Drebrin: A new oncofetal biomarker associated with prognosis of lung adenocarcinoma

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A B S T R A C T

Objectives: With the aim of searching for novel oncofetal tumor biomarkers of lung adenocarcinoma other than carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP), we developed a strategy involving monoclonal antibodies generated from embryonic tissue of miniature swine.

Materials and methods: Using immunohistochemistry, we selected suitable hybridoma clones that were reactive against swine fetal lung but not adult lung using tissue microarray loading of human normal lung, lung cancer, and fetal and adult swine tissues.

Results: The selected clones included several that were uniquely reactive against both swine fetal lung and human lung adenocarcinoma, and protein microarray revealed that the antigen they recognized was “drebrin” (DBN1). We then examined the association between the pattern of drebrin expression and the clinicopathological characteristics of lung adenocarcinoma using surgically resected samples of human lung adenocarcinoma. Two hundred formalin-fixed and paraffin-embedded tumor samples were immunostained for drebrin using clone B246, one of the clones that were reactive against drebrin. The cases were divided into those with strong (n = 85) and weak (n = 115) drebrin expression. In terms of disease-free survival, cases showing strong drebrin expression had a significantly poorer prognosis than those with weak drebrin expression (p = 0.033).

Conclusion: The present findings indicate that “drebrin” is a unique oncofetal protein that can be applied as a new biomarker of lung adenocarcinoma.

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1. Introduction

Lung cancer is one of the most common human malignancies worldwide and has a poor prognosis. As most lung cancer patients are diagnosed at an advanced stage, the prognosis remains poor. Adenocarcinoma is the most frequent histological subtype and its incidence is increasing [1]. Although the introduction of drugs that can target EGFR mutation, ALK translocation, etc. has increased the survival time of patients, advanced adenocarcinoma still shows progression and is associated with a high mortality rate [2]. Therefore, it is very important to establish reliable biomarkers of lung adenocarcinoma that can be used for detection of primary and recurrent tumors.

Tumor biomarkers are a major tool for diagnosis of cancer, both clinically and pathologically. Serum tumor biomarkers have been commonly used to support cancer diagnosis and for assessment of the effects of therapy. Tumor biomarkers are classified into four categories: (i) oncofetal antigens, (ii) carbohydrate antigens as components of glycolipids or glycoproteins, (iii) oncogene and anti-oncogene-associated proteins, and (iv) miscellaneous, including tissue-specific enzymes, cytoskeletal proteins, etc. [3]. Serum levels of lung cancer biomarkers, such as CEA, cytokeratin 19, squamous cell carcinoma antigen, pro-gastrin releasing peptide and neuron-specific enolase have been routinely assessed in clinical medicine.
On the other hand, various immunohistochemical tumor biomarkers have been employed for support of pathological diagnosis and are helpful for distinguishing histological types of lung cancer and some histological subtypes of lung adenocarcinoma. Examples of these include napsin A, thyroid transcription factor 1, p-63, p-40 (ΔNp-63), cytokeratin 5/6, chromogranin A, synaptophysin, neural cell adhesion molecule and hepatocyte nuclear factor-4α [8–12], TTF-1 as a marker of lung adenocarcinoma and cytokeratin 7/20 is used for distinguishing primary from metastatic adenocarcinomas [13,14], whereas Ki-67 is a useful proliferation marker, and positivity for p53 is a certain biomarker of malignancy [15,16]. Among these various tumor biomarkers, oncofetal protein is unique, being an antigen that is expressed during gestation, shows considerably reduced expression or loss after birth, but re-increases during neoplastic growth [3]. Since they are not normally expressed in human adult tissues, embryonic and/or fetal proteins, such as CEA and insulin-like growth factor II mRNA-binding protein 3 (IMP3), are extremely specific tumor biomarkers [4,17].

Few experimental attempts have been made to identify new oncofetal tumor biomarkers systematically. One of the reasons for this is the ethical objection to the use of human fetal tissues as experimental materials. On the other hand, the use of swine in biomedical research, particularly in the fields of surgery and physiology, has become well established [18]. Since swine mRNA shows more than 80% homology with human mRNA, embryonic swine tissue is an ideal material for detection of human embryonic biomarkers. We have recently established a new biomarker of lung adenocarcinoma, dimethylarginine dimethylamino-hydrolase 2 (DDAH2), which is expressed in the tumor stroma. The monoclonal antibody reactive against DDAH2 was generated from embryonic miniature swine tissue [19].

In the present study, we modified our strategy for systematic identification of new oncofetal biomarkers of lung adenocarcinoma using monoclonal antibodies generated from nuclear fractionation of fetal lung tissues of miniature swine and human lung adenocarcinoma tissue. Finally, we detected “drebrin” (DBN1) as a novel and useful oncofetal biomarker.

2. Materials and methods

2.1. Monoclonal antibody production (Supplementary Fig. S1)

Normal fetuses of CLAWN strain miniature swine (Kagoshima Miniature Swine Research Center, Kagoshima, Japan) at 7-weeks gestation were removed surgically from maternal swine. The fetal lungs were then resected and minced, immersed in the dispersion medium (supplementary Materials and Methods. 1). The nuclear fraction was isolated from swine fetal lung cells (LysoPure Nuclear and Cyttoplasmic Extractor Kit, Wako Osaka, Japan), and the isolated fraction was stored at −80°C. Nuclear fractions from three cases of human lung adenocarcinoma were prepared similarly. The protein concentration was determined (bicinchoninic acid protein assay, Thermo Fisher Scientific, Waltham, MA). For the first immunization, the nuclear fraction of swine fetal lung and an equivalent amount of Complete H-37 Ra adjuvant (Becton, Dickinson and Company, Franklin Lakes, NJ) were mixed and prepared as the first antigen. This adjuvant/antigen emulsion was then injected into the hock of 7-week-old BALB/c (CLEA Japan, Tokyo, Japan) mice on day-1. For the secondary immunization, the nuclear fractionation of human lung adenocarcinoma and incomplete Freund adjuvant (Becton, Dickinson and Company) were mixed and injected similarly on day-10. The hybridoma formation and cloning were performed using protocol reported previously [19].

2.2. Enzyme-linked immunosorbent assay (ELISA)

Goat anti-mouse ImmunoglobulinG (IgG) (Jackson ImmunoResearch, West Grove, PA) 5 μg/ml was coated in each well of a 96-well plate (Sumitomo Bakelite Co., Tokyo, Japan) and left at 4°C overnight. After blocking with 2.5% skim milk for 1-h, the hybridoma supernatant was added at room temperature for 30-min. After washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG Fc antibody (1:100,000, Abcam, Cambridge, United Kingdom) was added at room temperature for 30-min. After washing, TMB Substrate Solution (Thermo Fisher Scientific) added for 1-min. After stop reaction, the absorbance was measured at 450/570 nm.

2.3. Immunohistochemistry

Three-micrometer-thick sections were prepared from 10% formalin-fixed and paraffin-embedded blocks. Tissue microarray blocks comprised of miniaturized swine fetal and adult lung tissues, 22-cases of human lung cancer (17-cases of adenocarcinoma, 3-cases of squamous cell carcinoma, and 2-cases of large cell neuroendocrine carcinoma), and the corresponding human normal lung tissue from each carcinoma case. The unstained sections were deparaffinized and rehydrated. Antigen retrieval was performed using 10 mM Tris-EDTA buffer (pH 9.0) at 105°C for 10-min. Immunohistochemistry for screening antibodies of hybridoma supernatant was performed using a BenchMark ULTRA autostainer (Roche Diagnostics, Basel, Switzerland) with an ultraview DAB universal kit (Roche Diagnostics). Hematoxylin was used for counterstaining.

Immunohistochemistry for validation was performed using B246 (1.8 μg/ml) as a primary antibody. Two pathologists (SI and REH) evaluated all cases independently without prior knowledge of the clinicopathological characteristics. Results of immunohistochemistry for drebrin were evaluated in terms of the H-score (Supplementary materials and Methods. 2) [20].

2.4. Protein microarray

A HUProt(TM) Human Proteome Microarray v2.0 (CDI Laboratories, Baltimore, MD) service provided by Filgen Inc. (Nagoya, Japan) was used for analysis of the B246 antigen. The human proteome microarray was performed using protocols reported previously [21].

2.5. Western blotting

The Western blotting was performed using protocol reported previously [19]. GST-tag human drebrin E recombinant protein (Abnova, Taipei, Taiwan) was used as a positive control. The primary antibodies were B246 at 0.07 μg/ml, C220 at 0.2 μg/ml, C313 at 0.13 μg/ml, C344 at 0.1 μg/ml, a rabbit polyclonal anti-drebrin A antibody (DASZ) (1:100, IBL, Gunma, Japan), and a mouse monoclonal anti-beta actin antibody (1:5000, Sigma-Aldrich, St. Louis, MO) diluted 1:5000. The secondary antibodies were a HRP-conjugated goat anti-mouse IgG antibody (1:50,000, Abcam) or a HRP-conjugated goat anti-rabbit IgG antibody (1:100,000, Sigma-Aldrich).

2.6. Cell lines and culture conditions

The human lung adenocarcinoma cell lines A549 and PC-9 were purchased from RIKEN BRC (Ibaraki, Japan). A human lung adenocarcinoma cell line Calu-3 was purchased from the American Type Culture Collection (Manassas, VA). A549 was maintained in DMEM/F12 (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS). PC-9 was maintained in RPMI 1640 medium
(Thermo Fisher Scientific) supplemented with 10% FBS. Calu-3 was maintained in MEM alpha (Thermo Fisher Scientific) with 10% FBS. All cells were cultured at 37°C in a 5% CO2 incubator.

2.7. Patient selection

We selected 200-specimens of formalin-fixed and paraffin-embedded lung adenocarcinomas that had been surgically resected at the University of Tsukuba Hospital (Ibaraki, Japan) between 1999 and 2007. Follow-up information for all of the corresponding patients was available from the medical records. Informed consent for study of their materials had been obtained from all of the patients. The adenocarcinomas were classified according to the World Health Organization (WHO) classification of malignant tumors (fourth edition) and the UICC TNM classification of malignant tumors (seventh edition) [22,23].

2.8. Statistical analysis

Statistical analysis was performed using SPSS 24 (SPSS, Chicago, Illinois). Since the aim of the study was to clarify the correlation between drebrin expression and prognosis, we used drebrin expression and the outcome of the patients as variables for drawing the ROC curve. Correlations of clinicopathological features with drebrin expression were analyzed using the chi-squared test. Disease-free survival was compared with drebrin expression using the Kaplan-Meier method, and the significance of differences between survival curves was assessed using log-rank test. Statistical significance was defined as p<0.05.

3. Results

3.1. Screening for mouse monoclonal antibodies

A total of 484-hybridomas were generated, and by ELISA we pre-screened 284-clones that grew well and expressed the IgG subtype. From these 284-clones, we selected those reacting against both swine fetal lung and human lung adenocarcinoma by immunohistochemistry using tissue microarrays. Immunohistochemical analysis showed that 22-clones reacted with the nucleus and 89-clones reacted with the cytoplasm. We then selected 11-clones that were more reactive against swine fetal lung and human lung adenocarcinoma than against swine adult lung and human normal lung. Of these clones, five showed cytoplasmatic staining for human cancer cells and swine fetal lung tissue and two showed nuclear staining for human cancer cells and fetal lung tissue, although the staining patterns were slightly different. The remaining four clones showed clearer and stronger reactivity with the cytoplasm and cytomembrane of swine fetal lung and human lung cancer than with those of swine adult lung and human normal lung tissue. Among these four clones, we focused on one (B246) that reacted most strongly with human lung adenocarcinoma (10/15 cases 67%) and swine fetal lung (Fig. 1).

3.2. Antigen identification

The antigen that was reactive against B246 was examined using human proteome microarrays. As shown in Fig. 2, the antibody purified from clone B246 reacted strongly and specifically with “drebrin” protein. To confirm this result, we carried out Western blotting to examine the specificity of B246 against the human lung adenocarcinoma cell lines (PC-9, A549, and Calu-3) and swine fetal lung, employing human recombinant drebrin protein as a positive control. The positive signal for drebrin was detected in all samples except for swine adult lung (Fig. 3A). The polyclonal rabbit anti-drebrin A antibody (DAS2) specific for drebrin A was reactive against mouse brain, but not with the other samples (Fig. 3A). These results confirmed the findings of human proteome microarray analysis and indicated that B246 reacted against drebrin but was not specific for drebrin A. B246 and mouse monoclonal anti-drebrin A/E antibody (M2F6) reacted identically with various tissues (Supplementary Fig. S2). Three other clones (C220, C313 and C344) reacted similarly against drebrin by Western blotting (Fig. 3B). All four of the clones reacted with the immunoprecipitation samples using M2F6. In addition, M2F6 reacted with the immunoprecipitation samples obtained using the antibodies from clones B246, C220, and C344 (data not shown).

3.3. Expression of drebrin in resected human lung adenocarcinoma samples

Using immunohistochemistry, we investigated the clinical implications of drebrin expression in 200 surgically resected cases of lung adenocarcinoma. Drebrin positivity, i.e. A H-score of 10 or more, was detected in 156 (78%) of the cases. ROC curve analysis showed that a coordinate of 35 (sensitivity 0.515 and 0.379 (1-specificity)) was the minimum distance to the upper left corner, and this point was adopted as the cut-off point in this study. Scores below 35 were judged as weak expression, and those above 35 as strong expression (data not shown). Drebrin immunohistochemistry using B246 (purified antibody) delineated the samples into those with strong (n=85) and weak (n=115) drebrin expression according to the H-score, as described in Materials and Methods. Among invasive adenocarcinomas, strong drebrin expression was detected in 24 out of 50 lepidic adenocarcinomas (48%), 10 out of 21 acinar adenocarcinomas (47.6%), 15 out of 26 papillary adenocarcinomas (57.6%), 3 out of 4 micropapillary adenocarcinomas (75%), 21 out of 36 solid adenocarcinomas (58.3%), and 5 out of 21 invasive mucinous adenocarcinomas (23.8%) (Fig. 4A–F and Table 1). Interestingly, in several lung adenocarcinoma cases, drebrin was expressed more predominantly in the periphery than in the central core region (Fig. 4I–K). Among non-invasive lung adenocarcinomas, strong drebrin expression was detected in 2 out of 27 adenocarcinomas in situ (AIS) (7.4%) and 5 out of 15 minimally invasive adenocarcinomas (MIA) (33.3%) (Fig. 4G, H and Table 1).

We assessed the correlation between drebrin expression and clinicopathological features of patients using chi-squared test. Drebrin expression was significantly correlated with gender, pathological stage, lymph node status, pleural invasion, vascular invasion, lymphatic permeation, and histological subtype, but not with age. The Kaplan-Meier curves demonstrated a significant difference in disease-free survival between the groups with strong and weak drebrin expression (p = 0.033) (Fig. 5). High expression of drebrin was significantly associated with poorer outcome relative to low expression.

3.4. Expression of drebrin in resected human cancers of other organs

Using immunohistochemistry with tissue microarray loading, we also analyzed cancers of other organs for which no such data have been reported previously (Supplementary Table S1). Drebrin was expressed in gastric cancer (3/9), breast cancer (6/9), gallbladder cancer (4/9), pancreatic cancer (5/9), ovarian cancer (5/9), cervical cancer (2/9), endometrial cancer (9/9), seminoma (3/5), neuroblastoma (2/2), and hepatoblastoma (2/3).

4. Discussion

In the present study, we employed a unique strategy to search for novel oncofetal protein biomarkers involving immunization with two different antigens from swine fetal lung and human lung
Fig. 1. Immunohistochemical screening using hybridoma supernatant.Clone B246 showed a positive reaction with swine fetal lung (A) and human lung adenocarcinomas (B), but was negative with adult swine lung (C) and human normal lung (D).

Fig. 2. Results of human proteome microarray. Subarray of B246 (A) and negative control (B) with fluorescence detection at 532 nm excitation. Drebrin (DBN1) shows a strong interaction with clone B246.
adenocarcinoma. This led to the discovery of proteins that are commonly expressed in both swine fetal lung tissue and human lung adenocarcinoma but not in normal human lung tissue. This is thought to be a unique approach for investigating various oncofetal tumor markers systemically (Supplementary Fig. S1).

For this method, we developed four monoclonal antibodies of interest that were reactive against human lung adenocarcinoma and swine fetal lung tissue, but not human lung tissue. The four clones from which the antibodies were derived recognized the oncofetal protein, “drebrin”. Drebrin (developmentally regulated brain protein) was originally identified in the brain of 10-day chick (Gallus gallus) embryos [24, 25]. Drebrin is one of the actin binding proteins, being especially involved in the formation of neurites and protrusions of motile cells. There are two major isoforms, drebrin E (embryonic) and drebrin A (adult), which are generated by alternative splicing of the drebrin gene (DBN1) [26]. Drebrin A is considered to be a neuron-specific isoform expressed in mature neurons. It is mainly localized in dendritic spines and involved in the formation of neurites, and in synaptic plasticity [27]. Drebrin E, on the other hand, is a ubiquitous isoform expressed abundantly in cell bodies, and is involved in axonal growth during neural development through actin-myosin interactions [28]. The difference between drebrins E and A is due to an inserted sequence; the sequences are almost identical, except for an internal insert sequence in drebrin A. Our study (Fig. 3A) indicated that clone B246 might recognize a region common to both drebrins A and E. More detailed analysis of the specificity of the antibodies will be required. Although our initial goal was to detect oncofetal “nuclear protein”, drebrin is distributed to the membrane and/or cytoplasm, and not the nucleus. We suspect that the nuclear fraction had not been isolated completely, and that the membrane and cytoplasmic fraction might be carried over into the roughly isolated nuclear fraction. It is also possible that drebrin may bind to a transcriptional factor or nucleic acid easily, and thus carried into the nuclear fraction, since drebrin has been reported to bind spikar, which acts as a transcriptional co-activator for nuclear receptors in dendritic spines [29].

As drebrin was first detected in the field of neurology, most previous studies of drebrin expression and function have been in the neurobiology field. However, drebrin expression has also been recently reported in non-neuronal cells and tissues. In testis, drebrin E regulates actin filament bundles at both the apical and basal ectoplasmic specialization during spermatogenesis [30]. In T lymphocytes, drebrin binds F-actin and regulates recruitment of the chemokine receptor CXCR4 [31].

On the other hand, in the field of oncology, several studies of drebrin have been reported. Drebrin is involved in invasion and

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**Table 1**

Drebrin expression and clinicopathological features in patients with lung adenocarcinoma.

<table>
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<th>Weak</th>
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<tr>
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Drebrin immunohistochemistry using the B246 antibody on formalin-fixed and paraffin-embedded specimens of lung adenocarcinoma. B246 showed strong expression with lepidic (A), acinar (B), papillary (C), micropapillary (D), solid (E), invasive mucinous (F), adenocarcinoma in situ (G), and minimally invasive adenocarcinoma (H). Histological section of drebrin staining in a case of adenocarcinoma (I), and corresponding high-magnification images (J, K). Drebrin is expressed more predominantly at the periphery (J) than in central regions (K).

Migration of glioma or metastasis of colon cancer [32,33]. Drebrin also interacts with the growth factor progranulin and in bladder cancer is involved in invasion and anchorage-independent growth, thus regulating tumor formation [34]. In lung cancers, the drebrin gene has been reported as a genomic marker associated with the postoperative outcome of non-small cell lung cancer [35].

The level of drebrin expression increases stepwise from AIS (7.4%) to MIA (33.3%), and to invasive adenocarcinoma (78/158, 49%) (Table 1), and is significantly associated with prognosis (Fig. 5). Our results regarding the association between drebrin expression and prognosis of lung adenocarcinoma are similar to those obtained in a previous study of non-small cell lung carcinoma by genomic microarray analysis [35]. In the present study, however, we demonstrated drebrin over-expression in lung adenocarcinoma at the protein level. Based on these findings, we can speculate that drebrin may be associated with invasion of lung adenocarcinoma, as has been reported for glioma and bladder cancer. It is interesting that in several cases of lung adenocarcinoma drebrin was expressed more predominantly in the tumor periphery than in the central core (Fig. 4I–K). This finding may indicate that drebrin expression is associated with migration, being similar to the situation in glioma [32].
Our tissue microarray analysis showed that drebrin was expressed in various types of malignant tumors (Supplementary Table S2). Interestingly, drebrin was expressed strongly in neuroblastoma and hepatoblastoma. These results indicate that drebrin could be useful as a tumor biomarker for cancers in general, and it would be particularly interesting to study its expression in various types of blastoma, since drebrin is known to be a fetal protein.

In conclusion, we have demonstrated that drebrin is a novel biomarker of lung adenocarcinoma and a prognostic indicator. Like CEA, drebrin is an oncopetal biomarker and most normal human tissues (organs) are negative for drebrin. Therefore, it might be possible to use drebrin as a serum biomarker to detect lung adenocarcinoma and various other cancers. Bronchoalveolar lavage fluid and/or sputum might be more practical targets for use of drebrin as a biomarker in a clinical setting.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data


References


