Relationship between non-TRU lung adenocarcinomas and bronchiolar metaplasia - potential implication in their histogenesis -

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Summary. Lung adenocarcinomas (ADCs) have been roughly divided into two groups: the terminal respiratory unit (TRU) type and non-TRU type. These ADCs appear to develop through exclusive carcinogenetic pathways because of differences in their cellular morphologies and the profiles of protein expression and genetic alterations. The TRU type develops from atypical adenomatous hyperplasia as a precursor. On the other hand, the histogenesis of the non-TRU type has not yet been defined in detail. We herein investigated histopathological changes in the non-tumor lung tissues of patients with non-TRU-type ADCs in order to define their potential histogenesis. The non-TRU type preferentially occurs in patients with interstitial pneumonia, in whom tumors are located in honeycomb lesions and are associated with bronchiolar metaplasia (BM). Among patients without interstitial pneumonia, non-tumor lung tissues from non-TRU-type ADCs were often affected by multiple BM. In these cases, tumors often were associated with BM. Metaplastic cells adjacent to non-TRU-type ADCs ectopically expressed HNF-4α, a marker for non-TRU-type ADCs. These results suggest that the non-TRU type develops through distinct histogenesis, in which BM is implicated.

Key words: Non-TRU-type lung adenocarcinomas, Bronchiolar surface epithelial subtype, Bronchiolar metaplasia, Histogenesis

Introduction

Large variations have been reported in the histological appearance of lung adenocarcinomas (ADCs). These variations may be produced by differences in cancer progenitor cells and/or the wide spectrum of molecular genetic alterations. Recent studies have proposed that lung ADCs are roughly divided into two groups: the terminal respiratory unit (TRU) type and another (non-TRU type), based on their morphological features and immunohistochemical profiles (Yatabe et al., 2002; Sugano et al., 2013; Travis et al., 2015). The TRU type consists of club-shaped neoplastic cells resembling Clara cells (Club cells) (Winkelmann and Noack, 2010) and/or type 2 alveolar cells and is immunohistochemically positive for thyroid transcription factor-1 (TTF-1) (Rehm and Kelloff, 1991; Yatabe et al., 2002; Kunii et al., 2011). The TRU type accounts for up to 90% of lung ADCs and its clinicopathological characteristics have been defined in detail (Yatabe et al., 2005; Travis et al., 2015), whereas those of the non-TRU type currently remain unclear. Recent studies proposed that the non-TRU type is a subset of ADC that originates from bronchiolar surface epithelial cells (ciliated columnar epithelial cells) (Park et al., 2012; Sumiyoshi et al., 2014; Kim et al., 2016).

Kimula et al. originally named this group the bronchial surface epithelial (BSE) type (Kimula, 1978). The non-TRU/BSE type is immunohistochemically negative for TTF-1 and positive for Hepatocyte nuclear factor 4 alpha (HNF-4α) (Yatabe et al., 2002; Kunii et al., 2011; Sugano et al., 2013).

We recently reported that non-TRU/BSE-type ADCs developed at a significantly higher frequency in lungs affected by idiopathic interstitial pneumonia and were often associated with bronchiolar metaplasia (BM) in honeycomb lesions (Kojima et al., 2016), which is supported by other studies (Kunii et al., 2011; Masai et al., 2016). These findings prompted us to hypothesize that BM is a precancerous condition for non-TRU/BSE-type ADCs.

We herein investigated the relationship between non-TRU/BSE-type ADCs and BM in patients with and without interstitial pneumonia in order to verify this hypothesis.

Materials and methods

Patients

Subjects comprised 319 lung ADC cases that underwent surgery between 1998 and 2013. We intentionally collected as many non-TRU ADCs as possible (74 cases). We also randomly collected control cases including 212 TRU-type ADCs and 33 unclassifiable-type ADCs. Among these cases, 98 had interstitial pneumonia (80 idiopathic pulmonary fibrosis, 10 idiopathic non-specific interstitial pneumonia (NSIP), 5 collagen vascular disease-related NSIP, and 3 others), while 221 did not. The Ethics Committees of Kanagawa Prefectural Cardiovascular and Respiratory Center and Yokohama City University approved the research plan.

Histopathological examination

Hematoxylin and eosin (HE)-stained sections of lung ADCs and background non-tumor lung tissues were reviewed. ADCs were classified based on their cytological features into the TRU type, non-TRU/BSE type, and other unclassifiable type. The TRU type consists of low columnar/cuboidal neoplastic cells that display similar features to club cells and/or type 2 alveolar epithelial cells (Yatabe et al., 2005; Travis et al., 2015). The non-TRU/BSE type has been defined as tumors consisting of tall columnar epithelial cells that exhibit similar features to bronchial surface epithelial cells (Kimula, 1978). The other unclassifiable type includes poorly differentiated tumors with cytological features that are difficult to define. These tumors occasionally express some cellular differentiation markers (Sumiyoshi et al., 2014), TTF-1, HNF-4α, or MUC5AC and MUC6 (data not shown). However, since the expression of these markers was only focal or heterogenous, it was not sufficient to classify these poorly differentiated tumors into the TRU or non-

TRU/BSE type. Thus, these poorly differentiated tumors were unclassifiable in the present study.

The other ADCs were subtyped according to the World Health Organization Classification system (Travis et al., 2013).

The extent of BM in non-tumor tissues was roughly divided into diffuse and focal patterns. The diffuse pattern was defined as metaplastic lesions that appeared to extend with bridging between multiple acini, which was only found in patients with interstitial pneumonia. The focal pattern was detected in patients without interstitial pneumonia. We graded focal patterns into "frequent" and "occasional". Three to five low power fields (magnification of 12.5, objective lens (power 1.25) × eyepiece (power 10)) were scanned. An average frequency of more than or equal to 2 lesions of BM was defined as "frequent", while that less than 2 was defined as "occasional".

Immunohistochemistry

Tissue sections with the largest tumor areas were cut from formalin-fixed, paraffin-embedded tissue blocks. These sections were incubated with primary antibodies for TTF-1 (SPT24; Nichirei, Tokyo, Japan), HNF-4α (Perseus Proteomics Inc., Tokyo, Japan), anaplastic lymphoma kinase (ALK) (5A4; Santa Cruz Biotechnology, Inc., Tokyo, Japan), or nuclear protein p40 (BC28, Nichirei, Tokyo, Japan) (Bishop et al., 2012). Immunoreactivity was visualized using the Envision detection system (DAKO), and nuclei were counterstained with hematoxylin. According to previously described criteria, positivity for HNF-4α or TTF-1 expression was defined when >25% of tumor cell nuclei were stained (Kunii et al., 2011).

Confirmation ALK gene rearrangement

ALK gene rearrangement was exclusively examined for tumors immunohistochemically positive for ALK protein expression by fluorescent in situ hybridization, as described elsewhere (Kimura et al., 2012).

Search for EGFR and KRAS mutations

Tumor tissue was macroscopically (or, if necessary, microscopically) dissected from fresh frozen tissue or formalin-fixed paraffin-embedded tissue sections. DNA was extracted from tissue sections using a DNeasy blood & tissue kit (QIAGEN, Valencia, CA) or NucleoSpin tissue DNA extraction kit (Takara-MACHEREY-NAGEL, Kyoto, Japan). Fragments of the Kirsten rat sarcoma (KRAS) oncogene (exon 2) and epidermal growth factor receptor (EGFR) oncogene (exons 18, 19, 20, and 21) were amplified using a polymerase chain reaction (PCR). The resultant products were purified and subjected to a DNA sequencing analysis using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). The specific primers used for this

experiment have been described elsewhere (Kojima et al., 2016; Matsumura et al., 2016).

Statistical analysis

Pearson's χ^2 test was used to analyze categorical variables. All analyses were performed using JMP 9.0.2 (SAS Institute, Cary, NC, USA).

Results

Histological appearances and immunohistochemical profiles of the TRU type, non-TRU/BSE type, and other unclassifiable type

Representative photographs showing histological appearances and TTF-1/HNF-4α immunohistochemical expression in the different types of ADCs are presented (Fig. 1). Neoplastic cells in TRU-type ADCs had a club-like shape that displayed similar features to epithelial cells in respiratory bronchioles or alveoli, which expressed TTF-1, but not HNF-4α (Fig. 1, Table 1). On the other hand, neoplastic cells in non-TRU/BSE-type ADCs showed the tall columnar shape that displayed similar features to bronchial surface epithelial cells, and generally expressed HNF-4α, but not TTF-1 (Fig. 1, Table 1). Other unclassifiable ADCs were poorly differentiated. The expression patterns of TTF-1 and HNF-4α varied; both were occasionally expressed or lost (Fig. 1, Table 1).

Pathological features of non-TRU/BSE-type ADCs

Most non-TRU/BSE-type ADCs showed the mucinous or acinar pattern, which often had KRAS mutations (Table 1). They preferentially occurred in males, smokers, and patients with interstitial pneumonia (Table 2). In contrast, most TRU-type ADCs showed the lepidic pattern, had EGFR mutations (Table 1), and preferentially occurred in females, non-smokers, and patients without any specific lung disease (Table 2).

Relationship between non-TRU/BSE-type ADCs and BM in patients with interstitial pneumonia

Non-TRU/BSE-type ADCs developed predominantly in the lower lobes (Table 2) and correlated with the diffuse BM of honeycomb lesions (Fig. 2, Table 3) in patients with interstitial pneumonia. This result confirmed our previous finding (Kojima et al., 2016) and implicated BM in the development of non-TRU/BSE-type ADCs.

Relationship between non-TRU/BSE-type ADCs and BM in patients without interstitial pneumonia

BM also occurred at varying frequencies in patients without any specific lung disease. In order to investigate the relationship between non-TRU/BSE-type ADCs and BM in patients without interstitial pneumonia, the frequency of BM was evaluated in the non-tumor lung tissues of lung ADC patients without interstitial pneumonia (a representative photograph of lung affected by multiple BM is shown in Fig. 3). The frequency of BM in non-tumor lung tissues was significantly greater in patients with non-TRU/BSE-type ADCs than in those

Table 1. Pathological characteristics in different cytological subtypes.

	TRU (212)	Non-TRU/BSE (74)	Other (33)	P-value
Histological subtype				<0.0001
Lepidic	53.3% (113)	5.4% (4)	0% (0)	
Acinar	29.3% (62)	23.0% (17)	24.2% (8)	
Solid	2.8% (6)	0% (0)	75.8% (25)	
Papillary	10.8% (23)	0% (0)	0% (0)	
Micropapillary	3.8% (8)	0% (0)	0% (0)	
Mucinous	0% (0)	71.6% (53)	0% (0)	
Differentiation marker				<0.0001
TTF1/HNF4α +/-	92.5% (196)	0% (0)	63.6% (21)	
+/+	7.5% (16)	14.9% (11)	9.2% (3)	
-/-	0% (0)	0% (0)	24.2% (8)	
-/+	0% (0)	85.1% (63)	3.0% (1)	
Oncogenic mutations				<0.0001
EGFR	53.8% (114)	2.7% (2)	15.1% (5)	
KRAS	5.2% (11)	40.5% (30)	9.1% (3)	
ALK	1.4% (3)	0% (0)	6.1% (2)	
None	39.6% (84)	56.8% (42)	69.7% (23)	

TRU, terminal respiratory unit; BSE, bronchial surface epithelium; +, positive; -, negative. P-values are calculated with the chi-squared test.

Table 2. Clinical characteristics in different cytological subtypes.

	TRU (212)	Non-TRU/BSE (74)	Other (33)	P-value
Age				0.4279
<65	37.7% (80)	32.4% (24)	45.5% (15)	
≥65	62.3% (132)	67.6% (50)	54.5% (18)	
Mean (range)	67.4 (38-84)	67.8 (43-84)	66.2 (29-87)	
Gender				0.0055
Male	59.9% (127)	71.6% (53)	84.9% (28)	
Female	40.1% (85)	28.4% (21)	15.1% (5)	
Smoking history				0.0054
Light/Non	46.2% (98)	31.1% (23)	18.2% (6)	
Heavy	53.8% (114)	68.9% (51)	81.8% (27)	
PYI (Mean ± SD)	509±667	589±560	855±619	
Location (lobe)				<0.0001
Upper/Middle	63.7% (135)	24.3% (18)	36.4% (12)	
Lower	36.3% (77)	75.7% (56)	63.6% (21)	
Lung disease				<0.0001
IP	19.3% (41)	39.2% (29)	57.6% (19)	
COPD	5.2% (11)	5.4% (4)	0% (0)	
Mixed	1.9% (4)	5.4% (4)	3.0% (1)	
None	73.6% (156)	50.0% (37)	39.4% (13)	

TRU, terminal respiratory unit; BSE, bronchial surface epithelium; PYI, pack year index; SD, standard deviation; IP, interstitial pneumonia; COPD, chronic obstructive lung disease; Mixed, mixed lesion of COPD and IP. P-values are calculated with the chi-squared test.

with TRU-type ADCs (Table 4). The direct association between tumors and BM was further investigated among patients whose non-tumor lung tissue had BM. Tumors with diameters less than 20 mm were exclusively examined because it was difficult to detect this relationship in larger tumors. Non-TRU/BSE-type ADCs were frequently associated with BM (Fig. 4), while TRU-type ADCs were not (Table 5).

Histopathological and immunohistochemical characterization of BM associated with non-TRU/BSE-type ADCs

BM is characterized by the migration of mucous and ciliated/non-ciliated columnar epithelial cells to alveolar septa and generally consists of a mixture of the two. Metaplastic epithelia showed a bi-layered architecture

Table 3. Direct relationship between tumors and honeycomb lesions in patients with interstitial pneumonia.

	TRU (45)	Non-TRU/BSE (33)	Other (20)	P-value
Associated	22.2% (10)	90.9% (30)	40.0% (8)	<0.0001
Not associated	77.8% (35)	9.1% (3)	60.0% (12)	

TRU, terminal respiratory unit; BSE, bronchial surface epithelium. P-values are calculated with the chi-squared test.
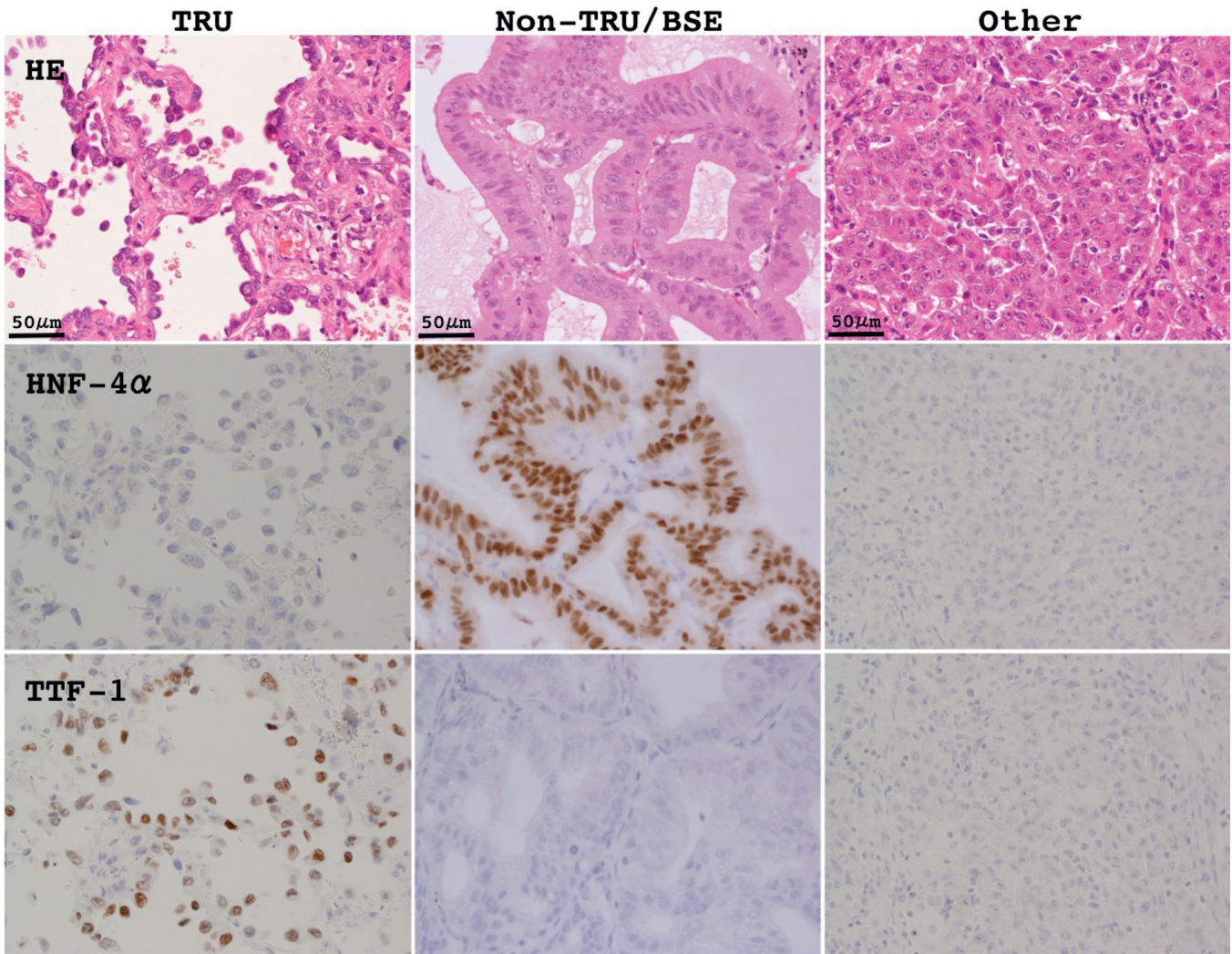

Fig. 1. Representative photographs of TRU-type, non-TRU/BSE-type, and other unclassifiable-type adenocarcinomas showing histological appearances and HNF-4α and TTF-1 expression: In a TRU-type tumor, club-shaped tumor cells replace alveolar cells (left upper panel, hematoxylin and eosin (HE) stain), which are immunohistochemically negative for HNF-4α (right middle panel) and positive for TTF-1 (right lower panel). In a non-TRU/BSE-type tumor, tall columnar neoplastic cells without cilia extend along alveolar septa (center upper panel, HE stain), which are immunohistochemically positive for HNF-4α (center middle panel) and negative for TTF-1 (center lower panel). In another unclassifiable tumor, polygonal neoplastic cells grow forming irregular acini or solid nests (right upper panel, HE stain), which are immunohistochemically negative for HNF-4α (right middle panel) and TTF-1 (right lower panel).
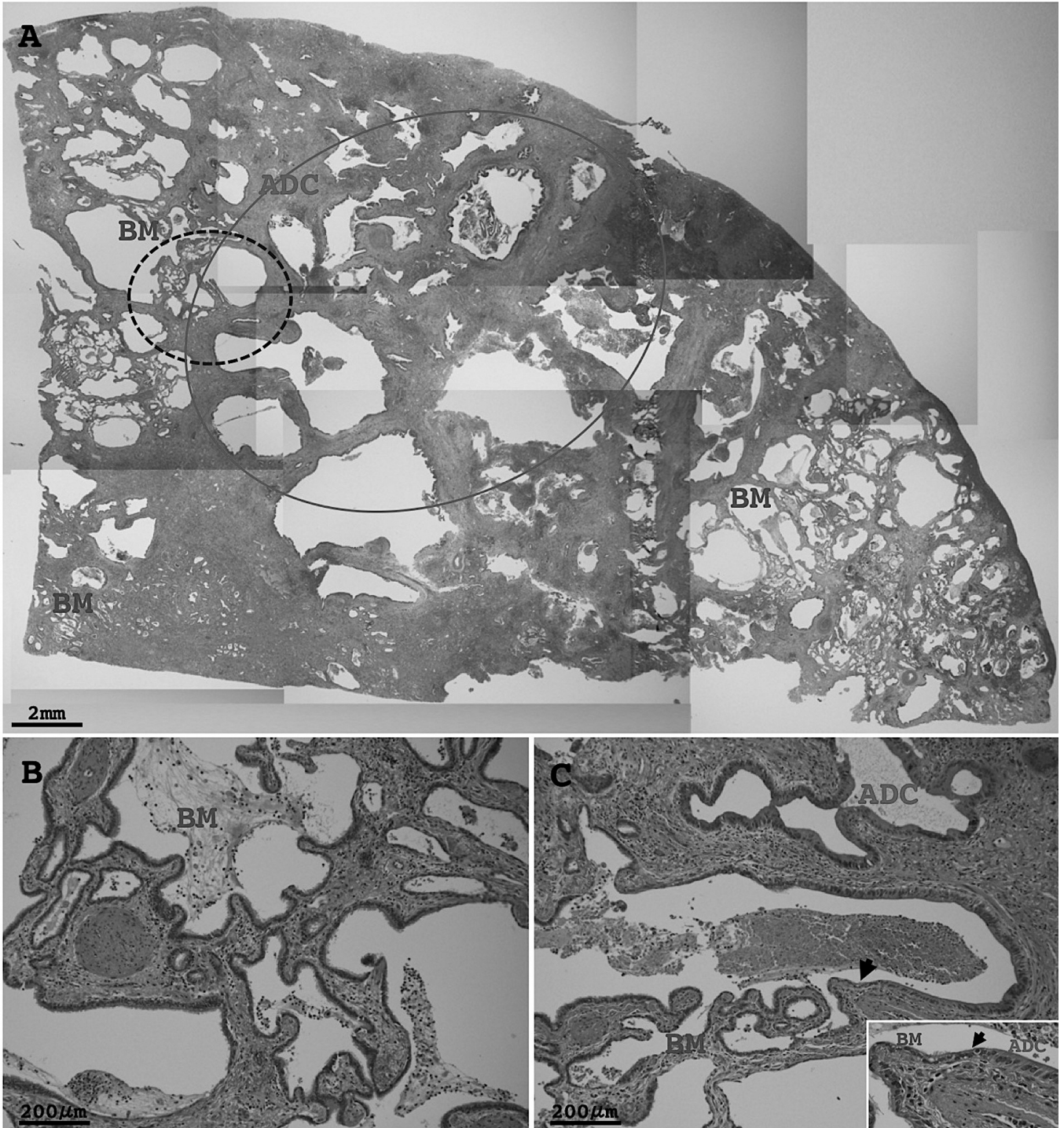
Fig. 2. A case of non-TRU/BSE-type adenocarcinoma occurring in interstitial pneumonia (honeycomb lesion). **A.** A scanning view (hematoxylin and eosin stain) is shown. Bronchiolar metaplasia (BM) expands throughout this section. The area of adenocarcinoma (ADC) is roughly circled (solid line circle). ADC associates with BM in the area circled by a dotted line (dotted line circle). **B.** A representative photograph showing BM surrounding ADC. **C.** A close view of a boundary between ADC and BM. An arrow indicates the front of neoplastic epithelia. An inset shows a close-up image of the area around the front (arrow).

Table 4. Frequency of bronchiolar metaplasia in non-tumor lungs among patients without interstitial pneumonia.

	TRU (167)	Non-TRU/BSE (41)	Other (13)	P-value
Frequent	4.2% (7)	26.8% (11)	7.7% (1)	0.0004
Occasional	19.8% (33)	26.8% (11)	15.4% (2)	
None	76.0% (127)	46.4% (19)	76.9% (10)	

TRU, terminal respiratory unit; BSE, bronchial surface epithelium. Three to five low power fields (magnification of x12.5) of non-tumor lungs were scanned. An average frequency of more than or equal to 2 lesions of bronchiolar metaplasia was defined as "frequent", while that less than 2 was defined as "occasional", and no bronchiolar metaplasia as None. P-values are calculated by the chi-squared test.

Table 5. Direct relationship between tumors and bronchiolar metaplasia among patients without interstitial pneumonia.

	TRU (14)	Non-TRU/BSE (6)	Other (2)	P-value
Associated	0.0% (0)	66.7% (4)	0.0% (0)	0.0013
Not associated	100.0% (14)	33.3% (2)	100.0% (2)	

TRU, terminal respiratory unit; BSE, bronchial surface epithelium. Patients who had bronchiolar metaplasia in their non-tumor lungs were selected. Tumor size less than 20 mm was exclusively analyzed for association with metaplasia. P-values are calculated by the chi-squared test.

consisting of basal and apical cells. Basal cells generally expressed TTF-1 and p40, whereas apical cells typically expressed TTF-1, but not p40 (Fig. 5). Metaplastic cells adjacent to non-TRU/BSE-type ADC occasionally lost

Table 6. Relationship between bronchiolar metaplasia and clinical factors among patients without interstitial pneumonia.

	Frequent (19)	Occasional (46)	None (156)	P-value
Age				0.0069
<65	26.3% (5)	26.1% (12)	48.7% (76)	
≥65	73.7% (14)	73.9% (34)	51.3% (80)	
Mean (range)	69.5 (55-80)	70.5 (56-83)	64.9 (29-84)	
Gender				0.9286
Male	52.6% (10)	52.2% (24)	55.1% (86)	
Female	47.4% (9)	47.8% (22)	44.9% (70)	
Smoking history				0.4833
Heavy	57.9% (11)	53.2% (24)	45.5% (71)	
Light/Non	42.1% (8)	47.8% (22)	54.5% (85)	
PYI (Mean ± SD)	299±337	349±453	292±646	

PYI, pack year index; SD, standard deviation. Three to five low power fields (magnification of x12.5) of non-tumor lungs were scanned. An average frequency more than or equal to 2 lesions of bronchiolar metaplasia was defined as "frequent", while that less than 2 was defined as "occasional", and no bronchiolar metaplasia as None. P-values are calculated by the chi-squared test.
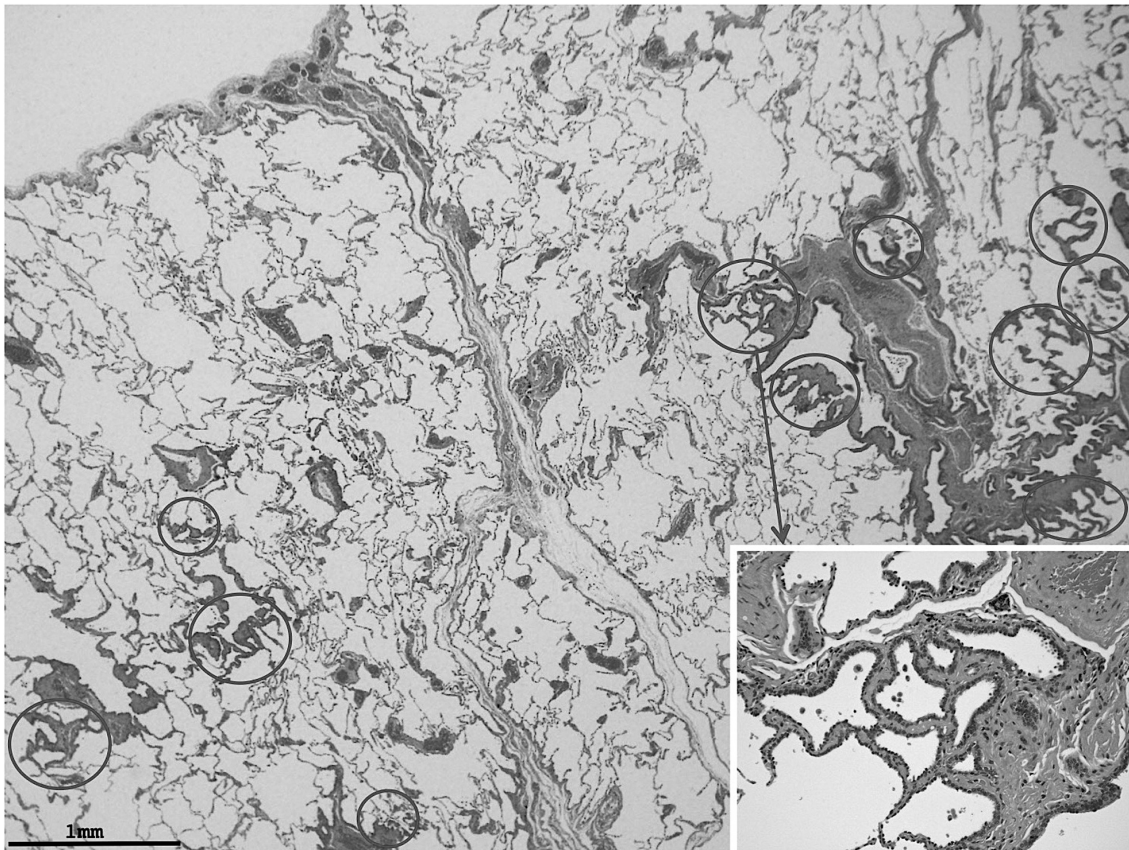

Fig. 3. A representative photograph of lung tissue affected by multiple bronchiolar metaplasia in a case without interstitial pneumonia: Multiple bronchiolar metaplasia has a scattered distribution (circles) (hematoxylin and eosin stain). An inset shows a close-up image of bronchiolar metaplasia (arrow).

cilia and nuclear polarity, and also acquired the weak ectopic expression of HNF-4α (Fig. 5).

Clinical characterization of patients with frequent BM among those without interstitial pneumonia

BM was frequently detected in elderly patients; however, no correlation was observed between the frequency of BM and gender, smoking habit (Table 6), or specific occupation (data now shown).

Discussion

The purpose of the present study was to define the histogenesis of non-TRU/BSE-type ADCs. We herein investigated pathological changes in non-tumor lungs
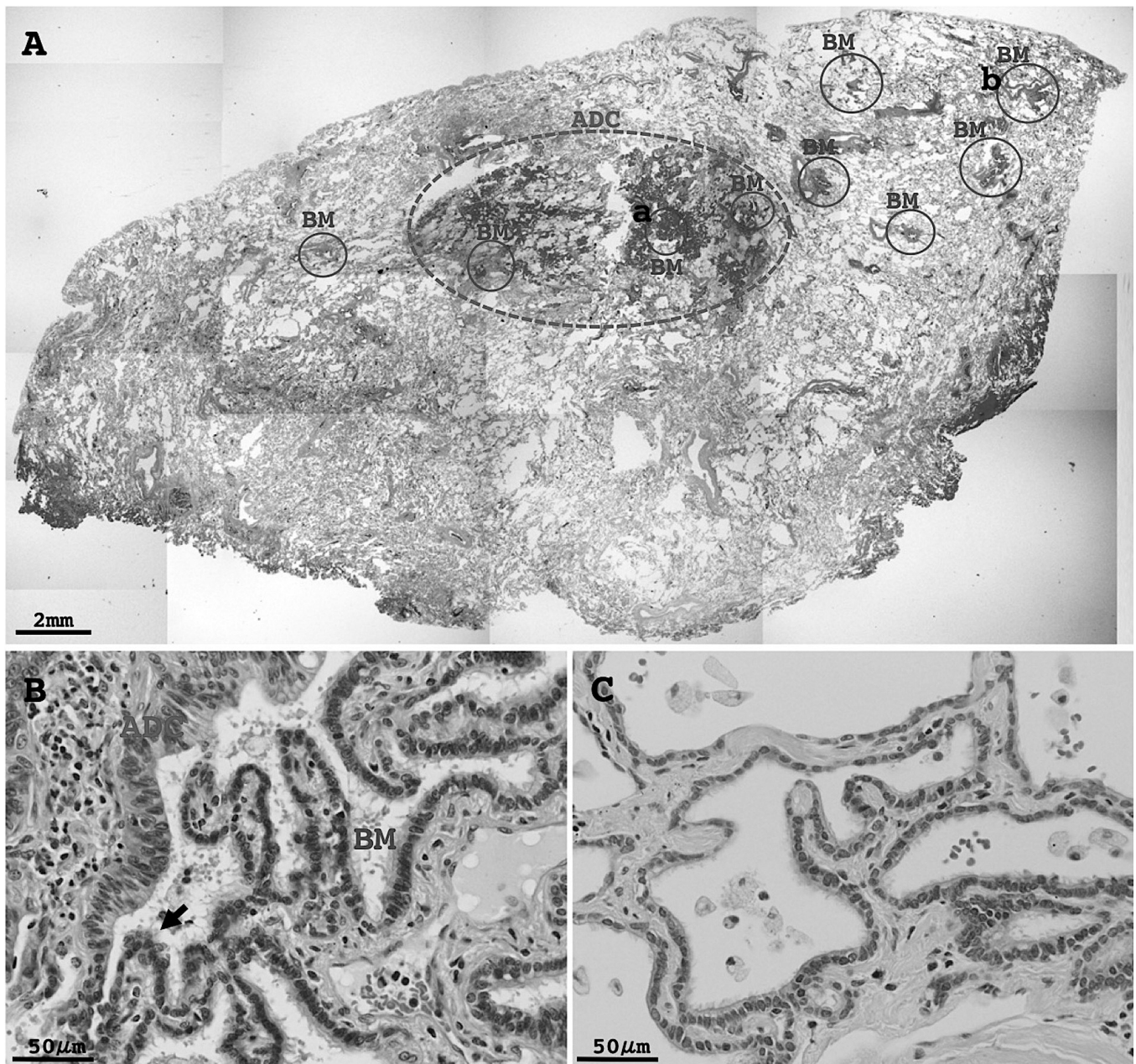

Fig. 4. A case of non-TRU/BSE-type adenocarcinoma occurring in a lung not affected by interstitial pneumonia. **A.** A scanning view (hematoxylin and eosin stain) is shown. Bronchiolar metaplasia (BM) has a scattered distribution (solid line circles). The area of adenocarcinoma (ADC) is roughly circled (dotted line circle). ADC associates with BM in the circle indicated with "a". **B.** A close view of the association between ADC and BM in the circle indicated with "a". An arrow indicates the front of neoplastic epithelia. **C.** A close view of BM in the circle indicated with "b".
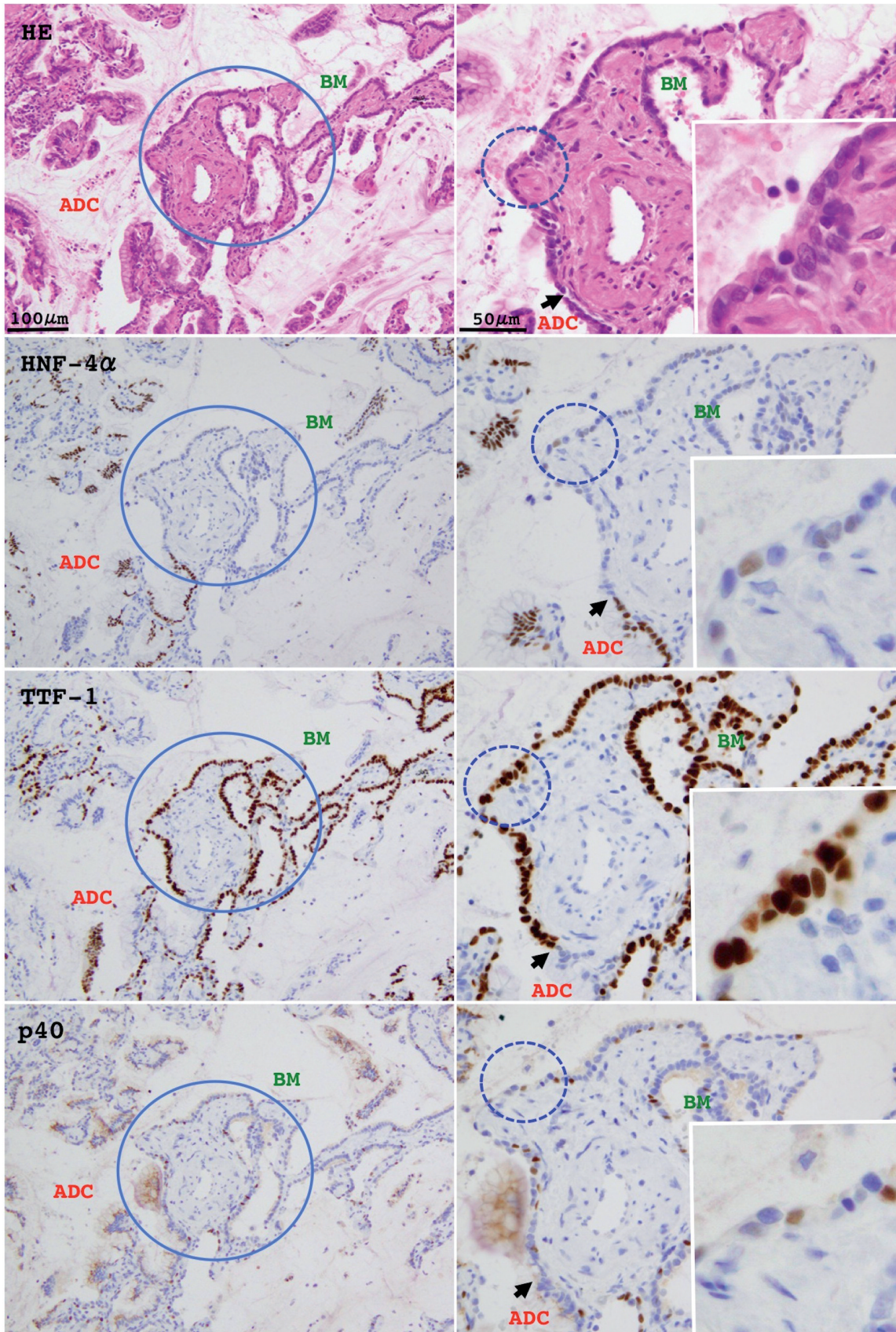
Fig. 5. Representative photographs of the transition between non-TRU/BSE-type adenocarcinoma and bronchiolar metaplasia: This transition between adenocarcinoma (ADC) and metaplasia (BM) is circled with a solid line (solid line circle) in the left panels. The area circled is expanded in the right panels and the front of neoplastic epithelia is indicated by an arrow. Among the right panels, BM adjacent to ADC (dotted line circles) is expanded in the insets. Ciliated low columnar cells extend along alveolar walls in the BM side, whereas non-ciliated tall columnar cells extend in the ADC side (the top panels, hematoxylin and eosin stain). Cells in BM adjacent to ADC occasionally lose cilia and nuclear polarity (the inset in the left top panel). ADC cells are positive for HNF-4α (the second panels) and negative for TTF-1 (the third panels), whereas BM cells are mostly negative for HNF-4α (the second panels) and positive for TTF-1 (the third panels). Cells in BM adjacent to ADC are weakly positive for HNF-4α (an inset in the left second panel). The ADC side entirely loses nuclear p40-positve basal cells, whereas the BM side retains these cells (the bottom panels).

among lung ADC patients. Non-TRU/BSE-type ADCs predominantly developed in honeycomb lesions and frequently connected to BM lining honeycomb spaces among patients with interstitial pneumonia. Non-TRU/BSE-type ADCs also preferentially developed in lungs affected by multiple BM of an unknown etiology among patients without any specific lung disease. These results suggest that non-TRU/BSE-type ADCs develop through distinct histogenesis, in which BM may be implicated.

Metaplasia is a precancerous condition in certain types of cancers, e.g., gastric adenocarcinoma develops from gastric intestinal metaplasia and squamous cell carcinoma from squamous cell metaplasia in different organs such as the bronchus, esophagus, and uterine cervix (Meyer and Liebow, 1965; Fujii et al., 2012). A recent molecular biological study suggested that metaplasia is a re-programming process in which tissue stem cells accumulate, and, thus, it results in increased susceptibility to neoplastic transformation (Fujii et al., 2012).

On the other hand, Coon et al. reported the up-regulation of sonic hedgehog gene (SHH) expression in BM in usual interstitial pneumonia (Coon et al., 2006). SHH, which is a secretory growth factor that is essential to morphogenesis in embryonal development, also has an impact on carcinogenesis by promoting cancer cell growth (Nielsen et al., 2004; Coon et al., 2006). Thus, BM is not simply heterotopic tissue regeneration, it is a pathological state associated with some molecular alterations. Our result that BM acquires the ectopic expression of HNF-4α is consistent with this notion. Additional alterations, such as the loss of TTF-1 expression due to gene mutations (Hwang et al., 2016), may lead to the development of non-TRU/BSE-type ADCs.

Airway epithelial cells are continuously exposed to external stresses including tobacco smoke, allergic antigens, infectious microorganisms, and air pollution. Severe stress may injure airway epithelial cells and induce different types of metaplastic regeneration, such as squamous cell metaplasia, mucous cell metaplasia, and ciliated and non-ciliated columnar cell metaplasia. Previous studies investigated the significance of different types of metaplasia in a number of airway diseases including pneumoconiosis, idiopathic interstitial pneumonia, lung cancers, and infectious diseases (Berkheiser, 1959, 1963; Miyamoto et al., 1987; Rehm and Kelloff, 1991; Rehm and Lijinsky, 1994; Liu et al., 2011). BM including mucous cell metaplasia and ciliated/non-ciliated columnar cell metaplasia, which are generally not well demarcated and intermingle, has been implicated in pathological conditions that damage terminal/respiratory bronchioles or alveolar ducts, such as pneumoconiosis, respiratory bronchiolitis interstitial lung disease, and hypersensitivity pneumonitis (Berkheiser, 1959, 1963; Miyamoto et al., 1987; Rehm and Kelloff, 1991; Rehm and Lijinsky, 1994; Liu et al., 2011). However, its significance in the development of

lung cancers has not yet been defined. Our research is unique because we exclusively analyzed the relationship between BM and different cytological types of ADCs, and implicated BM, particularly in the development of non-TRU/BSE-type ADCs.

It is important to note that BM was also detected in patients without any specific lung disease. Its occurrence was weakly associated with age, but not with a smoking habit or specific occupation. Undefined chronic trans-airway stimuli and/or genetic backgrounds may be involved in the production of BM. Its synergistic effect with smoking may lead to the development of non-TRU/BSE-type ADCs. Further studies are warranted in order to elucidate the potential causes and mechanisms underlying the production of BM.

Recent studies identified a number of genetic alterations in lung ADCs (Soda et al., 2007; Kohno et al., 2012, 2015; Seki and Kohno, 2015; Travis et al., 2015). A comprehensive analysis of genetic alterations and comparison of mutation profiles between non-TRU/BSE-type ADCs and BM is expected in order to further confirm our hypothesis.

In summary, this study describes the relationship between cytological subtypes of lung ADCs and BM. The results obtained suggest that non-TRU/BSE-type ADCs develop through distinct histogenesis in which BM appears to be a precancerous condition.

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