

Identification of DAB2 and Intelectin-1 as Novel Positive Immunohistochemical Markers of Epithelioid Mesothelioma by Transcriptome Microarray Analysis for its Differentiation from Lung Adenocarcinoma

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Abstract

There is currently no absolute immunohistochemical positive marker for the definite diagnosis of malignant mesothelioma. Therefore, the aim of this study was to identify novel positive markers of malignant mesothelioma. Whole genome gene expression analysis was performed using RNA extracted from formalin-fixed paraffin-embedded tissue sections of epithelioid mesothelioma (EM) and lung adenocarcinoma (LAC). Gene expression analysis revealed that disabled homolog 2 (DAB2) and Intelectin-1 had significantly higher expression in EM compared to that in LAC. The increased mRNA expression of DAB2 and Intelectin-1 was validated by RT-PCR of RNA from tumor tissue and protein expression was validated by western blotting of five mesothelioma cell lines. The utility of DAB2 and Intelectin-1 in the differential diagnosis of EM and LAC was examined by an immunohistochemical study of 75 cases of epithelioid mesothelioma and 67 cases of lung adenocarcinoma. The positive rates of DAB2 and Intelectin-1 expression in EM were 80.0% and 76.0%, respectively, and 3.0% and 0%, respectively, in LAC. Immunohistochemically, the sensitivity and specificity of DAB2 was 80% and 97% and those of Intelectin-1 were 76% and 100% for differentiation of EM from LAC. In conclusion, DAB2 and Intelectin-1 are newly identified positive markers of mesothelioma and have potential to be included in future immunohistochemical marker panels for differentiation of EM from LAC.

Key words

DAB2, Intelectin-1, gene expression analysis, immunohistochemistry, epithelioid mesothelioma, lung adenocarcinoma

Introduction

Malignant mesothelioma is a rare and fatal malignant tumor (1). In Japan, the mesothelioma death rate is increasing, approaching 1500 deaths in 2015, increased from 500 in 1995 according to Vital Statistics data published by the Ministry of Health, Labour and Welfare, Japan (2). Similarly, the death rate due to mesothelioma is increasing globally, including the UK and Ireland (3), USA and other developing countries (4). Asbestos exposure is the main risk factor for malignant pleural mesothelioma, including both occupational and environmental exposure. The time interval between first exposure to asbestos and diagnosis of mesothelioma is speculated to range from 20 to 50 years. Apart from the relatively long time it takes for asbestos to cause disease, delayed onset of symptoms can contribute to late-stage diagnosis and by then, the cancer spreads into the thoracic cavity and is more difficult to treat. Therefore, accurate diagnosis of mesothelioma is essential for its correct management.

A common site of origin of malignant mesothelioma is the pleura followed by other tissues including the peritoneum, pericardium and tunica vaginalis. Malignant mesothelioma is classified into three major histological subtypes: epithelioid, sarcomatoid, and biphasic as described in the 2015 World Health Organization (WHO) histological classification of tumors of lung and pleura, 2015 (5). Epithelioid mesothelioma (EM), which constitutes more than 60% of all mesothelioma, is the most common histological subtype, and has a relatively better prognosis than sarcomatoid or biphasic mesothelioma. EM shows various histological patterns including tubulopapillary, micropapillary, acinar, adenomatoid, and solid. As EM closely resembles other malignant tumors showing pseudomesotheliomatous growth patterns, such as those of primarily lung carcinoma, breast carcinoma, and cancer that affects the lining of internal organs, the diagnosis of malignant mesothelioma is challenging, both histopathologically and clinically. Currently, the final diagnosis of malignant mesothelioma requires thorough reviewing of clinico-radiological and pathological findings (gross examination and histological findings in tissue samples) with adequate

immunohistochemical and/or genetic analyses. As an immunohistochemical marker with absolute sensitivity and specificity is not yet available, the search for additional novel immunohistochemical markers is critical.

The aim of this study was to identify novel positive immunohistochemical markers by analysis of whole gene expression data using microarray gene chips. We performed gene expression analysis on epithelioid cells dissected from formalin-fixed paraffin-embedded (FFPE) tissue of EM and lung adenocarcinoma (LAC) and identified several novel genes that are differentially expressed between EM and LAC. Of these, we identified disabled homolog 2 (DAB2) and Intelectin-1 as potential novel positive immunohistochemical markers of EM for differentiation from LAC.

Materials and methods

Patients and histological samples

The materials included in this study were obtained from the archives of the Department of Pathology, Hiroshima University. The study group consisted of 75 patients with Epithelioid mesothelioma (EM) who had undergone thoracoscopic pleural biopsy, pleurectomy/decortication, extrapleural pneumonectomy, or autopsy between 2000 and 2016. Between 2005 and 2016, 67 lung adenocarcinoma (LAC) cases were also obtained via thoracoscopic surgical segmentectomy or lobectomy of lung harboring adenocarcinoma. All microscopic slides were reviewed and reclassified using the current WHO histological classification of tumors of lung and pleura, 2015 (6) by four pathologists (MK, KK, VJA, and YT). Pathological diagnosis of each case was confirmed by histological findings and an immunohistochemical marker panel recommended by Guidelines for Pathologic Diagnosis of Malignant Mesothelioma: 2012 Update of the Consensus Statement from the International Mesothelioma Interest Group (IMIG) (7) and current 2015 WHO histological classification of tumors of the lung, pleura, thymus and heart (6).

Anonymized tissue samples were provided by the Department of Pathology for gene

expression and immunohistochemical analysis. This study was carried out in accordance with the Ethics Guidelines for Human Genome/Gene Research enacted by the Japanese Government for the collection of tissue specimens and was approved by the institutional ethics review committee (Hiroshima University E-974).

Gene expression analysis

Identification of genes with marked difference between epithelioid mesothelioma and lung adenocarcinoma

Formalin-fixed paraffin-embedded sections from six EM cases and six LAC cases were used for gene expression analysis. RNA extraction for gene expression analysis was performed from papillary or solid growth of tumor cells in each specimen. Five 10 μ m thick FFPE tumor tissue sections, each approximately 1 cm in diameter, were processed for total RNA extraction using the Maxwell RSC RNA FFPE Kit (Promega KK, Tokyo, Japan) according to the manufacturer's protocol. Briefly, after deparaffinization and lysis with proteinase K, the samples were treated with DNase I for 15 min at room temperature. Following this, RNA purification was carried out according to the manufacturer's protocol using a Maxwell RSC automation instrument (Promega). RNA quality check and quantification was performed as described previously (8) and RNA with an absorbance ratio of ≥ 1.9 between 260 nm and 280 nm was used for microarray analysis. The Human Transcriptome 2.0 GeneChip Array (Affymetrix, Santa Clara, CA, USA) containing gene transcript sets of 44,699 protein coding and 22,829 non-protein coding clusters was used to analyze gene expression profiles. Total RNA was amplified and labeled with a 3' IVT Labeling Kit (Affymetrix) before hybridization onto the GeneChip. Briefly, 100 ng total RNA was amplified with GeneChip 3' IVT Pico kit (Affymetrix) to generate 30 μ g of SenseRNA according to the manufacturer's protocol. SenseRNA (25 μ g) was labeled with a 3' IVT Labeling Kit (Affymetrix) and hybridized to a Human Transcriptome 2.0 GeneChip (Affymetrix) as described previously (8). The data were analyzed using the Gene Expression Console Software (Affymetrix), and further statistical analyses were performed

using the Subio Software Platform (Subio, Amami-shi, Japan) to plot graphs and for fold change of expression and hierarchical clustering.

Validation of gene expression analysis

Real-time reverse transcriptase polymerase chain reaction

The same six cases of EM and LAC that were analyzed for gene expression profiling were used to validate the microarray expression data by mRNA expression. The relative mRNA expression of DAB2 and Intelectin-1 was assessed with SYBR Green-based real-time RT-PCR using GAPDH as a control. A total of 100 ng of RNA was used for mRNA expression with a VeryQuest SYBR Green one-step RT-PCR Master Mix (Affymetrix) using a Stratagene Mx3000P qPCR System (Agilent Technologies, Santa Clara, CA, USA). The primer pairs used for amplification of DAB2 and Intelectin-1 were DAB2-F: GTA GAA ACA AGT GCA ACC AAT GG, DAB2-R: GCC TTT GAA CCT TGC TAA GAG A, ITLN1-F: ACG TGC CCA ATA AGT CCC C, ITLN1-R: CCG TTG TCA GTC CAA CAC TTT C. Primers for GAPDH were GAPDH-F: ACA ACT TTG GTA TCG TGG AAG G, GAPDH-R: GCC ATC ACG CCA CAG TTT C. Data analysis was performed using the $\delta\delta$ CT method for relative quantification. Briefly, threshold cycles (CT) for GAPDH (control) and DAB2 and Intelectin-1 (samples) were determined in triplicate. The relative expression (rI) was calculated using the formula: $rI = 2^{-(CT_{\text{sample}} - CT_{\text{normal}})}$.

Western blotting

Total proteins were extracted from five commercially available mesothelioma cell lines (ACC-MESO-1, CRL-5915, ACC-MESO-4, CRL-5946, HMMME) using cell lysis protein extraction reagent (Cell-LyEX1 kit, TOYO B-Net, Tokyo, Japan). Approximately 25 μ g of protein was subjected to electrophoresis on a Novex 10% Bis-Tris gel using a Bolt mini gel tank (Thermo Fisher Scientific, Yokohama, Japan). The proteins were then transferred to a Hybond-P PVDF membrane (GE Healthcare, Buckinghamshire, UK) using a Mini Blot Module (Thermo Fisher Scientific). After treating with blocking buffer, the transfer membrane was incubated with anti-

DAB2 antibody (1:2000 rabbit polyclonal, catalog #HPA028888, Sigma-Aldrich, St. Louis, MO, USA), anti-Human Intelectin-1 (mouse monoclonal 3G9, Immuno-Biological Laboratories, Gunma, Japan, 1:2000) overnight at 4°C. This was followed by streptavidin-labeled anti-mouse or anti-rabbit secondary antibodies (Cell Signaling Technology, Tokyo, Japan) and Immunostar LD (Wako Pure Chemicals, Tokyo, Japan) as a chemiluminescent detection reagent. Anti-GAPDH antibody (rabbit polyclonal, Santa Cruz Biotechnology, CA, USA) was used as control. The blot membrane was captured by scanning with C-DiGit Blot Scanner (LI-COR) for detection of proteins of interest.

Immunohistochemical procedures and evaluation of expression of DAB2 and Intelectin-1

Immunohistochemistry was performed using 3 µm tissue sections prepared from the best representative FFPE blocks of EM and LAC cases. Immunohistochemical staining was performed using the Ventana Benchmark GX automated immunohistochemical station (Roche Diagnostics, Tokyo, Japan). Cell Condition buffer #1 at 95°C for 32 min (Roche Diagnostics) was used for antigen retrieval. The sections were then incubated with primary antibodies to calretinin (rabbit monoclonal, SP65, prediluted, Roche Diagnostics), podoplanin (mouse monoclonal, D2-40, Prediluted; Nichirei Bioscience, Tokyo, Japan), Wilms' tumor gene product (WT1) (mouse monoclonal, 6F-H12, 1:25; Dako, Glostrup, Denmark), DAB2 (rabbit polyclonal, catalog #HPA028888, 1:200; Sigma-Aldrich), and Intelectin-1 (mouse monoclonal, 3G9, 1:1000; Immuno-Biological Laboratories). Incubation with the secondary antibody and detection was performed with Ventana ultraView Universal DAB Detection Kit.

Immunoreactivity was scored as either negative (no immunostaining) or positive. Cells showing nuclear staining for calretinin and WT1, cytoplasmic staining for DAB2 and Intelectin-1, or membranous staining for podoplanin (clone: D2-40) were recorded as 'positive'. Positive immunoreactivity was further scored as 1+ for up to 10% of tumor cells showing positive immunostaining, 2+ for 10–50% positive tumor cells, and 3+ for more than 50% positive tumor cells. Statistical analyses were performed using Fisher's exact test. Sensitivity, specificity, positive

predictive value, negative predictive value, and accuracy rate were calculated using a simple 2×2 table.

Results

Differential gene expression and validation in epithelioid mesothelioma and lung adenocarcinoma

Out of the 44,699 protein coding and 22,829 non-protein coding transcripts on the Human Transcriptome 2.0 GeneChip Array, 902 statistically significant mRNA transcripts were differentially expressed, with a greater than 1.3-fold difference, between EM and LAC (**Figure 1**). Hierarchical clustering of 426 protein coding mRNA transcripts revealed 197 upregulated mRNA transcripts in EM, including CALB2, WT1, DAB2, and Intelectin-1, and 229 upregulated mRNA transcripts in LAC, including CEACAM6 and NAPSA (**Figure 2**; Supplementary Table S1).

Real-time RT-PCR showed relative mRNA expression of DAB2 and Intelectin-1 was significantly higher in EM than that in LAC (data not shown). Western blot analysis showed DAB2 and Intelectin-1 protein expression in all five commercially available mesothelioma cells lines with an electrophoretic band of 80 kDa with DAB2 and one or two electrophoretic bands in the range of 30–40 kDa with the Intelectin-1 antibody (**Figure 3**).

Immunohistochemical expression profiles in epithelioid mesothelioma and lung adenocarcinoma

The expression of positive mesothelioma markers are summarized in **Table 1** and the representative images for DAB2 and Intelectin-1 expression in EM and LAC are presented in **Figure 4** and **Figure 5** respectively. The staining pattern for each marker in two tumor types is described in the following sections.

DAB2 and Intelectin-1 expression

The expression of DAB2 and Intelectin-1 was localized in the cytoplasm of tumor cells in EM cases. Positive DAB2 expression was observed in 60 of 75 EMs (80.0%) and 2 of 67 LACs (3.0%). In half of EM cases, DAB2 immunoreactivity was generally strong and diffuse (score 3+). In

contrast, LACs showing DAB2 expression was focal (score 1+). In addition, DAB2 expression in alveolar macrophages in LACs was a helpful internal positive control. Positive Intelectin-1 expression was observed in 57 of 75 EMs (76.0%), with most of them showing score 3+, whereas none of the 67 LACs were positive for Intelectin-1.

Calretinin, D2-40, and WT1 expression

Positive calretinin expression was recorded for 74 of 75 EMs (98.7%) and 17 of 67 LACs (25.4%). In EMs, immunoreactivity was generally strong and diffuse (score 3+). In contrast, staining score in LACs was 1+ and 2+. There were no score 3+ cases in LACs. Positive D2-40 expression was observed in 71 of 75 EMs (94.7%), with most of them showing score 3+, whereas only seven LACs (10.4%) were focally positive (score 1+ and 2+) for D2-40. Positive WT1 expression was recorded in 62 of 75 EMs (82.7%), whereas none of 67 LACs (0%) were positive for WT1.

Sensitivity and specificity of each marker for differential diagnosis of epithelioid mesothelioma and lung adenocarcinoma

The sensitivity and specificity of each marker for the differential diagnosis between EM and LAC are shown in **Table 2**. Sensitivity of Intelectin-1 (76%) was lowest amongst five positive markers; however, its specificity (100%) was absolute. Sensitivity (80.0%) and specificity (97.0%) of DAB2 were nearly those of WT1. Specificity of calretinin (74.6%) was lowest amongst five markers.

Discussion

Pathologically, the role of immunohistochemistry in distinguishing pleural EM from LAC has received much attention especially in the last 20 years. Currently, there are many immunohistochemical markers available for distinguishing EM from LAC. Among these, calretinin, cytokeratin 5/6, podoplanin (D2-40), and WT1 are the preferred positive markers for EM. Carcinoembryonic antigen (CEA), MOC31 [epithelial-related antigen (ERA)], Ber-EP4, BG-8,

thyroid transcription factor-1 (TTF-1), claudin-4, and napsin-A are the preferred positive markers for LAC. The IMIG 2012 guidelines recommended the consideration of two mesothelial and two carcinoma markers, based on morphology at initial observation (7). In practice, immunohistochemical examination, most laboratories use calretinin, D2-40, and WT1 for diagnosis of EM. However, pathologists must interpret the results of staining by these markers carefully, as specificity of calretinin (74.6% in this study, 90-95% in IMIG 2012 guidelines) and D2-40 (88.9% in this study, up to 85% in IMIG 2012 guidelines) is not absolute; additionally, WT1 shows low sensitivity (82.7% in this study, approximately 90–100% in IMIG 2012 guidelines). Therefore, novel positive immunohistochemical markers, other than calretinin, D2-40, or WT1, are necessary for increasing the accuracy of EM diagnosis.

Recent development of molecular techniques enabled gene expression analysis from RNA extracted from archival FFPE tumor tissues using GeneChip technology. This method is very useful to find new diagnostic markers, especially in rare tumors, including malignant mesothelioma. We have recently reported the identification of a novel marker, MUC4, for differentiating pleural sarcomatoid mesothelioma from lung sarcomatoid carcinoma by analyzing gene expression data from a gene chip microarray (8). In this study, we performed gene expression microarray analysis to identify differentially expressed gene products in EM and LAC. We found that the expression of DAB2 and Intelectin-1 in EM was significantly higher than that in LAC and this was validated by real-time RT-PCR analysis of mRNA extracted from the same tissue source and western blot analysis of proteins extracted from mesothelioma cell lines. Immunohistochemical analysis showed that expression of DAB2 and Intelectin-1 in EMs was significantly higher than that in LACs. These novel positive mesothelial markers, DAB2 and/or Intelectin-1, contribute in accurate mesothelioma diagnosis, in addition to known positive markers (calretinin, D2-40, and WT1).

DAB2, a mitogen-responsive phosphoprotein, is expressed in normal ovarian epithelial cells, but is down-regulated or absent from ovarian carcinoma cell lines, suggesting its role as a tumor

suppressor (9). Decreased DAB2 expression has been reported in various human cancers, including esophageal (10), lung (11), ovarian (9), prostate (12), and breast (13) cancers. DAB2 downregulation in these cancers were reported partly due to miRNA targeting DAB2 (10, 14) or promoter hypermethylation (13, 15). However, the biological significance or expression of DAB2 has not yet been reported in malignant mesothelioma. In the present study, we found increased expression of DAB2 in EM compared to that in LAC by gene expression microarray analysis. We also confirmed this increased expression of DAB2 in EM by real-time RT-PCR and western blot. From the differential analysis of DAB2 expression between EM and LAC by immunohistochemical study, we found higher sensitivity and specificity in EM of more than 80%. In two of the 67 cases of LAC, DAB2 expression was identified in tumor cells but with a low immunoreactivity score. DAB2 expression in LACs was present in inflammatory cell infiltration, mainly macrophages; therefore, precaution must be taken by the physician when interpreting LAC results for DAB2 expression.

Human Intelectin-1, also known as omentin, is a galactose-binding lectin that is usually expressed in the heart and small intestine as a host defense lectin that binds to bacterial galactofuranose (16). Intelectin-1 is mainly expressed in the intestinal goblet cells and omentum, and occasionally in the thymus, bronchus, heart, liver, kidney collecting tubule cells, bladder umbrella and mesothelial cells (17). Recently, the overexpression of Intelectin-1 in human malignant pleural mesothelioma and its secretion into pleural effusions indicated towards it being a potential biomarker (17, 18). It was reported that Intelectin-1 was not expressed in various cancers, except in some mucus-producing adenocarcinomas (19). In the present study, we observed high expression of Intelectin-1 mRNA in EM, definite expression of Intelectin-1 in mesothelioma cell lines by western blot analysis, and in 57 of 75 mesothelioma tissue samples by immunohistochemical analysis. Additionally, we also found Intelectin-1 expression in non-neoplastic mesothelial lining cells and goblet cells in bronchi and bronchioles (data not shown). No Intelectin-1 expression was recorded in any LAC cases, confirming its potential as a novel positive mesothelial marker. The functional roles

of DAB2 and Intelectin-1 expression in EM need further analysis, which is beyond the scope of this study.

In conclusion, we identified two novel positive markers of EM, DAB2 and Intelectin-1, by using gene expression microarray analysis and confirmed their utility to differentiate EM from LAC by immunohistochemical study. Further validation of immunohistochemical staining of these markers by other institutes is warranted.

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Figure Legends

Figure 1. Scatter plot diagram showing differential expression of various genes between epithelioid mesothelioma and lung adenocarcinoma. Note, DAB2 and Intelectin-1 locate towards the epithelioid mesothelioma, in addition to previously known mesothelioma positive markers, CALB2 (calretinin) and WT1, while NAPSA (Napsin-A) and CEACAM6 (major gene for CEA), positive lung adenocarcinoma markers, locate towards lung adenocarcinoma.

Figure 2. Supervised hierarchical clustering of differentially expressed genes between epithelioid mesothelioma and lung adenocarcinoma. The hierarchical clustering of 426 protein coding mRNA transcripts revealed 197 upregulated mRNA transcripts in EM and 229 upregulated mRNA transcripts in LAC. See detailed data in Supplementary Table S1.

Figure 3. Western blot showing DAB2 and Intelectin-1 expression in mesothelioma cell lines. DAB2 expression is present in all five mesothelioma cell lines as a single band; however, Intelectin-1 expression is present as either a single or double band.

Figure 4. DAB2 and Intelectin-1 expression in epithelioid mesothelioma.

Various histomorphological patterns of epithelioid mesothelioma showing prominent expression of DAB2 (middle column) and Intelectin-1 (right column). Each row shows epithelioid mesothelioma with corresponding DAB2 and Intelectin-1 immunohistochemistry.

Figure 5. DAB2 and Intelectin-1 expression in lung adenocarcinoma.

Various histomorphological patterns of lung adenocarcinoma showing no expression of DAB2 (middle column) and Intelectin-1 (right column). Each row shows lung adenocarcinoma with corresponding DAB2 and Intelectin-1 immunohistochemistry. There is focal reactivity of DAB2 in alveolar macrophages and lymphocytes, which need to be interpreted with care.