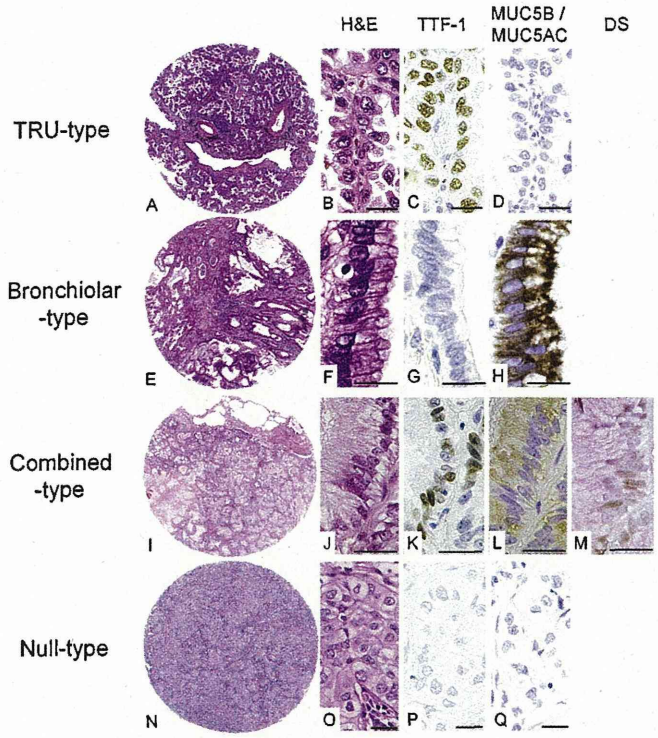

Fig. 1. Representative micrographs showing TRU-type adenocarcinoma (A–D), Bronchiolar-type adenocarcinoma (E–H), Combined-type adenocarcinoma (I–M), and Null-type adenocarcinoma (N–Q). A, E, I, and N: H&E staining of TMA cores of each of the above-mentioned subtypes. Cores were 2 mm in diameter. B, F, J, and O: high-magnification images of H&E staining of the above-mentioned subtypes. C, G, K, and P: high-magnification images of TTF-1 immunostaining of the above-mentioned subtypes. Most tumor cells in TRU-type and combined-type adenocarcinoma samples were positive for TTF-1 (nucleus, brown), whereas those in Bronchiolar-type and Null-type adenocarcinoma samples were negative for TTF-1. D, H, L, and Q: high-magnification images of MUC5B or MUC5AC immunostaining of the above-mentioned subtypes. Most tumor cells in Bronchiolar-type and Combined-type adenocarcinomas were positive for MUC5B and/or MUC5AC (cytoplasm, brown), whereas those in TRU-type and Null-type adenocarcinoma samples were negative for MUC5B and/or MUC5AC (D, H: MUC5B; L, Q: MUC5AC). M: double staining (DS) analysis of TTF-1 and MUC5AC expression in Combined-type adenocarcinoma samples revealed that the nucleus stained positive for TTF-1 (brown) and the cytoplasm stained positive for MUC5AC (pink) in the same tumor cells. Bar indicates 15 μm.

3. Results

3.1. Clinicopathological characteristics

The clinicopathological characteristics of the patients and tumors evaluated in the present study are summarized in Table 1. There were 125 (51.2%) men and 119 (48.7%) women, and the mean age was 65.8 years (range, 23–86 years). One hundred eleven patients (45.5%) had never smoked, and 133 patients (54.4%) were former or current smokers, with an average smoking index of 47.9 ± 38.4 pack-years. The mean tumor size was 26.1 ± 13.1 mm (range, 7–92 mm) and the number of tumors ≤30 mm in diameter was 181 (74.2%). The numbers of patients at each pathologic stage were as follows: IA, 130 (53.2%) patients; IB, 61 (25.0%) patients; IIA, 22 (9.0%) patients; IIB, 5 (2.1%) patients; and IIIA, 26 (10.7%) patients. TMA analysis detected the expression of TTF-1, MUC5B, and MUC5AC in 219 (89.6%), 75 (30.7%), and 33 (13.5%) of 244 cases, respectively (Fig. 1). Of the 244 cases, 236 cases were examined for *EGFR* and *KRAS* mutations. *EGFR* and *KRAS* mutations were detected in 114 (48.3%) and 27 (11.4%) cases, respectively. *EGFR* and *KRAS* mutations were mutually exclusive.

3.2. Correlation between TRU morphological features and clinicopathological factors in lung adenocarcinoma

Two hundred six (84.4%) cases in the present study were categorized as TRU subtype, and 38 (15.6%) cases were categorized as non-TRU subtype (Table 1). TRU morphology positively correlated with TTF-1 expression ($P < 0.001$) and *EGFR* mutations ($P < 0.001$). In contrast, non-TRU morphology positively correlated with smoker status ($P = 0.009$), pleural invasion ($P = 0.028$), MUC5B expression ($P < 0.001$), MUC5AC expression ($P < 0.001$), and *KRAS* mutations ($P < 0.001$).

3.3. Clusters grouped by expression of the proteins, EGFR mutations, and KRAS mutations: "specific type"

Next, we attempt hierarchical clustering analysis to apply to the protein expression and *EGFR* and *KRAS* mutations datasets (Fig. 2). Five clusters emerged from the analysis with similar patterns of

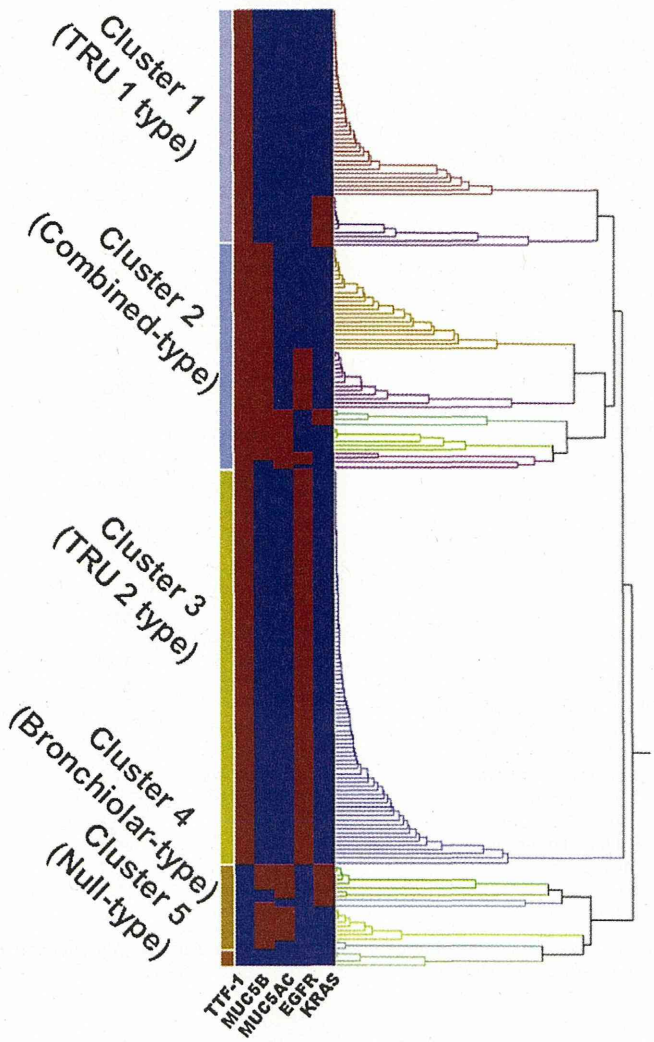

Fig. 2. Hierarchical cluster analysis based on the expression of TTF-1, MUC5B, and MUC5AC and on the presence of *EGFR*/*KRAS* mutations. Each row represents a different tumor, and each column indicates the primary antibody used for immunohistochemical analysis or the *EGFR*/*KRAS* genotype. Red and blue bars indicate positive and negative cases, respectively. Five groups emerged from the analysis as follows: Cluster 1 (TRU1-type) showing TTF-1(+), MUC5B(−), MUC5AC(−), EGFR(−); cluster 2 (Combined-type) showing TTF-1(+), MUC5B(+), and/or MUC5AC(+); cluster 3 (TRU2-type) showing TTF-1(+), MUC5B(−), MUC5AC(−), EGFR(+); cluster 4 (Bronchiolar-type) showing TTF-1(−), MUC5B(+) and/or MUC5AC(+); and cluster 5 (Null-type) showing TTF-1(−), MUC5B(−), MUC5AC(−), EGFR(−), KRAS(−).

Table 1
Characteristics of patients and Correlation between the parameter and TRU morphology.

	n	%	TRU morphology		*P*-Value
			TRU morphology	non-TRU morphology	
			206	38	
Sex					
Male	125	51.2	102	23	0.21
Female	119	48.7	104	15	
Age					
65.8 ± 9.7					
Under 65	109	44.6	94	15	0.481
Over 66	135	55.3	112	23	
Smoking status					
Never	111	45.5	100	11	0.009
Former/current	133	54.5	105	28	
Tumor size (mm)			26.1 ± 13.1		
30 mm or less	181	74.2	154	27	0.635
31 mm or over	63	25.8	52	11	
Stage					
IA	130	53.2	114	16	0.215
IB	61	25	49	12	
IIA	22	9	19	3	
IIB	5	2.1	5	0	
IIIA	26	10.7	19	7	
Tumor grade					
Well diff.	45	18.4	38	7	0.442
Moderately diff.	98	40.2	86	12	
Poorly diff.	101	41.4	82	19	
Lymphatic invasion					
Present	47	19.3	42	5	0.28
Absent	197	70.7	164	33	
Vascular invasion					
Present	61	25	52	9	0.837
Absent	183	75	154	29	
Pleural invasion					
PL0	178	72.9	156	22	0.028
PL1-3	66	27	50	16	
TTF-1					
Positive	219	89.8	198	22	<0.001
Negative	25	10.2	8	16	
MUC5B					
Positive	75	30.7	45	30	<0.001
Negative	169	69.3	161	8	
MUC5AC					
Positive	33	13.5	0	33	<0.001
Negative	211	86.5	206	5	
EGFR					
Mutated	114	48.3	106	7	<0.001
Wild	122	51.7	92	28	
KRAS					
Mutated	27	11.4	15	12	<0.001
Wild	209	88.6	183	23	
IASLC/ATS/ERS classification					
AIS (non-muc/muc)	8 (7/1)	3.3	7(7/0)	1 (0/1)	<0.001
MIA (non-muc/muc)	12 (10/2)	4.9	10 (10/0)	2 (0/2)	
Lepidic predominant	18	7.4	18	0	
Aci predominant	27	11.7	22	5	
Pap predominant	113	46.3	102	11	
Solid predominant	48	19.7	39	9	
MP predominant	10	4.1	8	2	
IMA	6	2.5	0	6	
Colloid	2	0.8	0	2	

Abbreviations: TRU, terminal respiratory unit; diff., differentiated; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; muc, mucinous; IMA, invasive mucinous adenocarcinoma.

protein expression and mutations as shown in the dendrogram. Among the clusters, all cases included in clusters 1 and 3 showed expression of TTF-1 and no expression of MUC5B or MUC5AC. *EGFR* mutations were detected in cluster 3, but not cluster 1. We designated these cluster groups as TRU1-type and TRU2-type, respectively, because of the results of correlations between morphological TRU type adenocarcinoma and protein expressions. In contrast, the cases included in cluster 4 all expressed MUC5B or MUC5AC, but not TTF-1, and did not harbor *EGFR* mutations. Additionally, about half of the cases (10 cases) in cluster 4 harbored *KRAS* mutations. This group was designated the Bronchiolar-type because bronchiolar epithelial cells and bronchial gland cells normally express MUC5B or MUC5AC, as described previously. Cluster 2 was designated as Combined-type because all cases co-expressed TTF-1 and MUC5B or MUC5AC, which was confirmed by double staining analysis (Fig. 1). Finally, the samples analyzed in cluster 5 did not express TTF-1, MUC5B, or MUC5AC and did not harbor *EGFR* or *KRAS* mutations; thus, this cluster was designated as Null-type.

3.4. Correlations between the "specific types of tumors" and the TRU morphology

Table 2 shows the correlations between the tumor types derived from cluster analysis and the TRU morphology. The morphological TRU cases included 61 TRU1-type cases, 93 TRU2-type cases, 40 Combined-type cases, 3 Bronchiolar-type cases, and 4 Null-type cases. In contrast, the morphological non-TRU cases included 1 TRU1-type case, 2 TRU2-type cases, 16 Combined-type cases, 16 Bronchiolar-type cases and no Null-type cases. Using IHC with whole-slide sections, 1 TRU-1-type case and 2 TRU-2-type cases among the morphologic non-TRU cases were positive for the anti-mucin antibodies. TRU1/TRU2-type cases, as defined by cluster analysis, were more likely to show the TRU morphology, whereas Bronchiolar-type cases were more likely to show the non-TRU morphology. Combined-type cases exhibited an intermediate morphology between TRU1/TRU2-type cases and Bronchiolar-type cases.

3.5. Correlations between the "specific type of tumors" and survival

Next, we investigated the associations between survival and the "specific type of tumors", as defined by cluster analysis. The mean clinical follow-up period was 49.7 months (range, 0.4–125 months). Fig. 3 shows survival curves for each tumor type. Because there were no significant differences in either DFS or OS between TRU1-type and TRU2-type tumors (data not shown), we merged these two types into the TRU-type. The 5-year DFS rate was significantly higher in patients with TRU-type tumors (75.2%), followed by patients with the Combined-type (61.7%) and Bronchiolar-type tumors (53.6%; Fig. 3a). The 5-year DFS rate was the lowest for patients with Null-type tumors (40.0%). Patients with TRU-type tumors had statistically better prognoses compared to those with Combined-type ($P=0.024$) or Null-type ($P=0.022$) tumors. As demonstrated by the OS curves in Fig. 3b, patients with TRU-type tumors had significantly better prognoses than those with Combined-type, Bronchiolar-type, or Null-type tumors ($P=0.002$). The prognosis for patients with Combined-type tumors tended to be better than that for patients with Bronchiolar-type tumors; however, the difference in OS analysis was not significant ($P=0.240$).

Table 3 shows the results of univariate and multivariate analyses of the clinicopathological factors examined in this study. Based on univariate results, multivariate analyses were performed using the Cox proportional hazards model. The results indicated that only stage and specific type remained significantly associated with DFS; stage 2 or 3 patients had an increased risk for recurrence compared with stage 1 patients (hazard ratio [HR] = 5.976; 95% confidence interval [CI], 3.319–10.823; $P<0.001$); the presence of non-TRU-type tumors indicated an increased risk for recurrence compared with the presence of TRU-type tumors (HR = 1.785; 95% CI, 1.041–3.063; $P=0.035$). Moreover, smoking status, stage, and specific tumor type were significantly associated with OS. Patients who had never smoked were at decreased risk of overall death compared with current/former smokers (HR = 0.357; 95% CI, 0.175–0.692; $P=0.002$). Additionally, stage 2 or 3 patients were at increased risk of overall death compared with stage 1 patients (HR = 7.527; 95% CI, 3.973–14.547; $P<0.001$), and patients with non-TRU type tumors were at increased risk of overall death compared to those with TRU-type tumors (HR = 1.928; 95% CI, 1.084–3.421; $P=0.025$).

4. Discussion

The clinicopathological characteristics of non-TRU type lung adenocarcinoma are not well defined in contrast to TRU type lung adenocarcinomas that are more prevalent in Asian females, never smokers, and patients with TTF-1-positive tumors and a relatively high incidence of *EGFR* mutations [25]. In this study, we demonstrated the significant characteristics of non-TRU type adenocarcinomas by analyzing the expression of mucins and TTF-1 and evaluating *EGFR* and *KRAS* mutations.

Yatabe et al. [25] speculated that the normal cellular counterpart of non-TRU type adenocarcinoma cells is bronchial surface epithelial and glandular cells. Few studies, however, have described the mucinous morphology of these tumors. Recently, Park et al. [26] found lung adenocarcinomas with transition foci from normal ciliated columnar cells to mucous columnar cell metaplasia, dysplasia, adenocarcinoma in situ, and finally invasive adenocarcinoma, suggesting that mucous columnar cell metaplasia and dysplasia may represent a non-TRU type adenocarcinoma pathway. In addition, Kunii et al. [18] reported that most TTF-1-negative lung adenocarcinomas are mucinous lesions with prominent expression of hepatocyte nuclear factor-4a (HNF-4a) and MUC5AC. Furthermore, Sugano et al. [27] demonstrated that HNF-4a expression strongly associated with invasive mucinous adenocarcinoma and concluded that HNF-4a-positive lung adenocarcinoma is not a TRU type adenocarcinoma. HNF-4a is a nuclear transcription factor that is expressed in the liver, kidneys, and gastrointestinal tract [28]. These findings indicate that non-TRU type adenocarcinoma can originate from the bronchial epithelium or submucosal glands and exhibits a gastric-mucin phenotype. In the present study, the non-TRU morphology was positively correlated with MUC5B expression, MUC5AC expression, and *KRAS* mutations, whereas the TRU morphology was correlated with TTF-1 expression and *EGFR* mutations. Our findings here supported the conclusion that non-TRU type adenocarcinomas are characterized mainly by mucinous-type morphology and expression of gastric-type mucins.

Many researchers believe that lung adenocarcinomas with TTF-1 expression belong to the TRU type adenocarcinoma category. However, in this study, we demonstrated the existence of a type of lung adenocarcinoma that co-expresses TTF-1 and gastric-type mucins (MUC5B and/or MUC5AC), which we assigned with the term "Combined-type" adenocarcinoma. This type of adenocarcinoma has not been previously reported. Thus, we question whether we should classify this special type of lung adenocarcinoma into the TRU type or non-TRU type category. In this study, we identified five specific subtypes using cluster analysis. Among these subtypes, cluster 2 was characterized by co-expression of TTF-1 with MUC5B and/or MUC5AC. This type of adenocarcinoma represented a subtype that was morphologically intermediate between the TRU type and non-TRU type tumors. Moreover, patients with this type of tumors had statistically poorer prognoses compared to those with TRU-type (TRU1/TRU2 type) tumors. Some studies have analyzed lung adenocarcinoma subtypes by unsupervised hierarchical clustering based on gene expression profiling [7,29–31] Takeuchi et al. reported three distinct pulmonary adenocarcinoma subtypes [7]. Among these, two TRU subtypes were identified: TRU-a and TRU-b. The TRU-b subtype was strongly associated with BAC features, well-differentiation, EGFR mutations, and better prognosis, whereas the TRU-a subtype showed intermediate characteristics between the TRU-b and Non-TRU subtype. Shibata et al. reported two distinct subtypes using gene expression profiling: the alveolar-type and bronchiolar-type [29], and they described a potentially transitional subgroup between these subtypes. The Combined-type, which we described in our study, could correspond to the specific subtype that Takeuchi et al. and Shibata et al. speculated to be a transitional subtype between the TRU-type and non-TRU-type. We speculate that determining MUC protein expression is the key to identifying the transitional subtype. Basically, MUC5AC and MUC5B are secreted gel-forming mucins and are expressed by surface goblet epithelial cells and

Table 2
Correlation between specific tumor types by cluster analysis and TRU morphology.

	n	Cluster 1 (TRU 1 type)	Cluster 2 (Combined-type)	Cluster 3 (TRU 2 type)	Cluster 4 (Bronchiolar-type)	Cluster 5 (Null-type)
TRU morphology	201	61	40	93	3	4
non-TRU morphology	35	1	16	2	16	0

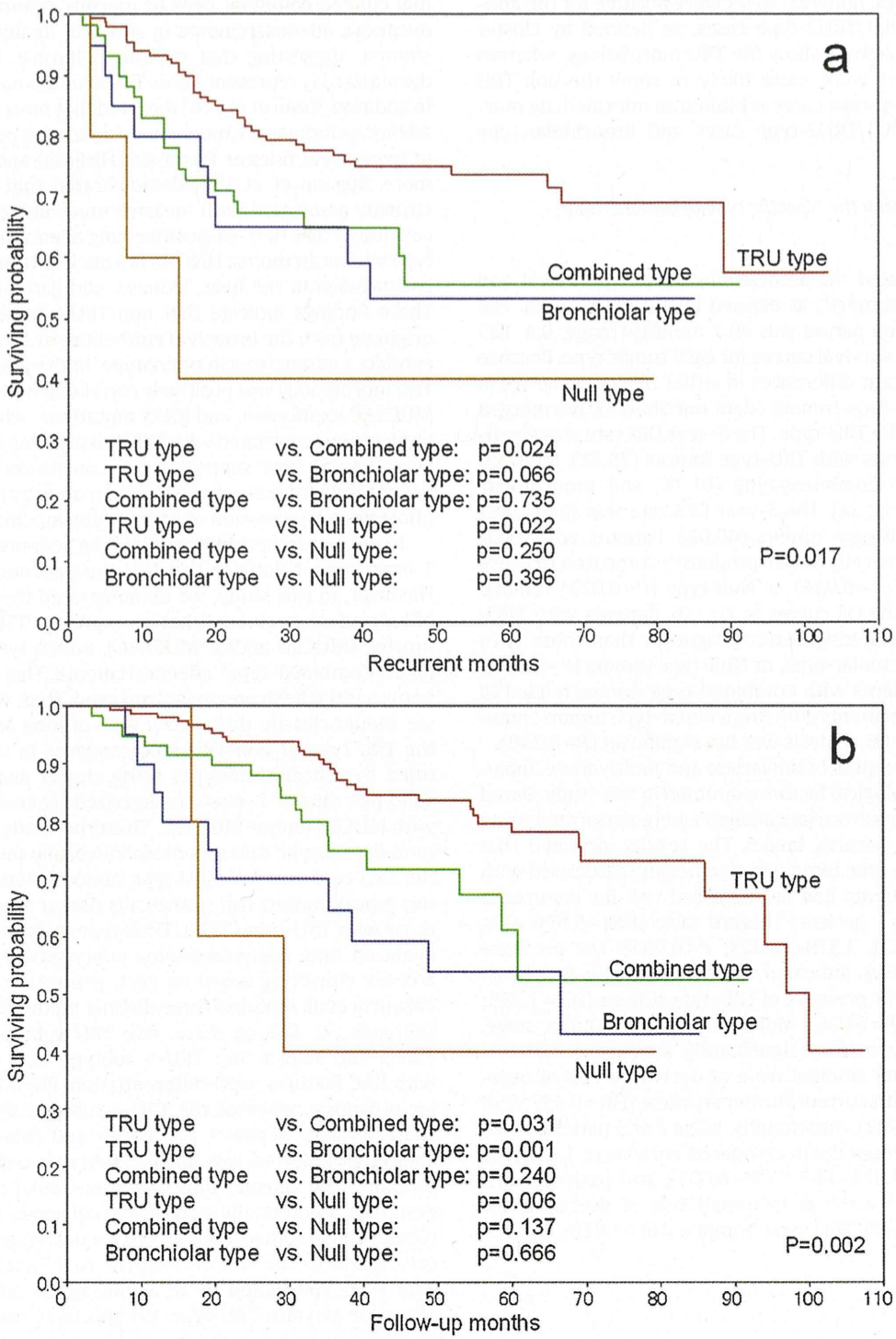

Fig. 3. (a) Kaplan–Meier analysis of DFS for specific tumor types according to the cluster analysis. (b) Kaplan–Meier analysis of OS for specific tumor types according to the cluster analysis.

Table 3
Summary of univariate and multivariate analyses of clinicopathological parameters.

Parameter	DFS univariate			DFS multivariate		
	HR	95% CI	*P*-Value	HR	95% CI	*P*-Value
Smoking status (never vs. current or former)	0.580	0.353–0.932	0.024	0.863	0.475–1.540	0.623
EGFR mutations (mutated vs. wild-type)	0.578	0.350–0.937	0.026	0.815	0.447–1.451	0.490
Tumor grade (poorly diff. vs. well diff./moderately diff.)	2.719	1.670–4.491	<.0001	1.495	0.770–2.887	0.233
Stage (stage 2–3 vs. stage 1)	7.119	4.454–11.435	<.0001	5.976	3.319–10.823	<.0001
Pleural invasion (present vs. absent)	2.203	1.373–3.495	<.0001	1.200	0.686–2.073	0.518
Lymphatic invasion (present vs. absent)	2.498	1.516–4.018	<.0001	1.515	0.518–1.747	0.893
Vascular invasion (present vs. absent)	2.363	1.463–3.760	<.0001	1.440	0.749–2.754	0.271
Specific type (non-TRU type vs. TRU type)	1.954	1.223–3.111	0.005	1.785	1.041–3.063	0.035
Parameter	OS univariate			OS multivariate		
	HR	95% CI	*P*-Value	HR	95% CI	*P*-Value
Smoking status (never vs. current or former)	0.330	0.180–0.576	<.0001	0.357	0.175–0.692	0.002
EGFR mutations (mutated vs. wild-type)	0.505	0.287–0.863	0.008	0.971	0.505–1.821	0.929
Tumor grade (poorly diff. vs. well diff./moderately diff.)	3.804	2.211–6.762	<.0001	1.870	0.897–3.915	0.094
Stage (stage 2–3 vs. stage 1)	9.542	5.635–16.470	<.0001	7.527	3.973–14.547	<.0001
Pleural invasion (present vs. absent)	2.312	1.374–3.846	0.002	1.630	0.894–2.956	0.109
Lymphatic invasion (present vs. absent)	2.577	1.489–4.336	0.001	0.821	0.428–1.550	0.546
Vascular invasion (present vs. absent)	2.835	1.696–4.712	<.0001	1.650	0.829–3.307	0.153
Specific type (non-TRU type vs. TRU type)	2.320	1.387–3.883	0.001	1.928	1.084–3.421	0.025

Abbreviations: TRU, terminal respiratory unit; diff., differentiated; HR, hazard ratio; CI, confidence interval.

mucous cells of the submucosal glands in normal airways, but are not expressed in alveolar epithelial cells [10–12,32]. Thus, we hypothesized that this tumor type was biologically different from typical TRU type tumors despite being positive for TTF-1 and could progress along either the terminal respiratory epithelial phenotype or the central mucinous epithelial phenotype. Further investigations are necessary in order to clarify this assumption.

Additionally, we discovered a Null-type tumor that was poorly differentiated; patients with these tumors, although few in number, had poorer prognoses. Interestingly, based on our multivariate analyses, non-TRU-type tumors (including Combined-type, Bronchiolar-type, and Null-type) conferred an increased risk of recurrence and overall death compared with TRU-type tumors, indicating that the non-TRU group had a poor prognosis independent of tumor grade. Thus, our study suggested that non-TRU type adenocarcinomas were a heterogeneous group and could provide the basis for selecting the most appropriate treatment for patients.

5. Conclusion

The present study demonstrated that non-TRU type adenocarcinoma correlated with MUC5B expression, MUC5AC expression, and *KRAS* mutations and were associated with a poorer prognosis than TRU type adenocarcinomas. Additionally, there are three distinct subtypes of non-TRU type adenocarcinomas. There are no effective treatments for patients with non-TRU type lung adenocarcinoma, while patients with TRU type lung adenocarcinoma can be treated with EGFR inhibitors. Therefore, the characterization of non-TRU-type lung adenocarcinomas described herein may be helpful in selecting appropriate patients for specific treatments that will hopefully be developed in the future.

Conflict of interest statement

All authors have no potential conflicts of interest.

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HER2 status in lung adenocarcinoma: A comparison of immunohistochemistry, fluorescence in situ hybridization (FISH), dual-ISH, and gene mutations

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Fluorescence in situ hybridization
Dual in situ hybridization
Trastuzumab

ABSTRACT

Objectives: While novel anti-human epidermal growth factor receptor 2 (HER2) agents have recently been developed, no definite criteria have been proposed as indications for the use of these agents in patients with lung cancer. Here, we tested HER2 alterations by using four methods and explored the concordance of these methods to improve our understanding of the accuracy of HER2 testing methods.
Materials and methods: We analyzed HER2 protein expression by immunohistochemistry (IHC) and HER2 amplification by fluorescence in situ hybridization (FISH) and dual in situ hybridization (DISH) by using a tissue microarray comprising lung adenocarcinoma specimens from 243 consecutive patients. The presence of mutations in the *EGFR*, *KRAS*, and *HER2* genes were also determined.
Results: Positive IHC (score 3+) was observed in six cases (2.5%). Amplification of *HER2* was observed in five cases (2.1%) by FISH and in nine cases (3.7%) by DISH. HER2 expression by IHC and gene amplification by FISH were significantly associated ($P < 0.001$). The overall concordance between FISH and DISH by amplification status was 96.7% ($P < 0.001$). One hundred nine tumors (49.9%) had *EGFR* mutations, 25 (11.2%) had *KRAS* mutations, and six (2.7%) had *HER2* mutations. All of these mutations were mutually exclusive. Cases having *HER2* mutations were positively correlated with cases having *HER2* amplification ($P < 0.001$). Two of six cases with *HER2* mutations showed amplifications by FISH and DISH tests.
Conclusion: HER2 protein overexpression, gene amplification, and gene mutations appeared to be uncommon in lung adenocarcinoma. Cases with *HER2* mutations tended to show *HER2* gene amplification. The results indicated that *HER2* gene amplification and mutations should be tested to determine whether patients are eligible for administration of new anti-HER2 agents. In addition, DISH was better than FISH for detection of cases with *HER2* amplification.

1. Introduction

Lung cancer remains the leading cause of cancer-related mortality worldwide [1]. The discovery of mutations in the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, as well as fusions involving anaplastic lymphoma kinase (ALK), have led to marked changes in the treatment of patients with lung adenocarcinoma, the most common type of lung cancer [2–5]. However, the mortality rate in patients with lung cancer remains high, and novel therapies, as well as more effective diagnostics, are necessary.

Human epidermal growth factor receptor 2 (HER2) is a membrane-bound tyrosine kinase belonging to the *ERBB* (or *EGF*) family. The *ERBB2* gene (also called the *HER2* gene), which encodes HER2 protein, has been a recognized proto-oncogene in human cancers since it was found to be amplified in breast cancers and gastric cancer more than two decades ago [6,7]. The use of HER2-targeted agents, such as trastuzumab, has improved outcomes for HER2-positive breast and gastric cancers [7–9]. Because trastuzumab has failed to provide clinical benefits to lung cancer patients harboring

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HER2 protein expression or gene amplification [10–13], clinical research on the use of HER2-targeting agents in lung cancer has slowed down. However, recent studies have again begun to investigate the use of novel anti-HER2 drug in lung cancer patients [14]. Because the efficacies of these novel drugs in patients with fluorescence in situ hybridization (FISH)-positive lung cancer have not been clearly studied, more work is needed to determine appropriate diagnostic techniques and indications for the use of these agents.

In this study, we analyzed HER2 alterations in patients with lung adenocarcinoma using a tissue microarray comprising 243 lung adenocarcinoma specimens. HER2 protein expression was examined by immunohistochemistry (IHC); *HER2* gene amplification was examined by FISH, which remains the "gold standard" for identification of *HER2* gene alterations; and *HER2* gene amplification was examined by dual in situ hybridization (DISH), an alternative to FISH. The presence of mutations in the *EGFR*, *KRAS*, and *HER2* genes was also determined. Following these analyses, we determined the concordance of these methods in our sample set.

2. Materials and methods

2.1. Patient selection and histological evaluation

Between January 2001 and December 2007, 337 consecutive patients with lung adenocarcinomas underwent pulmonary resection at Kyoto University Hospital. Patients were excluded if they had multiple primary lung cancers, underwent chemo- or radiotherapy before surgery, underwent incomplete resection, or lacked complete follow-up data retrieved from the Thoracic Surgical Database. All resected specimens were formalin-fixed, sectioned, and stained with hematoxylin and eosin (H&E) in the conventional manner. Slides (1–17 per patient, average = 3.4) were reviewed by two pathologists (AY, SS), blinded to patient outcomes. All cases were classified according to International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) criteria [15]. Tumor staging was performed according to the seventh edition of the TNM classification of the International Union Against Cancer [16].

2.2. Tissue microarray (TMA)

A portion of the present cohort was described in our previous report [17]. Briefly, after case selection described above, paraffin-embedded tumor blocks with sufficient tissue were selected for preparation of a TMA. The most representative region of the tumor was selected based on the morphology of the H&E-stained slide. Tissue cores were punched out from each donor tumor block using thin-walled 2-mm stainless steel needles (Azumaya Medical Instruments Inc., Tokyo, Japan), and cores were arrayed in a recipient paraffin block. Non-neoplastic lung tissue cores from selected patients were also arrayed in the same block. All subsequent tests (IHC, FISH, and DISH) were performed on serially cut 4-μm paraffin-embedded tissue sections.

2.3. HER2 IHC

For HER2 protein detection, the HercepTest kit (DAKO, Glostrup, DK) was used according to manufacturer's instructions. HER2 scoring was performed according to the four-step scale (0, 1+, 2+, 3+) outlined in the HercepTest manual for breast cancer. HER2 scores were determined by two independent pathologists (AY, SS), as shown in Fig. 1A and B.

2.4. HER2 FISH and DISH

FISH testing was performed using the PathVysion HER2 DNA Probe (Abbott Molecular, Abbott Park, IL, USA) according to the following standard manufacturer's protocols. At least 20 cells per case were analyzed by two pathologists independently. Cases with ratios between 1.8 and 2.2 (equivocal range) were reviewed, and an additional 20 cells were recounted to confirm their status as amplified or not amplified. Either high-level amplification (numerous loose or tight clusters of *HER2* signals, atypically large signals, or a *HER2*/centromere 17 ratio >5.0) or low-level amplification (*HER2*/centromere 17 ratio >2.0 and <5.0) were considered FISH-positive (Fig. 1C).

DISH was performed using the INFORM Dual ISH HER2 kit (Ventana Medical Systems) according to the US Food and Drug Administration (FDA)-approved protocol [18]. DISH-positive cases were determined by the same method as that used for FISH (Fig. 1D).

2.5. Detection of mutations in EGFR, KRAS, and HER2

Some of the mutation analysis has been reported in our previous studies [19–21]. Briefly, mutations in the *EGFR* (exons 18–21) and *HER2* (exon 20) genes were also studied using polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP), as reported previously. Mutations in *KRAS* were investigated using modified mutagenic PCR-restriction enzyme fragment-length polymorphism (RFLP), as reported previously [22,23]. Updated data from patients with *HER2* mutations detailed in previous reports was merged.

2.6. Statistical analysis

Chi-square and Fisher's exact tests were used to analyze categorical data. Survival rates were calculated using the Kaplan–Meier method, and the differences were analyzed using the log rank test. All statistical tests were two-sided at a 5% level of significance. For statistical analysis, the JMP statistical software package, version 8 (SAS Institute Inc, NC, USA) was used.

3. Results

3.1. Clinicopathological characteristics

Of the 337 lung adenocarcinomas resected from 2001 to 2007, we conclusively evaluated the HER2 status for 243 cases. Clinicopathological information of the 243 cases is listed in Table 1. There were 128 men and 115 women in this analysis cohort, and the mean age was 65.9 years (range, 23–88 years). The mean tumor size was 26.9 ± 13.8 mm (range, 7–92 mm). One hundred eleven patients (56.6%) had never smoked, 66 patients (27.1%) were former smokers, and 66 patients (27.1%) were current smokers. Data describing HER2 IHC, *HER2* FISH, *HER2* DISH, *HER2* mutations, *EGFR* mutations, and *KRAS* mutations were available for 243, 243, 243, 220, 222, and 222 patients, respectively. Positive IHC (score 3+) was observed in six cases (2.5%). Amplification of HER2 was seen in five cases (2.1%) by FISH and nine cases (3.7%) by DISH. One hundred nine tumors (49.9%) had *EGFR* mutations, 25 (11.2%) had *KRAS* mutations, and six (2.7%) had *HER2* mutations. All of these mutations were mutually exclusive. Younger age, smaller size of tumor, well-differentiated tumors, and *EGFR* mutations were associated with significantly better prognoses, whereas there was no association between prognosis and HER2 status (Table 1).

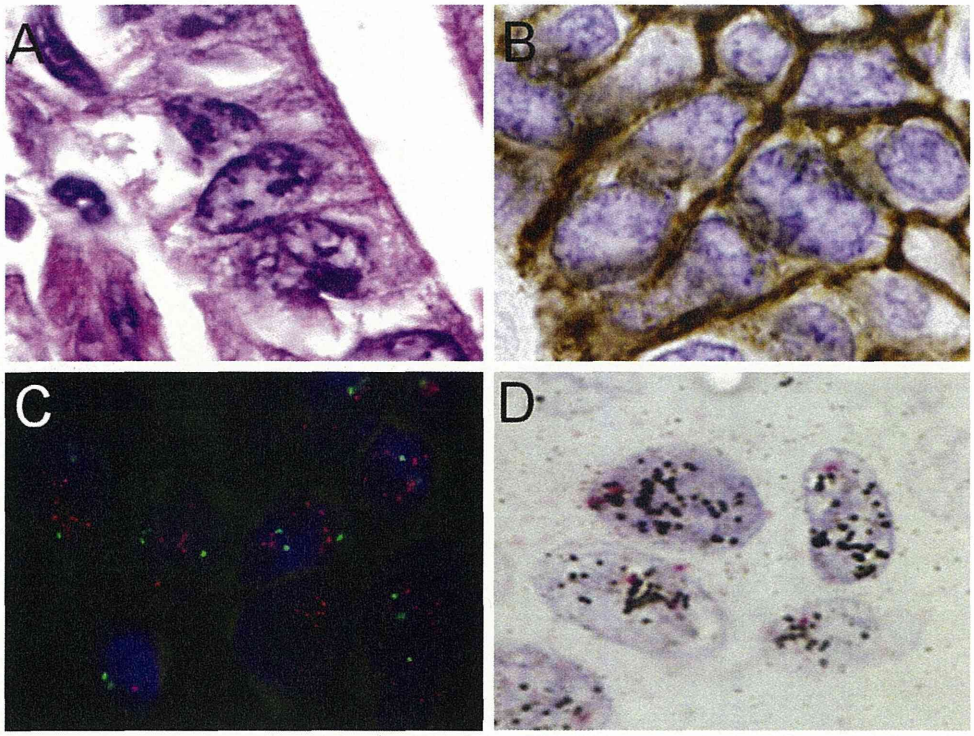

Fig. 1. Representative features of HER2 alterations. (A) Hematoxylin and eosin staining; (B) IHC for HER2 showing cell membranes of tumor cells were strongly positive for anti-HER2 antibody staining (score 3+); (C) FISH image showing high-level amplification of the *HER2* probe (red signals) compared to chromosome 17 centromere probe (green signals): (D) DISH image showing high-level amplification of the *HER2* probe (black signal) compared to the chromosome 17 centromere probe (red signals). Original magnification 1000×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. *HER2 expression versus HER2 amplification*

Both IHC and FISH were interpretable on the same tissue spot in 243 TMA cores. One hundred and three tumors (42.3%) proved HER2 IHC negative (score 0), whereas one hundred forty tumors (57.3%) had some degree of HER2 protein expression; 1+ in 103 tumors (42.3%), 2+ in 31 tumors (12.7%), and 3+ in 6 tumors (2.5%) (Table 1). A half of cases (3 tumors) of strong HER2 expression (3+) showed *HER2* gene high-level amplification. However, only one (3.2%) of 31 tumors with 2+ HER2 immunostaining had gene amplification. Conversely, two (1.9%) of 103 tumors with 1+ HER2 immunostaining had low-level gene amplification. Of 103 tumors with score 0 HER2 immunostaining, no *HER2* gene amplification was found. Protein expression and gene amplification were significantly associated ($P < 0.001$).

3.3. *Concordance of between FISH and DISH*

When tumors were categorized according to American Society of Clinical Oncologists/College of American Pathologists (ASCO/CAP) criteria [18] as amplified, equivocal, or not amplified, a concordance rate of 96.7% was found between FISH and DISH ($P < 0.001$; Table 2). All tumors with *HER2* amplification detected by FISH also showed amplification by DISH. Of the amplified cases, two showed high-level *HER2* amplification and the other two showed low-level *HER2* amplification with both methods. Of ten tumors characterized as *HER2* equivocal by DISH, eight showed no amplification with FISH. By regression analysis, the *HER2*/centromere 17 ratio evaluated by DISH was significantly higher than that by FISH ($y = 0.819x + 0.097$; $r^2 = 0.693$; Fig. 2).

3.4. *Association among HER2 tests*

Fig. 3 shows the relationships among the HER2 test results depicted as a Venn diagram in 220 patients. Two of six patients with *HER2* mutations showed amplifications by FISH and DISH. Conversely, three of five patients with *HER2* amplifications by FISH and DISH did not harbor *HER2* mutations. Of six patients with HER2 expression by IHC (3+), only one patient had *HER2* mutations. *HER2* mutated cases tended to have *HER2* amplification confirmed by FISH/DISH ($P < 0.001$), whereas there was no correlation between HER2 protein overexpression by IHC and *HER2* mutation ($P = 0.392$; Table 3).

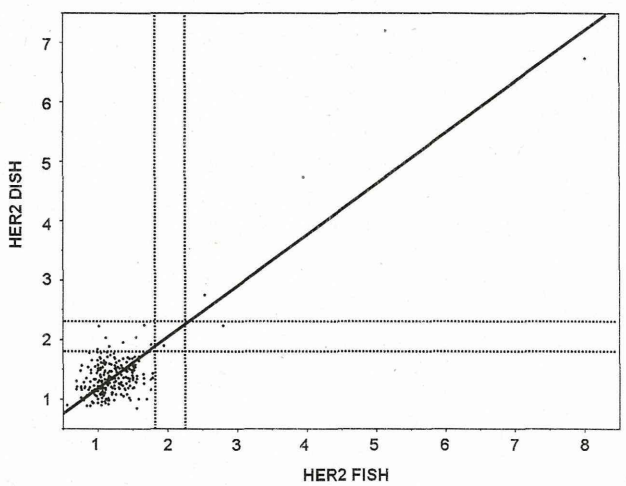

Fig. 2. Correlations of *HER2*/centromere 17 ratios as measured by FISH and DISH. The vertical and horizontal dotted lines encompass the equivocal ratios defined by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines. The solid line was determined by linear regression and shows that FISH resulted in lower estimates of *HER2*/centromere 17 ratios than DISH. Additionally, eight cases that were equivocal with DISH were normal with FISH ($y = 0.819x + 0.097$; $r^2 = 0.693$).

Table 1
Characteristics of patients.

Patients	*n*	5 years DFS rate (%)	*P* value
Median age, 65.9 ± 9.9 (range: 23–88)			
65 or younger	107	73.2	0.026
66 or older	136	58.3	
Gender			
Male	128	61.2	0.136
Female	115	69.4	
Smoking status			
Current	66	53.3	0.078
Former	66	67.7	
Never	111	69.9	
Tumor size, mm (range), 26.9 ± 13.8 (range: 7–92 mm)			
30 mm or smaller	172	75.9	<0.001
31 mm or larger	71	49.5	
Tumor grade			
Well differentiated	40	97.2	<0.001
Moderately differentiated	100	66.2	
Poorly differentiated	102	52.8	
IASLC classification			
AIS	7	100	<0.001
MIA	10	100	
Lepidic	18	93.7	
Acinar	30	61.8	
Papillary	115	67.1	
Micropapillary	8	25	
Solid	47	48.3	
Others	8	58.3	
Stage			
IA	125	88.4	<0.001
IB	54	63.3	
IIA	20	30.8	
IIB	5	20	
IIIA	27	18.5	
IIIB	1	0	
IV	11	24.2	
HER2 IHC			
0	103	71.3	0.121
1+	103	57.1	
2+	31	73.5	
3+	6	50	
HER2-FISH			
Not amplified	238	65.6	0.070
Amplified	5	40	
HER2-DISH			
Not amplified	237	65.9	0.115
Amplified	9	50	
HER2-mutations			
Wild	214	67.2	0.598
Mutated	6	80	
EGFR-mutations			
Wild	113	62.6	0.049
Mutated	109	72.6	
KRAS-mutations			
Wild	197	67	0.660
Mutated	25	69.8	

Abbreviations: 5 years DFS, 5 years disease-free survival.

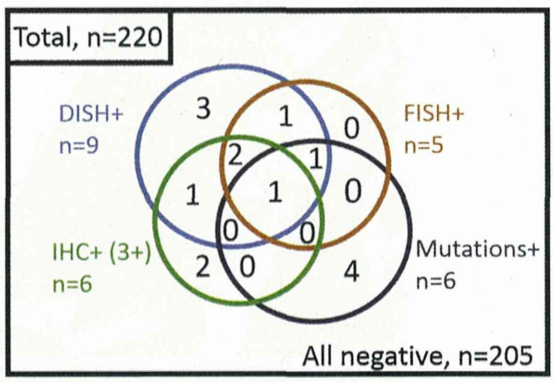

Fig. 3. Venn diagram illustrating relationships among IHC (green circle), DISH (blue circle), FISH (orange circle), and mutations (gray circle) in HER2 in patients with lung adenocarcinoma ($n = 220$). Diameters of each circle are roughly proportional to the number of positive cases for each test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In the last decade, clinical trials investigating the use of trastuzumab treatment in lung cancer patients with HER2 alterations have not been successful [11,13]. However, several novel anti-HER2 agents, including neratinib, dacomitinib, lapatinib, and afatinib, have recently been developed [14], and patient selection for the use of these novel agents tends to be based on the presence of *HER2* mutations. However, no definite criteria have been developed to indicate which patients are eligible for treatment with these drugs. Here, we investigated the concordance among IHC, FISH, and DISH and showed that HER2-positive cases were limited by every test. Additionally, we found positive correlations between *HER2* amplification by FISH/DISH and *HER2* mutation, but not HER2 protein expression.

HER2 overexpression in non-small cell lung cancers (NSCLCs), as assessed by IHC, is found in 2–6% of cases (with 3+ expression) in the literature [13,24–29]. In a meta-analysis of 40 published studies, a few papers showed an association between IHC and FISH [27,30,31]. Moreover, the number of cases in those studies was limited. Thus, the results from those papers are generally considered to be controversial. Recently, Grob et al. [32] reported that HER2 positivity by FISH was more frequently seen in IHC-positive cases. Our findings were similar to their results. Therefore, if we select patients for anti-HER2 therapy based on *HER2* amplification by FISH, as in breast cancer and gastric cancer, IHC may be relevant for detecting candidate case. On the other hand, our analysis of the association between IHC and mutations showed that only one of six cases with *HER2* mutations was IHC-positive (3+), and no significant concordance was observed between *HER2* mutations and IHC positivity. In contrast, cases with *HER2* mutations tended to present with amplification confirmed by FISH/DISH. Few papers have demonstrated an association between mutation status and amplification by FISH [31,33–36]. Arcila et al. found no *HER2* amplified cases in 11 lung cancer patients with activating *HER2* mutations [33], while Li et al.

Table 2
Concordance of FISH and DISH for HER2 amplification status.

DISH	FISH				
	Not amplified (<1.8)	Equivocal (1.8–2.2)	Low-level amplified (2.2–5)	High-level amplified (>5)	Total
Not amplified (<1.8)	229	0	0	0	229
Equivocal (1.8–2.2)	8	1	1	0	10
Low-level amplified (2.2–5)	0	0	2	0	2
High-level amplified (>5)	0	0	0	2	2
Total	237	1	3	2	243