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Non-terminal respiratory unit type lung adenocarcinoma has three distinct subtypes and is associated with poor prognosis

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ABSTRACT

Objectives: The characteristics of non-terminal respiratory unit (TRU) type lung adenocarcinoma are still unclear. The aim of the present study was to characterize non-TRU type lung adenocarcinoma.
Materials and methods: We analyzed the expression of mucins MUC5B and MUC5AC, as well as thyroid transcription factor-1 (TTF-1), using a tissue microarray comprising lung adenocarcinoma specimens from 244 consecutive patients. The presence of mutations in *EGFR* and *KRAS* were also determined.
Results: TTF-1, MUC5B, and MUC5AC were detected in 219 (89.8%), 75 (30.7%), and 33 cases (13.5%), respectively. Cluster analysis of protein expression profiles and *EGFR* and *KRAS* mutations yielded five groups of tumors as follows: TRU1-type [TTF-1(+), MUC5B(−), MUC5AC(−), *EGFR* mutations(−)]; TRU2-type [TTF-1(+), MUC5B(−), MUC5AC(−), *EGFR* mutations(+)]; Combined-type [TTF-1(+), MUC5B(+), and/or MUC5AC(+)]; Bronchiolar-type [TTF-1(−), MUC5B(+) and/or MUC5AC(+)]; and Null-type [TTF-1(−), MUC5B(−), MUC5AC(−), *EGFR* mutations(−), *KRAS* mutations(−)]. TRU-type tumors, which include TRU1- and TRU2-type tumors, were significantly associated with TRU morphology, whereas Bronchiolar-type tumors were associated with non-TRU morphology. Combined-type cases exhibited intermediate morphologies between TRU-type and Bronchiolar-type cases. TRU-type was associated with significantly better prognosis, followed by Combined-type, Bronchiolar-type, and Null-type (disease-free survival [DFS] $P = 0.017$; overall survival [OS], $P = 0.002$). Multivariate analyses indicated that non-TRU type tumors, which include Bronchiolar-, Combined-, Null-type tumors, were significantly correlated with poorer prognoses for DFS (hazard ratio = 1.785; 95% CI, 1.041–3.063; $P = 0.035$) and OS (hazard ratio = 1.928; 95% CI, 1.084–3.421; $P = 0.025$).
Conclusion: This study revealed three distinct subtypes of non-TRU type adenocarcinomas. Additionally, non-TRU type tumors were associated with worse prognoses than TRU type tumors. The results presented here may be useful for select patients should appropriate therapies become available.

1. Introduction

Lung cancer is the leading cause of cancer death worldwide [1], and adenocarcinoma is the most common histologic subtype of primary lung cancer [2,3]. Yatabe et al. [4–7] proposed the existence of a distinct subset of lung adenocarcinomas arising from a terminal respiratory unit (TRU), which develops in periphery of the lung parenchyma is similar in cell morphology to type II pneumocytes or Clara cells, is positive for the expression of thyroid transcription factor-1 (TTF-1), and harbors mutations in the gene encoding the epidermal growth factor receptor (*EGFR*). The clinicopathological characteristics of these tumors are generally well defined.

In contrast, few studies have focused on non-TRU type adenocarcinomas. For example, Yatabe et al. [4] reported that non-TRU type adenocarcinomas originate centrally, are TTF-1 negative, are solid in morphology and poorly differentiated, and are often necrotic. Other researchers have reported that a relatively high proportion of mucinous-type lung adenocarcinomas, particularly mucinous adenocarcinoma in situ (AIS), can be classified as non-TRU type adenocarcinomas, which do not express TTF-1 [8,9]. Mucinous AIS is characterized by the presence of mucous columnar cells, which

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are similar to mucinous cells of the bronchi-bronchiolar epithelium, and evidence suggests that it is a precursor of non-TRU type adenocarcinoma. The mucin core proteins MUC5B and MUC5AC are expressed in goblet-type epithelial cells in normal airways and serve as specific markers for these cells [10–12]. Therefore, we reasoned that MUC5B and MUC5AC may be candidate markers for non-TRU type adenocarcinoma.

In the present study, we analyzed the expression of MUC5B, MUC5AC, and TTF-1 in resected lung adenocarcinomas using immunohistochemistry to better define non-TRU type adenocarcinoma. The presence of *EGFR* and *KRAS* mutations was also determined.

2. Patients and methods

2.1. Patient selection and histologic 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. Tumor staging was performed according to the 7th Edition of the TNM classification of the International Union Against Cancer [13].

All resected specimens were formalin-fixed, sectioned, and stained with hematoxylin and eosin (H&E) in the conventional manner. Periodic acid Schiff (PAS) and Alcian-blue stains were performed to detect mucins. Elastic stains were also performed to detect invasion of the pleura or vessels. Slides were reviewed by two pathologists (AY, SS), who were blinded to patient outcomes. First, we attempted to divide the lung adenocarcinomas into TRU type and non-TRU type according to previous studies [4–7]. Because some of the adenocarcinomas exhibited a mixture of cytologic subtypes, we categorized the tumor as non-TRU type when morphologic resemblance to mucinous columnar cells of bronchi-bronchiolar epithelium and/or bronchial glandular cells was seen. All cases were classified according to IASLC/ATS/ERS criteria [14]. Findings of significant prognostic factors for lung adenocarcinomas prompted further analyses of lymphatic invasion, vascular invasion, pleural invasion, and/or tumor grade, which were assessed according to the IASLC/ATS/ERS criteria [14].

2.2. Tissue microarray (TMA)

A portion of the present cohort was described in our previous report [15]. Briefly, after case selection described above, paraffin-embedded tumor blocks with sufficient tissue were selected to prepare a TMA. The most representative region of the tumor was selected based on the morphology of the H&E-stained slide. Tissue cores measuring 2 mm in diameter were punched out from each donor tumor block using thin-walled stainless steel needles (Azumaya Medical Instruments Inc., Tokyo, Japan), and core were arrayed in a recipient paraffin block. Non-neoplastic lung tissue cores from selected patients were also arrayed in the same block.

2.3. Immunohistochemistry (IHC)

A standard two-step technique was implemented, using polymeric conjugates as secondary antibodies for MUC5B and MUC5AC [16], and the standard avidin–biotin–peroxidase complex technique was used to detect TTF-1. Primary anti-mucin antibodies were as follows: anti-MUC5B (H-300, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-MUC5AC (CLH2, Novocastra, New Castle Upon Tyne, UK). We performed immunohistochemistry using the SPT24 antibody clone (Novocastra), which was recently reported to be one of the most sensitive antibodies against TTF-1 [17–19]. IHC was performed using an auto-immunostainer (Benchmark, Ventana Medical Systems, Tucson, AZ, USA), according to the manufacturer's instructions. We confirmed that TTF-1 was expressed in the alveolar epithelial cells, bronchial epithelial cells, and focal cells of the bronchial submucosal glands in normal lung tissue. We also confirmed that MUC5B and MUC5AC were expressed in the goblet cells of the bronchial epithelium and bronchial submucosal glands in normal lung tissue.

Scoring was based on the distribution and intensity of staining according to previous study [20]. The sums of the distribution and intensity scores were expressed as total scores. Here, a total score of 0 was regarded as a negative result. Alveolar epithelial, bronchiolar epithelial, and bronchial gland cells in the same TMA section were used as internal controls. Immunostaining was scored independently by two investigators (AY, SS), and when the scores differed, a consensus decision was made by viewing the specimen with a multiheaded microscope.

In general, compared to TTF-1 expression, mucin expression could be heterogeneous within individual tumors. To avoid false-negative results (cases showing TTF-1(+), MUC5B(−), and MUC5AC(−) in TMA sections, but TTF-1(+), MUC5B(+), and/or MUC5AC(+) in whole-slide sections), we performed IHC using anti-mucin antibodies with whole-slide sections for such cases.

To detect co-expression of TTF-1 and MUC5B or MUC5AC in the same cells, double immunostaining was carried out. Briefly, sections were immunostained with mouse monoclonal anti-TTF-1 antibodies using a standard avidin–biotin–peroxidase complex technique with horseradish peroxidase (HRP) and DAB on an automated stainer. Sections were then incubated with rabbit monoclonal anti-MUC5B or anti-MUC5AC antibodies and visualized with alkaline phosphatase and a fuchsin substrate system on the automated stainer.

2.4. Somatic EGFR and KRAS mutations

EGFR and *KRAS* mutations were detected using published methods [21,22]. Briefly, a section from each tumor was frozen immediately, and a part of the section was observed microscopically to confirm that the sample included sufficient numbers of tumor cells. Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) was then employed to detect mutations within exons 18, 19, 20, and 21 of *EGFR* [21,22]. For detecting *KRAS* mutations, the mutagenic PCR-restriction enzyme fragment length polymorphism method was used according to a published method [23]. Because *KRAS* mutations were previously detected in codon 12, but not codon 13 [23,24], we only assayed for codon 12 mutations [21,22].

2.5. Statistics

Chi-square and Fisher's exact tests were used to analyze categorical data. Hierarchical cluster analysis was conducted using the Ward's minimum variance method. Tissue samples were clustered based on protein expression profiles and *EGFR* and *KRAS* mutations. The factors evaluated by univariate and multivariate analyses to assess their impact on overall survival (OS) and disease-free survival (DFS) rates were as follows: sex, age, smoking status, tumor size, stage, tumor grade, lymphatic invasion, vascular invasion, pleural invasion, *EGFR* status, and *KRAS* status. The survival rates were calculated using the Kaplan–Meier method, and the differences were analyzed using the log rank test. Multivariate analysis was performed using Cox's proportional hazards model. All statistical tests were two-sided at a 5% level of significance. Data analysis and summary graphs were generated using the JMP statistical software package, version 8 (SAS Institute, Cary, NC, USA).