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# XAGE-1a and XAGE-1d are potential biomarkers of lung squamous cell carcinoma

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ABSTRACT

*Background:* Lung cancer is the leading cause of cancer deaths worldwide. We evaluated the diagnostic potential of sera XAGE-1a and XAGE-1d in lung cancer, both of which are variants of the X antigen family, member 1.
*Methods:* The expression levels of XAGE-1a and XAGE-1d in cell lines were determined using western blot analysis. Competitive ELISA was used to analyze XAGE-1a and XAGE-1d levels in culture supernatants and sera from 194 lung cancer patients and 194 healthy sex- and age-group-matched controls. To evaluate the diagnostic performance of these proteins, we also analyzed carcinoembryonic antigen (CEA) and cytokeratin 19 fragment (CYFRA 21–1) in culture supernatants and 388 sera using commercial ELISA kits.
*Results:* XAGE-1a and XAGE-1d proteins were expressed in both breast cancer and lung cancer cell lines, but they were only secreted by the latter. The areas under the curves (AUCs) for XAGE-1a and XAGE-1d were 0.787 and 0.806, respectively. The cutoff values (sensitivity, specificity) for XAGE-1a and XAGE-1d were 1.62 ng/ml (0.866, 0.572) and 2.51 ng/ml (0.871, 0.613), respectively. The diagnostic performance was improved for patients with squamous cell carcinoma. The AUC values for XAGE-1a and XAGE-1d for patients with squamous cell carcinoma versus a group containing all healthy participants and patients with any illness other than squamous cell carcinoma were similar to those for CEA and CYFRA 21–1. Better performance (AUC: 0.914) for all patients was obtained when using a combination of four markers (Random Forest).
*Conclusions:* Sera XAGE-1a and XAGE-1d are potential biomarkers for lung cancer; they display a diagnostic performance comparable to that of CEA or CYFRA 21–1. Further studies are needed to evaluate the diagnostic and prognostic potential of XAGE-1a and XAGE-1d in lung cancer.



## 1. Introduction

Lung cancer is the leading cause of cancer death worldwide (http://www.who.int/publications/en/). Lung cancers are generally classified into small-cell lung cancer (SCLC), which comprises 20% of lung cancers, and non-small-cell lung cancer (NSCLC). SCLC initially responds well to chemotherapy but commonly recurs with resistance. NSCLC comprises a diverse array of histological subtypes, including adenocarcinoma, bronchioloalveolar, squamous, anaplastic and large-cell carcinomas [1]. The high mortality rate in lung cancer patients is a reflection of poor prognosis. Despite the technical improvements in lung cancer therapy that have been achieved in the past few decades, the overall five-year survival rate after diagnosis remains <20%.

Screening approaches for the early detection of lung cancer in high-risk individuals, including chest X-rays [2] and more advanced spiral computed tomography scans [3,4], may lack diagnostic sensitivity and specificity; whether these approaches reduce mortality remains to be determined through large, randomized prospective trials [5]. DNA methylation can also be used as a biomarker for early detection as well as monitoring and as a target for chemoprevention. However, the biology underlying these processes requires further research [6]. Molecular and genetic studies on pre-invasive and early invasive cancers that seek to identify biomarkers in biological fluids for the early detection of lung cancer and chemoprevention should include clinical trials [7–11].

Although carbohydrate antigens 12–5 (CA125), 19–9 (CA199), 15–3 (CA153), cytokeratin 19 fragment (CYFRA 21–1), carcinoembryonic antigen (CEA) and neuron-specific enolase (NSE) are common serum markers for tumor diagnosis [12,13], none of these markers are perfect in terms of sensitivity and/or specificity [14–19]. Therefore, the search for novel biomarkers for the early diagnosis of lung cancer remains an important task.

*XAGE-1* was originally identified by computer-based screening using an expressed sequence tag database to search for PAGE/GAGE-related genes [20]. The *XAGE-1* gene is located on the X-

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chromosome [21] and exhibits characteristics of a cancer/testis antigen [22]. Four transcript variants (*XAGE-1a*, *XAGE-1b*, *XAGE-1c* and *XAGE-1d* [23–25]) have been previously characterized, but the GenBank database contains only two transcript variants: *XAGE-1a* (formerly *XAGE-1b*) and *XAGE-1d*. *XAGE-1* transcripts are present in normal testes, Ewing's sarcoma, melanocytic tumors, prostatic cancer, lung cancer, breast cancer, ovarian cancer, gastric cancer, alveolar rhabdomyosarcoma and some epithelial tumors [23,24,26–28]. The expression of XAGE-1a protein in lung cancer has been examined immunohistochemically using an anti-XAGE-1a antibody [27,28], and XAGE-1 antibody production has been observed in sera obtained from patients with lung adenocarcinoma [27] and prostate cancer [26].

XAGE-1a and XAGE-1d proteins are composed of 81 and 69 amino acids, respectively, and they share the same amino terminus up to amino acid 32. Several reports showing that sera from lung cancer patients react with XAGE-1a [27,29] suggest that XAGE-1 proteins may be present in the extracellular space where they would be exposed to antibodies, even though XAGE-1 contains a nuclear localization signal [24]. Here, we show that XAGE-1a and XAGE-1d proteins are expressed in and secreted by lung cancer cell lines. Each variant was selectively analyzed using C-terminal-specific antibodies. The serum levels of XAGE-1a and XAGE-1d proteins in lung cancer patients and healthy controls were evaluated as diagnostic biomarkers for lung cancer.

## 2. Materials and methods

### 2.1. Cell culture

The following cell lines were used for this analysis: the human lung embryonic fibroblast cell lines WI-38 (Korean Cell Line Bank, Seoul, Korea) and IMR-90 (American Type Culture Collection, Manassas, USA); the lung adenocarcinoma cell lines A549 and NIH-H460; and the human breast carcinoma cell lines MCF7 and MDA-MB-453 (Dr. Young Woo Park, KRIBB). Cells were cultured in RPMI1640 supplemented with 10% FBS in a 5% $CO_2$ atmosphere at 37 °C. For the detection of proteins in culture supernatants, cells were plated at 5 x $10^5$ cells in 100 mm culture dishes and maintained in a 10 ml medium. Culture supernatants were collected every 12 h, and total protein levels were analyzed.

### 2.2. Western blot analysis

Cells were incubated at 37 °C for 48 h and lysed using an extraction buffer. A total of 10 μg of protein was analyzed using 15% SDS-PAGE and transferred to a nitrocellulose membrane (Whatman, Rotenburg, Germany). The membrane was then blocked using 5% skim milk/Tween 20/TBS and incubated with diluted (1:1,000) anti-XAGE-1a or -XAGE-1d goat antibody (Abcam, Cambridge, USA) overnight at 4 °C. Peroxidase-conjugated anti-goat mouse IgG (Stressgen Biotechnologies, Victoria, Canada) was added, and the samples were incubated for 2 h at room temperature. The SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc., Rockford, IL) was used as the detection reagent. β-actin was used as the loading control.

### 2.3. Serum samples

Serum samples were obtained from 194 lung cancer patients and 194 healthy sex- and age-group-matched individuals during a health examination before the analysis. Healthy donors were individuals without any malignant diseases. All of the sera were divided into aliquots and stored at −80 °C until analysis. Lung cancer patients were diagnosed by pathological examination, including histological methods. Serum analyses were performed independently in a different laboratory. Written informed consent was obtained from all of the patients for the use of their sera in this study. The study was approved by the institutional review board of the National Cancer Center (Goyang-si, Korea).

### 2.4. XAGE-1a and XAGE-1d detection in culture supernatants and sera

The protein levels of XAGE-1a and XAGE-1d in culture supernatants and sera were measured using a competitive immunoassay [30]. Biotinylated XAGE-1a peptide (biotin-PEAGEEQPQV) (Peptron, Daejeon, Korea) competes with the XAGE-1a peptide (CPEAGEEQPQV) or XAGE-1a protein in binding to the XAGE-1a-specific antibody. A 100 μl aliquot of culture supernatant or serum was mixed with 100 μl of biotinylated XAGE-1a peptide (40 ng/ml), added to a well of a 96-well microtiter plate coated with the anti-XAGE-1a antibody (1 μg/well) and then blocked with phosphate-buffered saline (PBS) containing 5% BSA. After incubation for 2 h at room temperature with shaking, the plate was washed five times with 200 μl/well of washing buffer (PBS containing 0.05% Tween-20). Streptavidin-HRP (Pierce, 1:10,000) was added, and the plate was incubated for 1 h. The plate was then washed five times, and TMB solution was added. Plates were read at 450 nm using a microplate spectrophotometer (Fusion-α, Packard Bioscience, Meriden, CT). The concentration of XAGE-1a was calculated using linear regression equations for the standard curve (Supplementary Fig. S1B and S1D) from the percentage bound (% $B/B_0$) using the following formula: % $B/B_0$ = absorbance of standard or sample/absorbance of $B_0 \times 100\%$. For the protein concentrations, the molecular weight ratios of the biotinylated peptides and proteins were considered (1:4.068 and 1:4.869 for XAGE-1a and XAGE-1d, respectively). For the measurement of XAGE-1d, XAGE-1d-peptide (CGFGFRRQGEDNT) (Abcam), biotinylated XAGE-1d peptide (biotin-GFGFRRQGEDNT) (Peptron) and an XAGE-1d-specific antibody were used. CEA and CYFRA 21–1 protein levels in the sera were analyzed using an ELISA kit (Monobind Inc., Lake Forest, USA) and an EIA kit (Fujirebio Diagnostics Inc., Gothenburg, Sweden), respectively, according to the manufacturers' protocols.

### 2.5. Statistical analysis

Because of their non-normal distributions, the differences between the medians of the groups were evaluated using Mann–Whitney *U*-tests. To evaluate the utility of XAGE-1a and XAGE-1d levels for cancer diagnosis, we constructed receiver operating characteristic (ROC) curves by calculating the sensitivities and specificities of each marker using the MedCalc statistical software package, ver 11.5 (Mariakerke, Belgium). AUC for ROC curves were compared according to the method described by Hanley et al. [31]. A $p < 0.05$ was considered statistically significant for all tests. We used six machine-learning algorithms to evaluate the performance of a combination of the four markers, including 2 Bayesian methods (Bayesian network and naïve Bayes) and 4 tree-based methods (BFTree, J48graft, random forest and simple CART). WEKA was used for multivariate analyses [32].

## 3. Results

### 3.1. Detection of XAGE-1a and XAGE-1d in cell lines

Many alternative transcripts have been reported for the *XAGE-1* gene. To investigate the cellular protein expression of XAGE-1a and XAGE-1d in human lung fibroblast cell lines, lung adenocarcinoma cell lines and breast cancer cell lines, whole-cell lysates were subject to western blot analysis. C-terminal-specific antibodies for XAGE-1a and XAGE-1d showed bands at ~9 kDa and ~7 kDa, respectively (Fig. 1A and B). This result was consistent with previous results showing the expression of *XAGE-1a* (formerly *1b*, 81 A.A.) and *XAGE-1d* but