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Original article

Cathepsin D as a potential prognostic marker for lung adenocarcinoma

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ABSTRACT

We previously identified cathepsin D as a possible marker for lung adenocarcinoma (AD). The purpose of the present study is to evaluate the correlation between cathepsin D expression and clinicopathological findings or prognosis. We conducted immunohistochemistry (IHC) to assess 150 AD tissues. For these 150 tumors, TTF-1 expression, *EGFR* and *KRAS* gene mutations, and *ALK* rearrangements had already been examined. Cathepsin D expression was detected in 44% (66 of 150, IHC score ≥1+) and 27.3% (41 of 150, IHC score ≥2+). Cathepsin D-positive (IHC score ≥2+) tumors were more poorly differentiated than cathepsin D-negative ones, while all lepidic predominant invasive adenocarcinomas showed no cathepsin D expression. Univariate analysis revealed a poor prognosis for cathepsin D-positive lung AD patients with an IHC score ≥2+ ($P = 0.044$). Cathepsin D expression was more frequent in TTF-1-negative than in TTF-1-positive ADs ($P = 0.034$), and more frequent in ADs with *EGFR* wild genotype than mutant *EGFR* ($P < 0.001$). Regarding AD patients with *ALK* rearrangements, 4 were positive for Cathepsin D, while 2 were negative. Cathepsin D expression is indicated to be a possible prognostic marker for lung AD and to correlate with a more poorly differentiated form.

Introduction

The molecular and histopathological features of lung cancer are important for understanding its potential for malignancy and sensitivity to therapies. Lung cancer comprises different subtypes with divergent and heterologous phenotypes; however, as yet, there is no accepted classification system for differentiating tumors on the basis of specific features. In working toward addressing this issue, a newly revised TNM Classification for Lung Cancer was published in 2009 [1]. This classification includes widely validated changes for the staging system [2]. Non-small-cell lung cancer (NSCLC) comprises about 80% of all lung cancers. Lung adenocarcinoma (AD) is the most common histologic subtype of lung cancer in most countries, accounting for almost half of all lung cancers [3]. TNM classification is an important prognostic factor for NSCLCs, including AD, and most patients with pathological (p) Stage I (pStage I) lung AD have a good prognosis. However, 20–30% of patients with pStage I lung AD do have a poor prognosis, and surgical therapy alone is an inadequate form of treatment for them [4]. The TNM classification thus cannot differentiate between these patients with pStage I lung AD, and a new predictive marker is needed to determine whether adjuvant therapy should be performed.

In a previous study, we reported that cathepsin D is expressed at a significantly higher level in lung AD compared with malignant pleural mesothelioma [5]. Cathepsin D is synthesized as a preproenzyme [6] and subsequently forms the intermediate active enzyme (48 kDa) by post-translational modifications. This molecule is divided into the 2-chain (34 and 14 kDa) mature enzyme after further cleavage occurs in the acidic lysosome [7,8]. Under normal conditions, less than 10% of cathepsin D escapes processing and is secreted [9]; however, cathepsin D is secreted aberrantly and excessively in various types of cancers [10,11], and is associated with increased cancer growth, invasion, and metastasis. Thus, this molecule might also be involved in lung AD progression, and may therefore be a useful prognostic marker for this form of cancer. Although previous reports have examined the correlation between cathepsin D expression and prognosis of patients with NSCLC by immunohistochemistry (IHC) [12,13], it is still controversial as to whether cathepsin D has any value as a prognostic indicator.

In the present study, we used IHC to evaluate cathepsin D expression in samples of tumor from patients with lung AD. We

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also analyzed the correlation between cathepsin D expression and clinical features in order to determine the appropriate criteria for evaluating cathepsin D expression. Additionally, we assessed the correlation of cathepsin D expression with thyroid transcription factor 1 (TTF-1) (a marker of lung AD) expression, epidermal growth factor receptor (*EGFR*) and *KRAS* mutation status and anaplastic lymphoma kinase (*ALK*) rearrangements involved in lung AD progression. These data will serve as background information for studies assessing the predictive value of molecular markers for sensitivity to cathepsin D-targeted therapy.

Materials and methods

Patient population

This study included 150 patients with lung invasive AD having undergone surgical resection at the National Cancer Center Hospital (Tokyo, Japan) between 1997 and 2003. An institutional review board approved this study.

All hematoxylin and eosin-stained or Elastica van Gieson stained slides and the immunohistochemical analyses available were reviewed by pathologists (T.M. and K.T.). Histologic diagnosis was based on the classification schema of the latest edition of the World Health Organization Classification [14], with the aid of immunohistochemical panels [15]. Furthermore, AD cases were graded on the basis of differentiation in a 3-tiered system. Briefly, well-differentiated tumors were consistent with predominant lepidic or papillary patterns, moderately differentiated tumors were consistent with a predominant acinar pattern, and poorly differentiated tumors were consistent with a solid growth pattern [16]. In addition, AD cases were classified according to International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) Classification of Lung Adenocarcinoma in Resection Specimens [17].

Tissue microarray construction

The most representative tumor areas were sampled for the tissue microarray (TMA). The TMAs were assembled with a tissue-array instrument (Azumaya, Tokyo, Japan). To reduce sampling bias due to tumor heterogeneity, we used 2 replicate 2.0-mm diameter cores from different areas of individual tumors.

Immunohistochemical analysis

For immunohistochemical staining, 4-μm-thick sections were routinely deparaffinized. The sections were exposed to 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity, and then washed in deionized water for 2–3 min. Heat-induced epitope retrieval was performed with citrate buffer solution (pH 6.0) (Muto Pure Chemicals Co., Japan). After the slides were allowed to cool at room temperature for about 30 min, they were rinsed with deionized water. The slides were then incubated with primary antibodies against cathepsin D (1:1000, 49/cathepsin D; Becton Dickinson, San Jose, CA) and TTF-1 (1:100, 8G7G3/1, Dako) for 1 h at room temperature. Immunoreactions were detected using the Envision-Plus system (Dako), and visualized with 3,3′-diaminobenzidine. Counterstaining was performed with hematoxylin.

IHC scoring system

We evaluated the immunostaining for cathepsin D at magnifications of 40× and 100×, using an Olympus BX40 microscope (Olympus, Tokyo, Japan). Immunoreactivity was classified on the basis of cytoplasmic staining intensity, and the following scoring system was used: negative (Score 0); weak intensity (Score 1), defined as positive immunoreactivity that was only detected at 100× magnification; moderate intensity (Score 2), defined as positive immunoreactivity that was easily detected at 40× magnification, but was weaker than that of intra-alveolar macrophages used as an internal positive control; strong intensity (Score 3), defined as positive immunoreactivity equal to, or more intense, than that of intra-alveolar macrophages. Immunoreactivity was defined as positive if ≥10% of tumor cells stained immunohistochemically positive for each score.

EGFR, KRAS mutational status, and ALK rearrangements

We detected 2 common *EGFR* mutations (deletions in exon 19 [DEL], and a point mutation at codon 858 in exon 21 [L858R]), as well as *KRAS* mutations (exons 1 and 2), using high-resolution melting analysis routinely performed at our institution [18]. *ALK* rearrangements were analyzed by immunohistochemistry, reverse transcription polymerase chain reaction, and/or chromogenic in situ hybridization assay [19].

Statistical analysis

A *t*-test for continuous variables and χ^2 tests for categorical variables were used. A *P* value of ≤0.05 was regarded as significant. Overall survival (OS) curves were calculated using the Kaplan–Meier method. Univariate survival analysis was performed with the log-rank test and Cox proportional hazard regression. The multivariate Cox model was subsequently used to evaluate variables with $P < 0.10$ as indicated by Wald's test. Statistical significance was set at $P \leq 0.05$.

Results

Cathepsin D expression status and clinicopathological findings in patients with lung AD

Representative cases of positive cathepsin D expression detected using IHC for lung AD are shown in Fig. 1a–c while negative cathepsin D expression for normal lung alveolar cells is shown in Fig. 1d. We evaluated all 150 cases of lung AD for cathepsin D immunoreactivity, using 2 criteria, namely, an IHC score ≥1+ or ≥2+. The cathepsin D expression rate for an IHC score of ≥1+ was 44% (66 of 150 cases), while the rate for a score of ≥2+ was 27.3% (41 of 150 cases).

The study cohort included 89 male and 61 female patients. The mean age at the time of diagnosis was 63.1 years (range, 37–82 years), and 84 of the patients were smokers. Lung AD tumors were grouped according to their histomorphology: 49 tumors were well differentiated, 52 were moderately differentiated, and 49 were poorly differentiated. With regard to the p-stage, 82 patients had lymph node metastasis while 100 patients had pStage I or II tumors (TNM 7th edition). Tumor size ranged from 1.1 to 9.0 cm (mean, 3.23 cm) (Table 1). The median follow-up time was 94.1 months (range, 2.3–146.9 months). The 5-year survival rate in this patient cohort was 63.1% (95% confidence interval, 59.2–67.1%), with 82 patients still alive at the time of this report.

We adopted the criterion of IHC score ≥2+ as indicative of positive cathepsin D expression, because cathepsin D expression and OS were only significantly correlated at this IHC score ($P = 0.044$). No significant differences were detected between cathepsin D expression and sex, age, smoking, lymph node metastasis, tumor stage, or tumor size (Table 1). Cathepsin D positive immunostaining was more frequently observed in poorly than in well-differentiated cases of AD ($P < 0.001$); poorly differentiated AD was significantly correlated with cathepsin D expression, compared to moderately