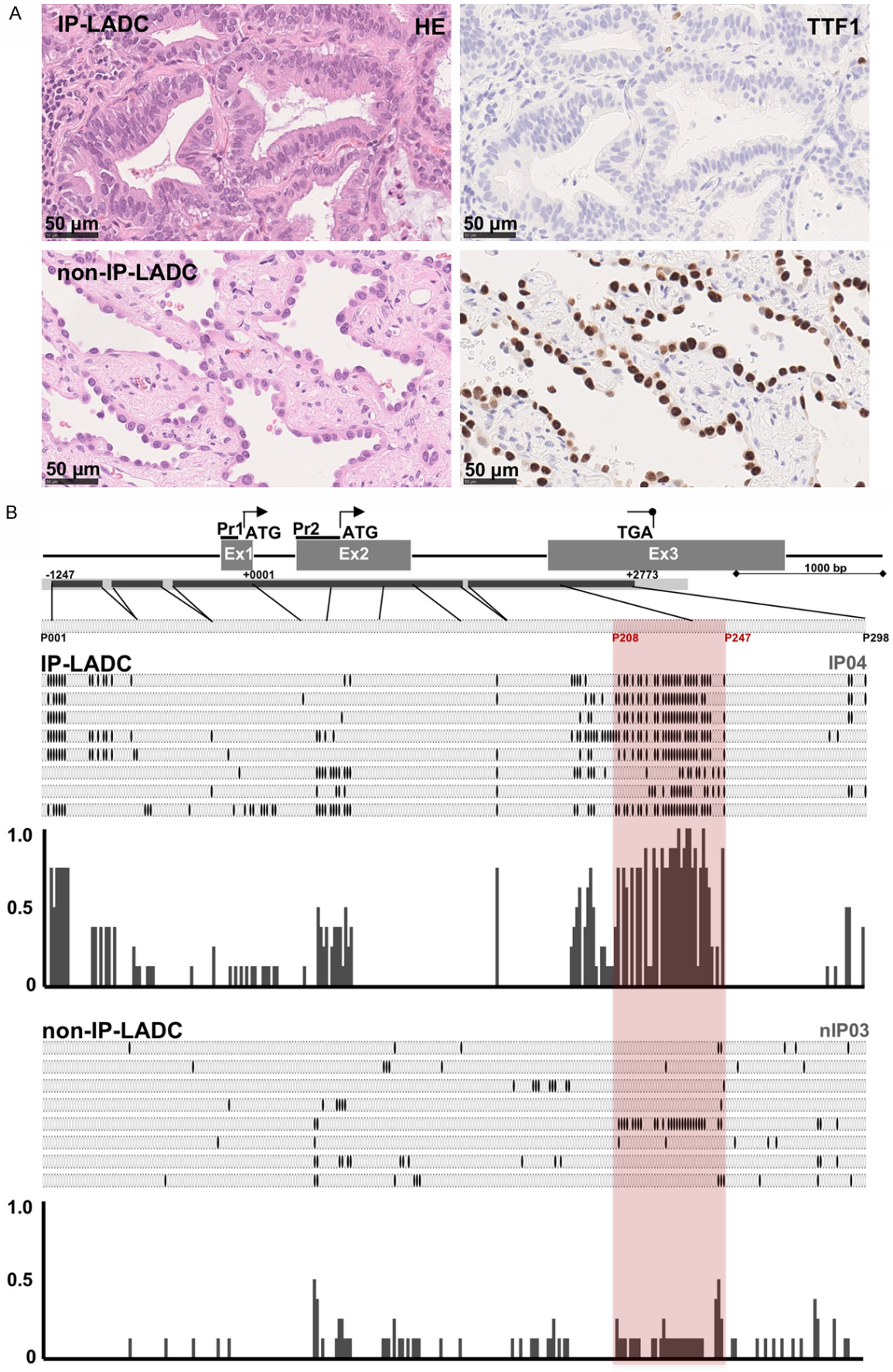
A
IP-LADC
HE
50 μm
TTF1
50 μm
non-IP-LADC
50 μm
50 μm
B
Pr1
ATG
Pr2
ATG
TGA
Ex1
Ex2
Ex3
-1247
+0001
+2773
1000 bp
P001
P208
P247
P298
IP-LADC
IP04
1.0
0.5
0
non-IP-LADC
nIP03
1.0
0.5
0

Figure 1. Representative histologic appearance, immunohistochemical expression of TTF-1, and *TTF-1* methylation (result from bisulfite sequence) in IP-related non-TRU LADC and IP-unrelated (non-IP) TRU-LADC. Representative IP-LADC shows non-TRU features: tall columnar, slightly mucin-producing (A: upper left panel, hematoxylin-eosin stain), and immunohistochemically negative for TTF-1 (A: upper right panel). Non-IP-LADC shows TRU features: club-shaped, hobnail (A: lower left panel, hematoxylin-eosin stain), and immunohistochemically positive for TTF-1 (A: lower right panel). The structure map of the *TTF-1* is shown (B: Ex, exon; Pr, promoter; the first codon (ATG) of exon 1 is defined as position +0001). CpG sites in a particular region (P208-P247) were found to be highly methylated in the IP-LADC in comparison to the non-IP LADC (B: second and fourth panels: back mark, methylated; white mark, unmethylated), and the methylation levels were significantly higher in the IP-LADCs than in the non-IP-LADCs (B: third and fifth panels: X-axis, CpG position; Y-axis, methylation level).

laser capture microdissection system (PALM-LMC system, Carl Zeiss, Jena, Germany).

Bisulfite conversion-based DNA sequencing

Methylation status in the TTF1 promoter region was analyzed according to a previously described method [13]. Briefly, genomic DNA was subjected to bisulfite conversion using the EpiTect Bisulfite kit (Qiagen, Valencia, CA). Almost the entire region of the *TTF-1* gene, including the promoter, was amplified using 10 primer sets described elsewhere [13], and the PCR products were subcloned into plasmids (pT7blue, Novagen, Darmstadt, Germany). Eight to ten clones were examined for methylation status of 298 CpG sites by Sanger DNA sequencing using the Big-dye terminator kit (version 3.1; Applied Bioscience, Foster City, CA). The methylation level was determined as the ratio of methylated clones to the total clones examined at each CpG site. The average methylation level was determined as the mean methylation level at all CpG sites in each case.

Statistical analyses

Differences in methylation levels between the groups were analyzed using the Wilcoxon/Kruskal-Wallis test. Dendrograms showing hierarchical clustering based on methylation levels were described using Ward's method. Relationships between the clusters and clinicopathological subjects were analyzed using the Chi-square test. Statistical significance was set at $P < 0.05$.

Results

Differences in TTF-1 methylation status and histologic subtypes and between IP-related LADC (IP-LADC) and IP-unrelated LADC (non-IP-LADC)

In our series of IP-LADCs, non-TRU LADC was more common, and *KRAS* mutations were consistently more frequent (Supplementary Table 1). Representative IP-LADC showed non-TRU features that were tall columnar, mucin-producing, and immunohistochemically negative for TTF-1 (**Figure 1A**). CpG sites in the *TTF-1* gene were highly methylated in the IP-LADCs compared to those in the non-IP-LADCs (**Figure 1B**), and the methylation levels were significantly higher in the IP-LADCs than in the non-IP-LADCs (**Figure 2A**). The difference in methylation levels between the IP-LADCs and non-IP-LADCs was remarkable in a particular region (P208-P247) (**Figure 2B** and **2C**). The dendrogram based on the methylation levels demonstrated that the IP-LADCs and the non-IP-LADCs could be differentially categorized into two clusters (cluster 1 and cluster 2, respectively) (**Figure 2D**). Interestingly, the LADCs belonging to cluster 1 were of the non-TRU type. These results suggest that hypermethylation at a particular region of the *TTF-1* gene could be distinctive of IP-LADC, especially non-TRU LADC, and it could be involved in their histogenesis.

Differences in TTF-1 methylation status between IP and non-IP lungs

We next examined the methylation status of IP lungs and non-IP healthy lungs to confirm this theory. Observation of *TTF-1* gene hypermethylation in IP lungs may help us to explain why LADC, particularly non-TRU type LADC, frequently developed in IP lungs. Representative results from bisulfite sequencing of the P208-P247 region (which was highly methylated in IP-related non-TRU LADC) in IP lungs and non-IP healthy lungs are shown in **Figure 3**. CpG sites in the region were highly methylated in the IP lungs than in the non-IP lungs (**Figure 3**), and the methylation levels were significantly higher in the IP lungs (**Figure 4A** and Supplementary Table 2). Differences were observed at almost all CpG sites in this region (**Figure 4B**).

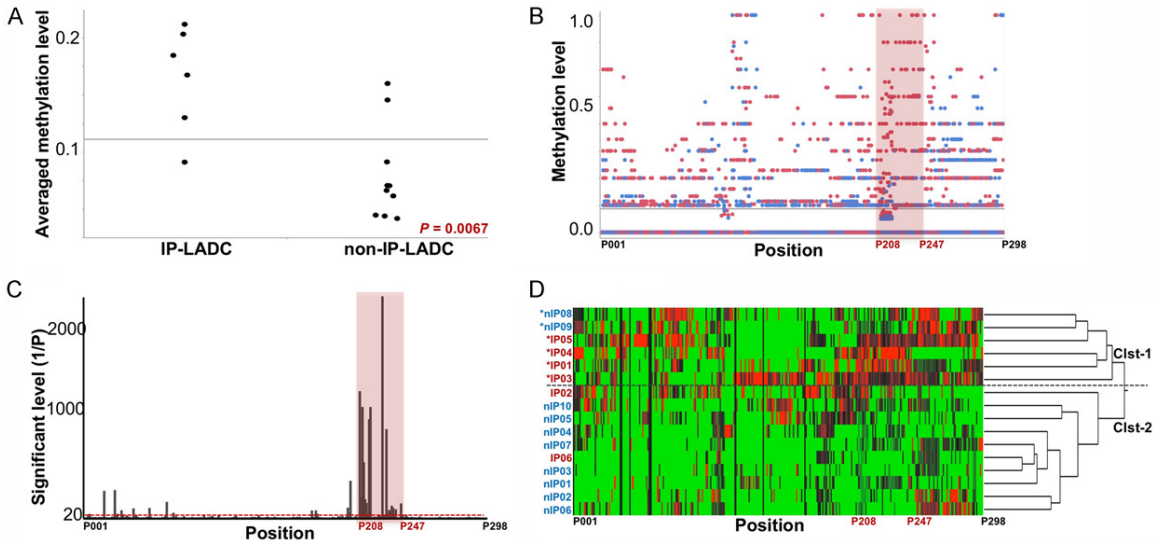

Figure 2. Differences in TTF-1 methylation status between IP-related LADC (IP-LADC) and IP-unrelated LADC (non-IP-LADC). Six IP-LADCs and ten non-IP LADCs were examined. The average methylation levels are found to be significantly higher in the IP-LADCs than the non-IP-LADCs (P = 0.0067, Wilcoxon/Kruskal-Wallis test) (A). A particular region (P208-P247) is more highly methylated in the IP-LADCs the pink-shadowed area in (B) (red circle, IP-LADC; blue circle, non-IP-LADC). The differences in methylation level between the IP-LADCs and non-IP-LADCs (reciprocals of *P*-value calculated by Wilcoxon/Kruskal-Wallis test at the CpG sites each) are remarkable in the particular region in the pink-shadowed area in (C). The dendrogram based on the methylation levels is described by Ward's method, where the IP-LADCs and the non-IP-LADCs can be differentially categorized into the cluster 1 (Clst-1) and cluster 2 (Clst-2), respectively (D: IP, IP-LADC; nIP, non-IP-LADC; * marks non-TRU LADC).

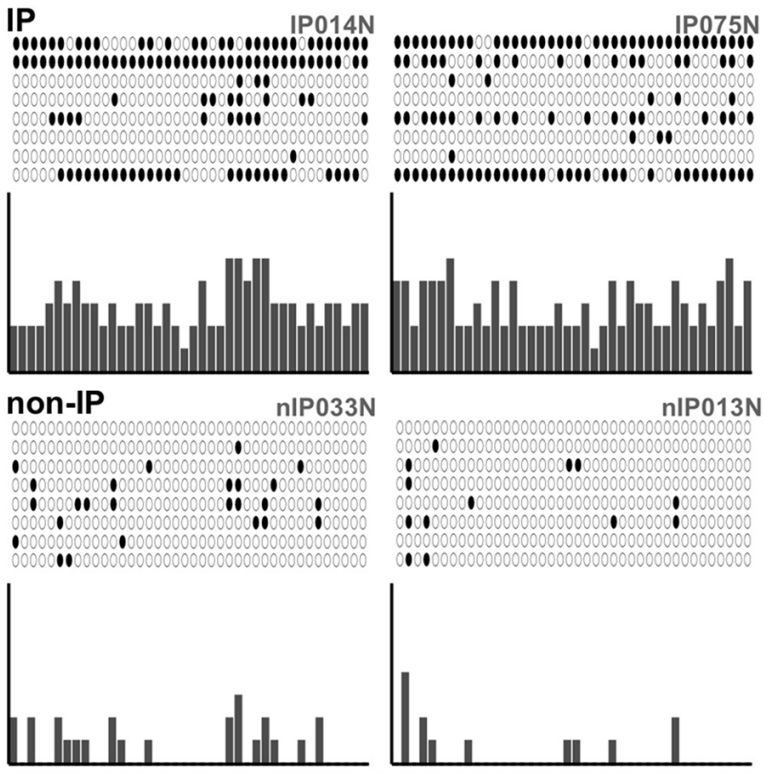

Figure 3. Representative results from bisulfite sequencing for the region P208-P247 (which was highly methylated in the IP-related non-TRU LADCs) in IP-lung and non-IP healthy lung (almost healthy non-tumor parts from surgically resected lungs of lung cancer patients). CpG site maps (back mark, methylated; white mark, unmethylated) and bar graphs (X-axis, CpG position; Y-axis, methylation level) are shown. The region is found to be highly methylated in the IP lung (upper panels) compared to the non-IP lungs (lower panels).

Some specific sites (P209-P217; P230-P240) were highly methylated in IP lungs (**Figure 4B** and **4C**). The dendrogram based on the methylation levels demonstrated that the IP lungs could be categorized into three different clusters (clusters A, B, and C, respectively), while all non-IP lungs were categorized in cluster C (**Figure 4C**). Cluster A was a randomly methylated group, where CpG sites were extensively but not highly methylated (**Figure 4C** and **Table 1**). Cluster B was a regionally methylated group,

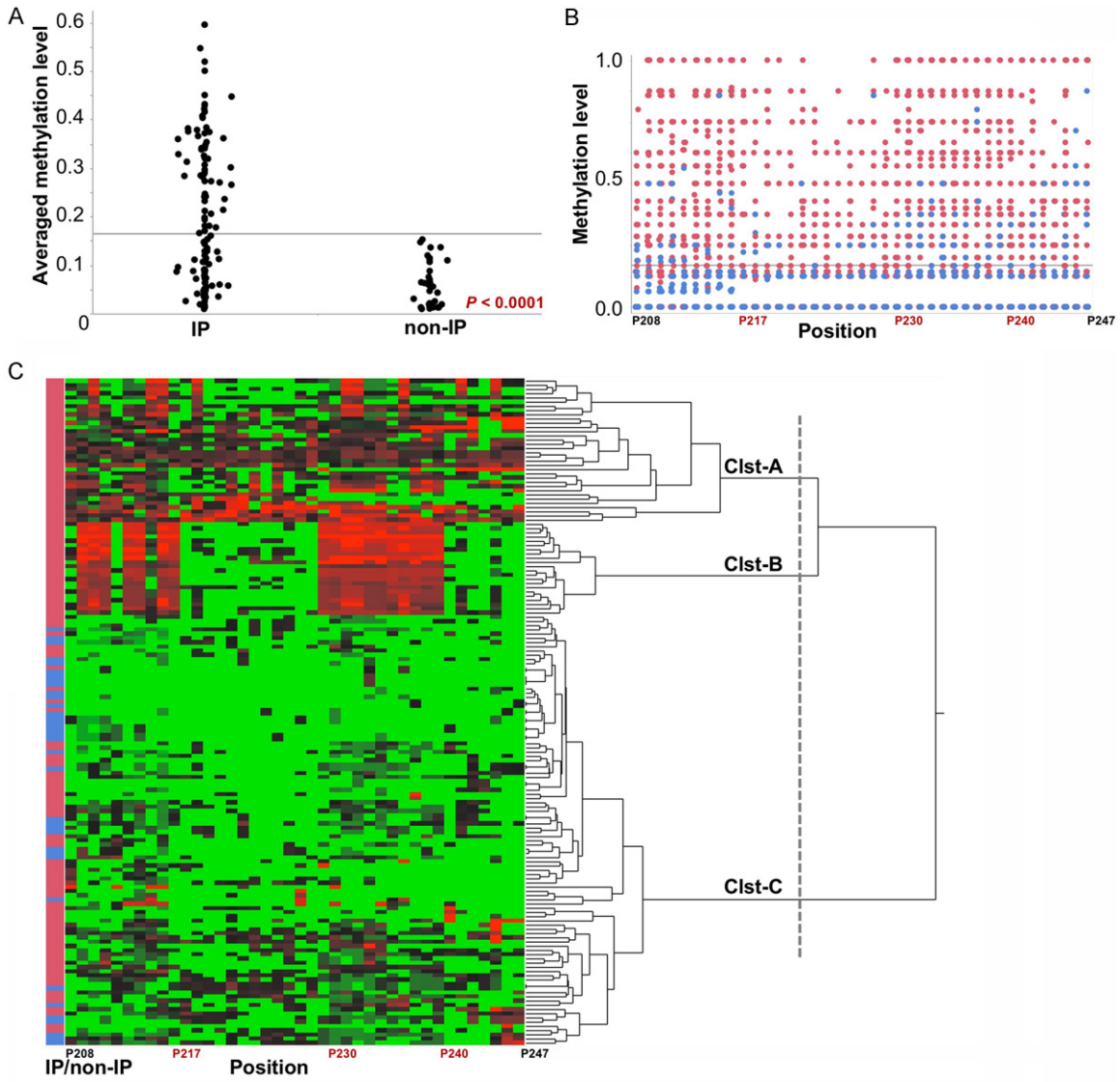

Figure 4. Differences in the methylation status of the susceptible region (P208-P247) between IP-lung and non-IP healthy lung (almost healthy non-tumor parts from surgically resected lungs of lung cancer patients). The averaged methylation levels are found to be significantly higher in the IP lungs than the non-IP lungs ($P < 0.0001$, Wilcoxon/Kruskal-Wallis test) (A). The differences are observed at almost all CpG sites in the region (B) (red circle, IP-LADC; blue circle, non-IP-LADC). Some sites (P209-P217; P230-P240) appear to be particularly highly methylated in the IP lungs. The dendrogram based on the methylation levels is described by Ward's method (C) (1/P represents reciprocals of *P*-values by Wilcoxon/Kruskal-Wallis test for differences between methylation levels at the CpG site each), where the IP lungs (and the non-IP lungs) can be categorized into the three different clusters (Clst-A, Clst-B, and Clst-C) (C: the extra color bar indicates IP (red) and non-IP (blue)).

where CpG sites were focally but highly methylated (**Figure 4C** and **Table 1**). Cluster C was a weakly methylated group, where the methylation levels were generally not very high (**Figure 4C** and **Table 1**).

Relationship between TTF-1 methylation profiles and lung cancer occurence

Among the clusters, lung cancers most frequently developed in cluster A, where all five LADCs were of the non-TRU type (**Table 1**). Representative histologic appearances from IP lungs belonging to cluster A, where non-TRU LADCs developed, are shown in **Figure 5**. One TRU LADC, one non-TRU LADC, and one squamous cell carcinoma developed in cluster B (**Table 1**). Three TRU LADCs developed in cluster C (**Table 1**). There were no significant differences in gender, age, smoking, and IP subtypes between the clusters (although there were sig-

Table 1. Clinicopathologic features of the three clusters described based on TTF-1 methylation levels in IP and non-IP lungs

	Cluster A (34)	Cluster B (22)	Cluster C (102)	
Disease status				P < 0.0001
IP	100.0 (34)	100.0 (22)	65.7 (67)	
non-IP	0 (0)	0 (0)	34.3 (35)	
TTF-1 methylation level				P < 0.0001
High (> 0.1)	97.1 (33)	100.0 (22)	8.8 (9)	
Low (≤ 0.1)	2.9 (1)	0 (0)	91.2 (93)	
Median (range)	0.29 (0.09-0.59)	0.35 (0.28-0.45)	0.06 (0.01-0.21)	
Gender				P = 0.2625
Male	29.4 (10)	50.0 (11)	42.2 (43)	
Female	70.6 (24)	50.0 (11)	57.8 (59)	
Age				P = 0.3494
Elder (> 70)	41.2 (14)	27.3 (6)	28.4 (29)	
Younger (≤ 70)	58.8 (20)	72.7 (16)	71.6 (73)	
Median (range)	68 (54-78)	67 (42-78)	67 (45-81)	
Smoking				P = 0.2484
Heavy (PYI > 100)	29.4 (10)	36.4 (8)	45.1 (46)	
Non/Light (PYI ≤ 100)	70.6 (24)	63.6 (14)	54.9 (56)	
Subtype*				P = 0.6753
IPF	20.6 (7)	13.6 (3)	22.4 (15)	
non-IPF	79.4 (27)	86.4 (19)	77.6 (52)	
Lung cancer concurrence*				P = 0.0064
TRU LADC	0 (0)	4.5 (1)	3.0 (2)	
non-TRU LADC	14.7 (5)	4.5 (1)	0 (0)	
Others	8.8 (3)	4.5 (1)	0 (0)	
None	76.5 (26)	86.5 (19)	97.0 (65)	

IP, interstitial pneumonia; IPF, idiopathic pulmonary fibrosis; TTF-1 methylation, average methylation frequencies from 40 CpG sites between positions 208 and 247; PYI, pack-year index. *P* values were calculated using the Chi-square test. *Correlations were analyzed restrictedly in patients with IP.

nificant differences in disease status (IP versus non-IP) and TTF-1 methylation level) (**Table 1**).

Comparison of TTF-1 methylation status between healthy bronchioles/alveoli, bronchiolar metaplasia lining honeycomb/cystic lesions, and IP-related non-TRU LADCs

Our results demonstrated that some populations of IP and non-TRU LADC lungs were sequential in *TTF-1* methylation profile and suggested that the methylation could be related to the histogenesis of non-TRU LADC in IP lungs. Recent studies have proposed that bronchiolar metaplasia could be a precursor for non-TRU LADC [9, 14, 15]. Thus, different levels of airway epithelia from healthy lungs, bronchiolar metaplasia lining honeycomb/cystic lesions, and IP-related non-TRU LADCs were separately collected by microdissection and examined for methylation of the susceptible region (P208-P247). Representative results from the two IP lungs, where non-TRU LADCs developed, are shown in **Figure 6**. In both cases, the regions in bronchiolar metaplasia were highly methylated compared to the alveoli/bronchioles from non-IP lungs (**Figure 6**). Bronchiolar metaplasia appeared between the alveoli/bronchioles and non-TRU LADCs with respect to methylation levels (**Figure 6**).

Discussion

We herein demonstrated that a particular region of *TTF-1* was highly methylated in IP-related LADCs, especially in non-TRU LADCs and in

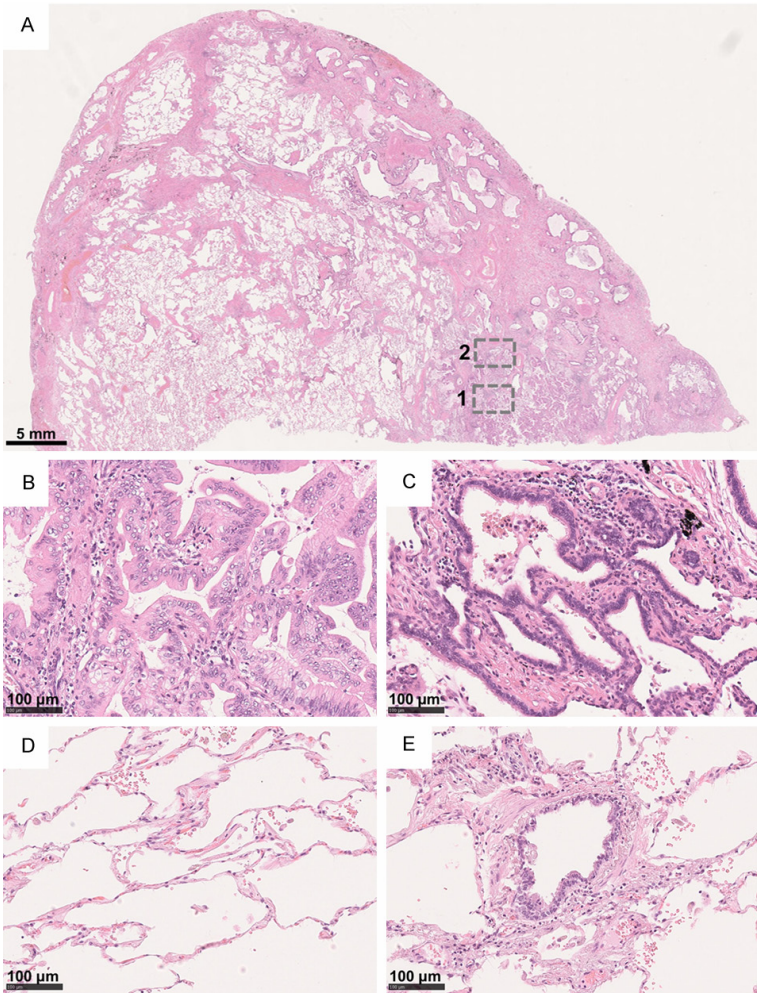

Figure 5. Histologic appearances of a representative case of IP lung belonging to cluster A that developed non-TRU LADC. A scanning view of a hematoxylin-eosin section shows diffuse fibrous thickening of alveolar septa with lymphoid cell infiltration, alveolar collapses along with interlobular septa, consequent traction bronchiolar ectasia, and occasional microscopic honeycomb/alveolar cystic changes, suggesting that this could be a lesion indeterminate for usual interstitial pneumonia according to the ATS/ERS/JRS/ALAT 2018 pathological criteria (A). A closer view of square 1 in panel A shows LADCs cells with a kind of non-TRU features are growing along thickened (partially broken) alveolar septa (B). A closer view of square 2 in panel A shows bronchiolar metaplasia is spreading along thickened alveolar septa (C). Representative histological appearances of bronchioles (D) and alveoli (E) from a non-IP healthy lung (almost healthy non-tumor parts from a surgically resected lung of a lung cancer patient) are shown as a reference (negative control).

bronchiolar metaplasia of IP lungs. The results supported the theory that non-TRU LADC may develop from bronchiolar metaplasia lining honeycomb/cystic lesions in IP lungs.

A previous study showed hypermethylation in a particular region of the *TTF-1* gene was related to the loss of TTF-1 expression in non-TRU LADCs [13]. Our results were similar to those of a previous study on the methylation levels and sites [13]. Interestingly, the highly methylated sites were in the intragenic region of *TTF-1* (P208-P247), which was not the promoter. We believe that this unique pattern could provide clues to search for precursor lesions of non-TRU LADCs. Therefore, we focused on this region and examined IP lungs for the methylation status of CpG sites. Although the levels and patterns of methylation varied among the cases, some of the IP lungs showed diffuse hypermethylation in this region, which were similar to non-TRU LADCs in the methylation pattern.

Previous studies have suggested that bronchiolar metaplasia lining honeycomb/cystic lesions could be a precursor for IP-related non-TRU LADCs [14, 15]. To further support this theory, metaplastic lesions were microscopically dissected and examined for *TTF-1* methylation. As shown in the representative results, some metaplastic epithelia showed methylation patterns similar to those of the non-TRU LADCs. However, bronchiolar epithelia (and alveoli) in healthy tissues did not show such hypermethylation patterns. Nevertheless, not all metaplastic lesions showed hypermethylation patterns. We examined 15 metaplastic lesions from three IP lungs; only two lesions showed hypermethylation patterns (data not shown). These results suggest that metaplastic epithelia could be accidentally affected by *TTF-1* methylation during chronic damage-regeneration processes in IP lungs, and methylation might not be a necessary event to generate bronchiolar metaplasia. Our previous study demonstrated that HNF4α-expressing cells

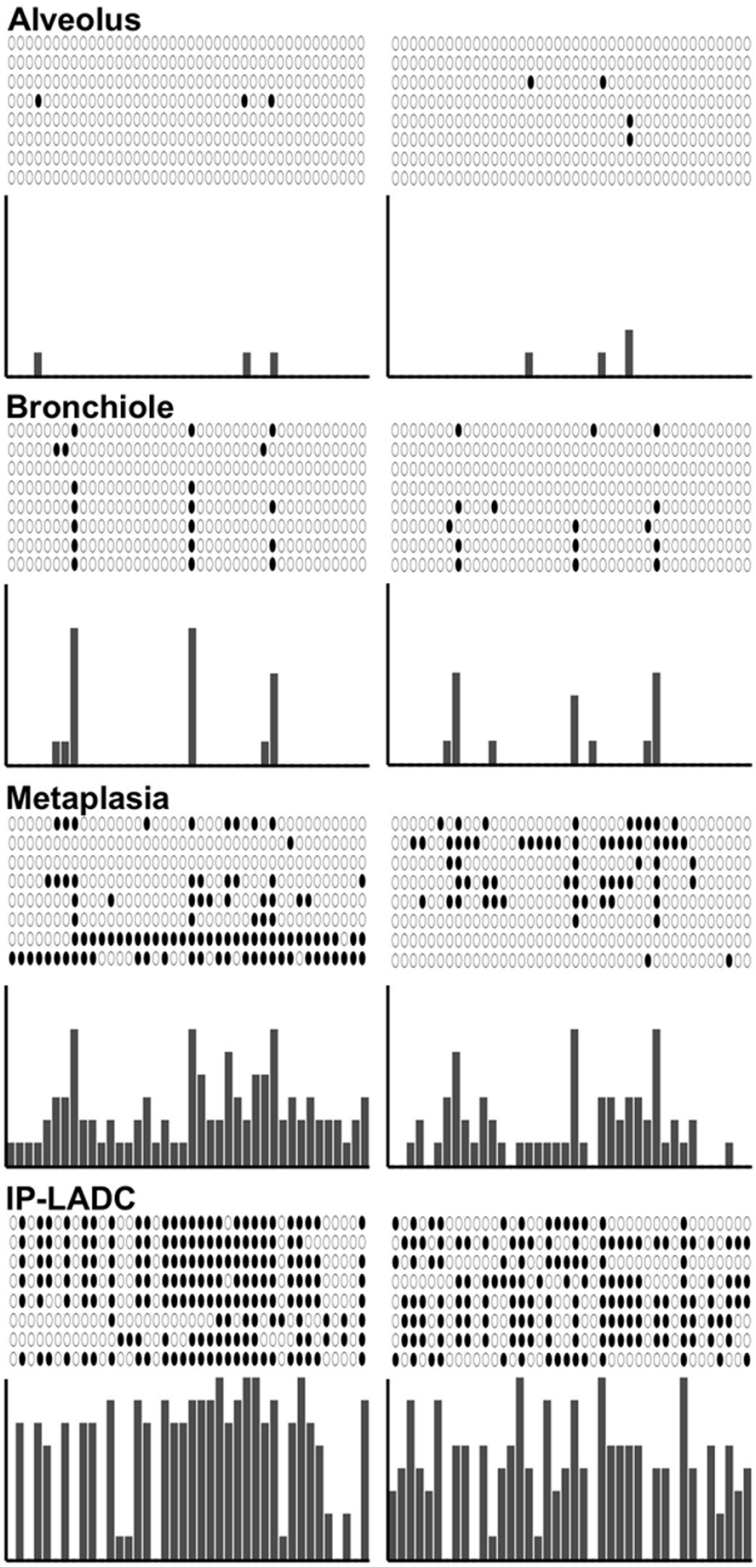

Figure 6. Representative results from bisulfite sequencing for the susceptible region (P208-P247) in alveoli, bronchiolar epithelia, metaplastic epithelia, and IP-related non-TRU LADCs. Different levels of airway cells from healthy lungs (almost healthy non-tumor parts from surgically resected lungs of lung cancer patients), bronchiolar metaplasia lining honeycomb/cystic lesions, and IP-related non-TRU LADCs were separately collected by microdissection and examined for the methylation status. CpG site maps (back mark, methylated; white mark, unmethylated) and bar graphs (X-axis, CpG position; Y-axis, methylation level) are shown. The region was found to be highly methylated in the IP-LADCs and metaplasia, but not so in the alveoli and bronchioles.

were found in bronchiolar metaplasia (where the proportion and levels of HNF4α expression varied among metaplastic lesions) [14, 15]. HNF4α, a key molecule for the gastric or enteric phenotype in non-TRU LADCs, is negatively regulated by TTF-1 [12, 16]; therefore, TTF-1 downregulation due to hypermethylation can lead to increased HNF4α expression. Thus, our results partially uncovered a mechanism by which HNF4α-expressing metaplastic cells, potential precursors for non-TRU LADC, appear in bronchiolar metaplasia. TTF-1-hypermethylation may participate in this process. It is also possible that secondary interstitial lesions could be susceptible fields for non-TRU LADC, in addition to the WHO-consensus idiopathic IPs., The absence of a significant relationship between IP subtypes and TTF-1 methylation levels may support this hypothesis.

Metaplasia is a precancerous condition in certain types of cancers. For example, gastric adenocarcinoma develops from gastric intestinal metaplasia, and squamous cell carcinoma develops from squamous cell metaplasia in different organs, such as the bronchus, esophagus, and uterine cervix [17]. In recent years, metaplasia has been considered a pathologic condition that can result from cer-

tain molecular alterations rather than merely an anomaly in heterotopic tissue regeneration [18, 19]. Our results were consistent with this notion, as some bronchiolar metaplasia exhibited *TTF-1* hypermethylation with patterns similar to those of non-TRU LADCs, suggesting that the methylation could be pathognomonic. It is of great interest to investigate bronchiolar metaplasia with such hypermethylation to elucidate the histogenesis of IP-related non-TRU LADC in detail. Comprehensive analyses of infrequent somatic mutations by deep-sequencing methods using next-generation sequencers [20] and comparisons of mutation profiles between metaplastic lesions and non-TRU LADCs are expected to confirm their sequential association further. These will help discover possible new carcinogenic stimuli.

We inevitably became interested in uncovering the factors related to *TTF-1* hypermethylation. However, we failed to find any relationship between the clusters based on methylation status and clinicopathological factors examined here, such as IP-subtypes, smoking history, sex, and gender. Thus, the underlying mechanisms of hypermethylation in IP lungs may not be simple, and inflammatory stimuli (various pathogens and allergens), regenerative cellular replication, exposure to particular carcinogens (smoking and certain air pollutants), and other unknown factors may be cooperatively involved.

In conclusion, this is the first study to demonstrate the similarity in *TTF-1* methylation profiles between bronchiolar metaplasia and IP-related non-TRU LADC and support their sequential association.

Acknowledgements

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Disclosure of conflict of interest

None.

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Supplementary Table 1. TTF-1 methylation status and clinicopathological features in the IP LADC and non-IP LADC

	IP LADC (6)	non-IP LADC (10)	
TTF-1 methylation level			P = 0.0134
High (> 0.1)	83.2 (5)	20.0 (2)	
Low (≤ 0.1)	16.7 (1)	80.0 (8)	
Median (range)	0.16 (0.09-0.21)	0.07 (0.04-0.16)	
Gender			P = 0.0736
Male	100.0 (6)	60.0 (6)	
Female	0 (0)	40.0 (4)	
Age			
Elder (> 70)	33.3 (2)	80.0 (8)	P = 0.0619
Younger (≤ 70)	66.7 (4)	20.0 (2)	
Median (range)	68 (57-70)	72 (64-79)	
Smoking			P = 0.3296
Heavy (PYI > 100)	83.2 (5)	60.0 (6)	
Non/Light (PYI ≤ 100)	16.7 (1)	40.0 (4)	
Subtype			P = 0.0134
TRU	16.7 (1)	80.0 (8)	
non-TRU	82.3 (5)	20.0 (2)	
Driver mutation type			P = 0.0239
EGFR	0 (0)	60.0 (6)	
KRAS	66.7 (4)	10.0 (1)	
None	33.3 (2)	30.0 (3)	

IP, interstitial pneumonia; LADC, lung adenocarcinoma; TTF-1 methylation, averaged methylation frequierncies are shown; PYI, pack year index; TRU, terminal respiratory unit; *P* values, calculated with the Chi square test.

Supplementary Table 2. TTF-1 methylation status and clinical features in the IP and non-IP lung

	IP (123)	non-IP (35)	
TTF-1 methylation level			P < 0.0001
High (> 0.1)	53.0 (64)	20.0 (0)	
Low (≤ 0.1)	48.0 (59)	100.0 (35)	
Median (range)	0.16 (0.01-0.59)	0.05 (0.01-0.15)	
Gender			P = 0.0598
Male	35.6 (78)	54.3 (19)	
Female	63.4 (45)	45.7 (16)	
Age			
Elder (> 70)	23.6 (29)	57.1 (20)	P = 0.0002
Younger (≤ 70)	76.4 (94)	42.9 (15)	
Median (range)	69 (42-78)	70 (51-81)	
Smoking			P = 0.0078
Heavy (PYI > 100)	35.0 (43)	60.0 (21)	
Non/Light (PYI ≤ 100)	65.0 (80)	40.0 (14)	

IP, interstitial pneumonia; TTF-1 methylation, averaged methylation frequierncies from 40 CpG sites between the poitions 208 to 247; PYI, pack year index. *P* values are calculated with the Chi square test.