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KYUKWANG CHUNG, NORITOSHI NISHIYAMA, HIDEKI WANIBUCHI et al. AGR2 as a Potential Biomarker of Human Lung Adenocarcinoma. Osaka City Medical Journal. 2012, 58, 13-24

Osaka City Med. J. Vol. 58, 13-24, 2012

AGR2 as a Potential Biomarker of Human Lung Adenocarcinoma

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Abstract

Background

The present study aimed to identify useful candidate biomarkers of lung adenocarcinoma for clinical diagnosis and treatment using proteomics technology.

Methods

We assessed frequently highly overexpressed proteins in 12 cases of lung adenocarcinoma compared with adjacent normal tissue samples by liquid chromatography tandem mass spectrometry (LC-MS/MS) coupled with isobaric tags for relative and absolute quantitation (iTRAQ) technology, and validated the expression of target proteins by immunohistochemistry in 268 lung adenocarcinoma cases. Protein expression and clinicopathological variables were compared statistically for the evaluation of novel biomarkers.

Results

One hundred seventy-seven proteins displaying significant quantitative changes compared with adjacent normal-appearing lung tissue were identified in more than 9 out of 12 lung adenocarcinoma patients. Based on the results of liquid chromatography tandem mass spectrometry, Ingenuity Pathway, and immunohistochemical analyses, anterior gradient homolog 2 (AGR2) (upregulated 9.9-fold) was selected as a potential biomarker of human lung adenocarcinoma. AGR2 was positive in 94% of lung adenocarcinoma patients. Negative AGR2 expression was associated with poor survival ($p=0.007$).

Conclusions

AGR2 is likely to become a biomarker for clinical applications.

Key Words: Proteomics; Lung adenocarcinoma; AGR2; Prognostic biomarker

Introduction

Lung cancer is the leading cause of cancer-related mortality, with an increasing incidence in Japan and worldwide. Despite advances in early diagnostic methods and treatment modalities,

Received October 11, 2011; accepted November 29, 2011.
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lung cancer is often diagnosed at an advanced stage and presents a poor prognosis. Early detection and complete surgical resection still remains the most successful curative therapeutic option. Non-small cell lung cancer (NSCLC) is a heterogeneous group and can be subdivided into three main histological subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, and adenocarcinomas are now the most common type of lung cancer. The TNM classification is the most important predictor of survival in NSCLC; however, occasionally patients staged according to the TNM classification have different survival times[1]. Thus, novel prognostic markers, such as ones that identify the subgroup of patients with a high risk of recurrence, are needed for better therapy design. Additionally, in early detection, the commonly available serum markers such as CEA, CA19-9, SCC, CYFRA21-1, NSE, and ProGRP have limited sensitivity and specificity[2]. Therefore, new candidate biomarkers for lung cancer have been sought in the hope of achieving early detection of the disease, improving diagnosis, predicting response, or monitoring recurrence after treatment. Although many candidate biomarkers for the management of patients with NSCLC have been proposed, larger prospective trials to validate them have not been undertaken and their clinical usefulness remains limited at present[3]. The discovery of new potential biomarkers is difficult because of the high degree of heterogeneity of NSCLC.

Proteomics can be defined as the comprehensive analysis of the proteome, which is composed of vital protein parts of a living organism. The development of accurate mass spectrometry, advances in of ionization technology, progression of powerful computing and completion of the human genome sequence have accelerated proteomics enormously in recent years. Beyond genomics, the next major challenge is the identification of novel proteins and to understand the structure, function, and interactions of proteins and other molecules. The main aim of clinical proteomics in the field of oncology is the discovery of novel diagnostic, prognostic, or therapeutic markers for future individualized therapy. With recent advances in mass spectrometry techniques, it is now possible to investigate protein expression profiles from biological specimens, healthy and diseased, over a wide range of molecular weights. Their relative expression abundances can even be quantified. Recently, isobaric tags for relative and absolute quantitation (iTRAQ™) reagents have been introduced (AB Sciex, Framingham, USA). The iTRAQ technology allows multiplexed relative quantitative proteomic analysis under the same experimental conditions and, in combination with liquid chromatography tandem mass spectrometry (LC-MS/MS) techniques, increases analytical accuracy and precision. Accordingly, the technology facilitates identification of markers with high selectivity, sensitivity, and specificity[4]. Accordingly, we recently demonstrated significant overexpression of novel biomarkers in rat and mouse liver preneoplastic lesions, which drive their transformation into hepatocellular carcinomas, using the QSTAR Elite LC-MS/MS system allowing multiple protein identification[5,6].

The aim of this study was to identify candidate biomarkers of lung adenocarcinoma in comparison with adjacent normal lung tissue samples, using proteomics coupled with iTRAQ technology. We also aimed to validate the candidates with immunohistochemical staining and identify correlations between the expression of the candidates and clinicopathological variables, thus identifying useful biomarkers for clinical diagnosis and treatment.

Methods

Institutional review board approval and informed consent

This study was approved by the ethics committee at Osaka City University Graduate School of Medicine. Written informed consent was received from all patients.

Patients and tissue samples

Patients were 268 cases with primary lung adenocarcinoma resected at Osaka City University Hospital from January 2004 to December 2008 who had no systemic chemotherapy before tumor resection. Diagnoses were performed by pathologists from the pathology department in our hospital according to the criteria of the World Health Organization and were staged according to the TNM classification of the International Union Against Cancer. The mean age at the time of surgery was 67 years (range: 20 to 93 years) and the median postoperative observation time was 24 months (range: 3 to 61 months). Clinical follow-up data, including disease-specific survival, were recorded from the day of surgery to the time of death or to the last follow-up observation. All lung tumors and adjacent normal lung tissue were obtained at the time of surgery, frozen immediately in liquid nitrogen, and stored at −80℃ until analysis.

LC-MS/MS analysis

Twelve cases were selected randomly among the subjects. The lung tumor and adjacent normal tissue samples of the 12 cases (20 mg each) were homogenized and dissolved in 500 μL of 9 M Urea, 2% CHAPS lysis buffer with protease inhibitors. In addition, the cell lysate was then treated by ultrasonication. After acetone precipitation, protein concentrations were measured by the BCA Protein Assay (Pierce, IL, USA). Reduction, alkylation, digestion, and subsequent peptide labeling of 50 μg protein for each sample were performed using the AB Sciex iTRAQ Reagent Multi-Plex Kit (AB Sciex, Concord, ON, Canada). The adjacent normal and lung tumor tissues of one subject were labeled with the iTRAQ 114 and 115 Da signature ion signal reagents, respectively, and in the same way, the adjacent normal and lung tumor tissues of another subject were labeled with 116 and 117 Da ion signals in MS/MS mode. The iTRAQ-labeled samples were loaded onto an ICAT cation exchange cartridge (AB Sciex). The peptides were eluted as eight fractions (1 mL KCl solution of 10, 30, 50, 70, 90, 110, and 200 mM) and the supernatant was evaporated in a vacuum centrifuge. Peptides of each faction were resuspended in 500 μL of solvent A (98% water/2% ACN/0.1% formic acid, v/v) and injected into Sep-Pak Light C18 cartridges (Waters Corporation, Milford, MA, USA). A double elution was performed with 1.5 mL of solvent A and B (30% water/70% ACN/0.1% formic acid, v/v) for desalting and concentrating. The supernatant was evaporated in a vacuum centrifuge and resuspended in 20 μL of 0.1% formic acid. Proteome analysis was performed on a DiNa-AI nano LC System (KYA Technologies, Tokyo, Japan) coupled to a QSTAR Elite Hybrid mass spectrometer (AB Sciex, Concord, ON, Canada) through a NanoSpray ion source (AB Sciex, Concord, ON, Canada). The separation of samples was performed isocratically (95(A)/5(B), v/v). A 5-μL injection loop was manufactured in-house by measuring an appropriate length of fused-silica capillary (32.0 cm, 30 μm id), and full loop injection was achieved by total injection of 5 μL via a six-port switching CN2 valve injector (KYA Technologies, Tokyo, Japan). The pump output (5 μL/min) was split before the injection port to a flow rate of 200 nL/min. The column effluent enters the spray chamber through a tapered stainless steel emitter and is directly electrosprayed into QSTAR System ion trap mass spectrometer in the positive mode for nanoESI-MS/MS analysis. The emitter position

was adjusted under a microscope and positioned in front of the orifice at a distance of 1.5 mm. Ion spray voltage was set between 1400 and 1600 V to achieve a stable current. Heated nitrogen drying gas (3.0 L/min, 200℃) was introduced into the spray chamber to aid in desolvation. No nebulizing gas was employed. Source voltage was adjusted such that the precursor ion generated maximized product ion signal from the tandem experiment. Each sample was run for 150 min.

MS/MS data were searched against the Swiss Protein database (HUMAN) using ProteinPilot™ software (version 2.0, AB Sciex, Concord, ON, Canada) with trypsin set as the digestion enzyme and methyl methanethiosulfonate as the cysteine modification. The search results were further processed by ProteinPilot™ software using the Paragon Algorithm for removal of redundant hits and comparative quantitation, resulting in the minimal set of justifiable identified proteins. All reported data were used at 95% confidence cut-off limit. Relative quantitation of peptides was calculated as a ratio by dividing the iTRAQ reporter intensity at 115.0, 117.0, m/z (lung tumor) by that at 114.0, and 117.0 m/z (adjacent normal tissue). The ratios of peptides that support the existence of one protein were averaged for protein relative quantitation. After the one simple t-test of averaged protein ratio against 1 to assess the validity of the protein expression changes, a p-value was reported. Protein ratios with a p-value less than 0.05 were considered reliable. Previously standard deviations of the protein ratio, which stem from technical variation, were reported to be less than 0.3 in 90% of iTRAQ experimental runs[7]. Therefore, expression changes greater than 1.6-fold or less than 0.625-fold of normalized expression levels were considered to be outside the range of technical variability.

Selection of candidate proteins for biomarkers and validation by immunohistochemistry

Proteins highly and frequently overexpressed in tumor tissue were noted as candidate biomarkers associated with lung adenocarcinoma. Immunohistochemical staining was performed by the ABC method using a primary rabbit monoclonal anterior gradient homolog 2 (AGR2) (clone ab76473, Abcam) primary antibody. After deparaffinization, gradual dehydration, and antigen retrieval, the endogenous peroxidase activity was blocked. The first antibody (diluted 1:1000 in 1.5% goat serum in PBS) was applied at 4℃ overnight. A biotinylated goat anti-rabbit IgG (diluted 1:200 in 1.5% goat serum in PBS) was applied as secondary antibody for 30 min, and avidin-biotin peroxidase complex was applied for 30 min. The peroxidase reaction was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide in Tris-buffered saline for 2 min. Hematoxylin was used for counterstaining. The immunostains were examined by two independent pathologists who analyzed clinicopathological data under the light microscope. Cells showing cytoplasmic staining were evaluated as positive. The staining for AGR2 was classified into three categories according to the percentage of carcinoma cells positive for AGR2: staining in >50% of tumor cells, strongly positive (scored as 2+); staining in 1 to 50% of tumor cells, weakly positive (1+); and staining in <1% of tumor cells, negative (0).

Statistical analysis

Statistical analysis was carried out using SPSS, version 17.0. Statistical significance of the associations between expression of AGR2 and various clinicopathological variables was evaluated using Fisher's exact and chi-square tests. Survival curves were calculated from the day of surgery to the relapse of death or to the last follow-up observation using the Kaplan-Meier

method, and differences in survival curves were assessed with the log-rank test. Multivariate analyses were calculated according to the Cox regression model to determine associations between clinicopathological variables and disease-specific mortality. P value <0.05 was considered statistically significant.

Results

LC-MS/MS analysis

After the labeling procedure and nano-LC-separation, derivatized peptides were identified and quantified by MS/MS analysis with QSTAR Elite hybrid mass spectrometer and iTRAQ reagents. Each sample was run twice and, in 12 measurements, the mean number of identified proteins in both runs was 256 (≧95% confidence, $p<0.05$). The LC-MS/MS and ProteinPilot analyses results of 12 patients were averaged and further analyzed by Ingenuity Pathway Analysis (IPA). The results are presented in Figure 1. Most of the proteins were overexpressed in the cytoplasm of tumor cells (Fig. 1A). One hundred seventy-seven proteins displaying significant quantitative changes compared with adjacent normal-appearing lung tissue were identified in lung adenocarcinomas of more than 9 patients out of 12 patients. These proteins

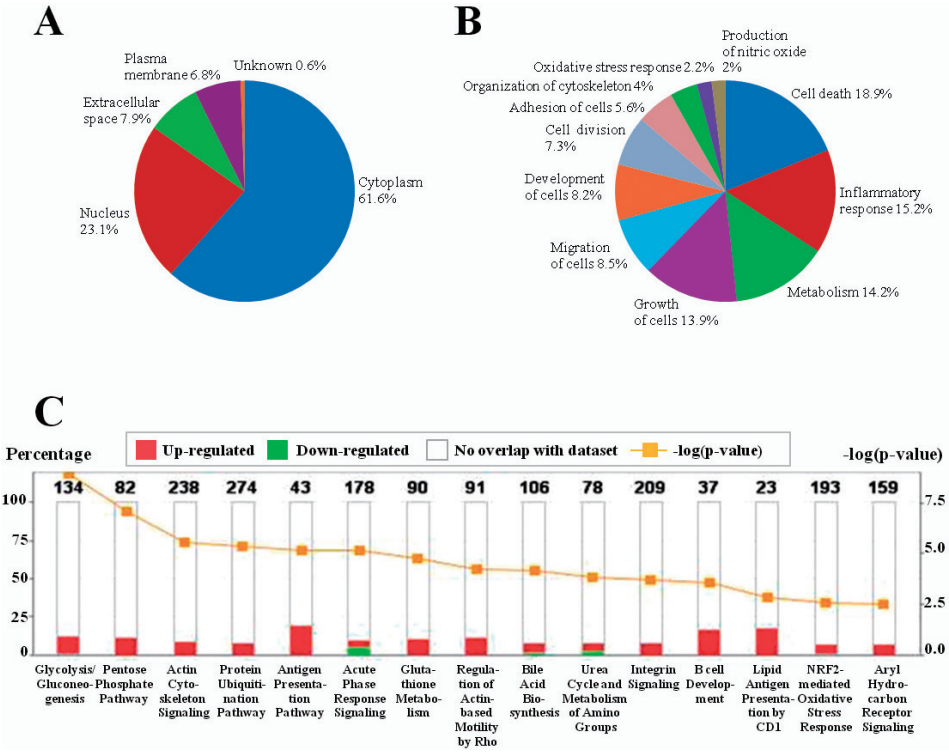

Figure 1. Ingenuity Pathway Analysis for alterations to protein expression in human lung adenocarcinomas of 12 patients. localization (A), biological process (B), and canonical pathways of identified proteins (C). All differentially identified proteins (100%) in lung adenocarcinoma were subdivided according to the localization and biological process as indicated by Ingenuity Pathway Analysis. The numbers of proteins for each localization and biological process were expressed as percentages. In Figure 1C, all differentially expressed proteins in lung adenocarcinoma were subdivided according to their participation in Canonical pathways as indicated by Ingenuity Pathways Analysis. In Figure 1C numbers of proteins with altered expression are represented as (−log (p value)). P value is calculated automatically by the Ingenuity Pathway Analysis based on the statistical analysis of the number of proteins participating in canonical pathways. No overlap with dataset means the number of proteins which were not implicated in canonical pathways.

Table 1. Proteins overexpressed in the lung adenocarcinomas of 12 patients

Protein name (symbol)	GI number	No. cases	Location	Type	Function	Fold change
anterior gradient homolog 2 (Xenopus laevis) (AGR2)	67462105	9	ES, C	Other	MS	9.9
prothymosin, alpha (PTMA)	135834	12	N	Other	TR	4.5
enolase 1, (alpha) (ENO)	119339	12	C	TR	TR	4.2
non-metastatic cells 2, protein (NM23B) (NME2)	127983	12	N	K	TR, NM	3.6
interleukin enhancer binding factor 3, 90kDa (ILF3)	62512150	9	N, C	TR	TR	2.9
signal transducer and activator of transcription 1, 91kDa (STAT1)	2507413	9	N	TR	TR	3.2
RNA binding motif protein, X-linked (RBMX)	23503093	9	N	Other	RNA S	3.8
synaptotagmin binding, cytoplasmic RNA interacting protein (SYNCRIP)	92090361	11	N, C	Other	RNA S-P	2.6
heterogeneous nuclear ribonucleoprotein L (HNRNPL)	133274	9	N, C	Other	RNA S-P	2.7
DEAD (Asp-Glu-Ala-Asp) box polypeptide 39A (DDX39A)	61212932	11	N	E	RNA S	2.9
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked (DDX3X)	3023628	9	C, N	E	RNA B	2.9
acidic (leucine-rich) nuclear phosphoprotein 32 family, member A (ANP32A)	730318	10	N, C	Other	T, RNA M	3.5
high-mobility group box 3 (HMGB3)	85701353	9	N	Other	DNA REP	10.6
parathymosin (PTMS)	135846	9	N, C	Other	DNA REP	2.8
splicing factor proline/glutamine-rich (SFPQ)	1709851	10	N, C	Other	DNA RR, TR	3.1
nucleophosmin (nucleolar phosphoprotein B23, numatrin) (NPM1)	114762	12	N	TR	DNA RR, ST	3.9
non-POU domain containing, octamer-binding (NONO)	67460768	10	C, N	Other	DNA RR, TR	3.3
ribosome binding protein 1 homolog 180kDa (dog) (RRBP1)	23822112	11	C	T	TRL	4.9
Tu translation elongation factor, mitochondrial (TUFM)	1706611	9	C	TR	TRL	3.0
CNDP dipeptidase 2 (metallopeptidase M20 family) (CNDP2)	23396498	9	C	P	PRO, M	2.6
leucine aminopeptidase 3 (LAP3)	124028615	11	C	P	PRO	3.0
coatomer protein complex, subunit alpha (COPA)	1705996	11	C	T	PT	4.3
thioredoxin domain containing 5 (endoplasmic reticulum) (TXNDC5)	29839560	10	C	E	T, AAP	4.1
coatomer protein complex, subunit gamma (COPG)	12229771	9	C	T	PT	3.2
archain 1 (ARCN1)	1351970	10	C, GA	Other	PT	3.9
cofilin 1 (non-muscle) (CFL1)	116848	12	N, C	Other	CS, ACS	3.1
tropomyosin 4 (TPM4)	54039746	12	C	Other	CS, ACS	2.6
coronin, actin binding protein, 1A (CORO1A)	1706004	11	C, IS	Other	CS, ACS	2.6
plectin (PLEC)	134044255	11	C	Other	CS, ACS	2.5
capping protein (actin filament) muscle Z-line, alpha 1 (CAPZA1)	1705650	9	C	Other	CS, ACS	2.9
coactosin-like 1 (Dictyostelium) (COTL1)	21759076	9	C	Other	CS, ACS	2.5
fascin homolog 1, actin-bundling protein (FSCN1)	2498357	10	C	Other	CS, ACS	2.8
myosin, heavy chain 9, non-muscle (MYH9)	6166599	12	C	E	CS	2.6
keratin 8 (KRT8)	90110027	12	C	Other	CS	3.5
keratin 18 (KRT18)	125083	12	C	Other	CS	2.8
keratin 19 (KRT19)	90111766	10	C	Other	CS	2.8
microtubule-associated protein 4 (MAP4)	20455500	12	C	Other	CS	2.6
tubulin, beta 6 (TUBB6)	68776070	11	C	Other	CS	2.7
caldesmon 1 (CALD1)	2498204	10	C	Other	CS	2.8
transgelin 2 (TAGLN2)	586000	12	PM, C	Other	CS	3.1
cytoskeleton-associated protein 4 (CKAP4)	74735614	10	C	Other	Unknown	3.6
S100 calcium binding protein A11 (S100A11)	1710818	12	C	Other	ST	5.1
S100 calcium binding protein A6 (S100A6)	116509	11	C	T	ST, AXO	3.3
ras-related C3 botulinum toxin substrate 2 (rho family) (RAC2)	131806	9	C	E	ST, ACS	2.6
IQ motif containing GTPase activating protein 1 (IQGAP1)	1170586	10	C	Other	ST, ACS	2.5
thymidine phosphorylase (TYMP)	67477361	10	ES, C	GR	NM, ANG	2.7
periostin, osteoblast specific factor (POSTN)	93138709	9	ES, C	Other	A, MIG, I	4.0

Localization: C, cytoplasm; N, nucleus; PM, plasma membrane; ES, extracellular space; GA, Golgi apparatus; and IS, immunological synapse.

Type: E, enzyme; K, kinase; T, transporter; TR, transcription regulator; GR, growth regulator; and P, peptidase.

Function: CS, cytoskeleton; ACS, actin cytoskeleton; M, metabolism; NM, nucleotide metabolism; RNA S, RNA splicing; RNA S-P, RNA splicing, processing; RNA B, RNA binding; RNA M, RNA metabolism; DNA REP, DNA replication; DNA RR, DNA repair; TRL, translation; PRO, proteolysis; ST, signal transduction; T, transport; PT, protein transport; TR, transcription regulator; ANG, angiogenesis; A, adhesion; MIG, migration; I, invasion; AAP, anti-apoptosis; AXO, axonogenesis; and MS, mucus secretion.

were related to transcription, translation, DNA replication and repair, metabolism, protein folding, proteolysis, cytoskeleton filaments reorganization, cell cycle control, cell growth, development, adhesion and migration, immune response, NRF2-mediated oxidative stress responses, and signal transduction (Fig. 1B). The upregulated proteins (>2.5-fold) in more than 9 patients detected by QSTAR Elite LC-MS/MS and IPA in lung adenocarcinomas compared with the adjacent normal tissue are presented in Table 1. We selected tumorigenesis-associated proteins based on the results of ProteinPilot analyses and IPA. Among the identified upregulated extracellular space and cytoplasmic proteins, those most frequently and highly overexpressed proteins in lung adenocarcinoma samples compared with adjacent normal tissue were selected as candidate biomarkers. They included anterior gradient homolog 2 (*Xenopus laevis*) (AGR2) (9.9-fold), ribosome binding protein 1 homolog 180kDa (dog) (RRBP1) (4.9-fold), coatomer protein complex, subunit alpha (COPA) (4.3-fold), thioredoxin domain containing 5 (endoplasmic reticulum) (TXNDC5) (4.1-fold), cytoskeleton-associated protein 4 (CKAP4) (3.6-fold), acidic (leucine-rich) nuclear phosphoprotein 32 family, member A (ANP32A) (3.5 fold), and non-POU domain containing, octamer-binding (NONO) (3.3-fold) (Table 1). The most highly and frequently overexpressed candidate protein, AGR2, was chosen as the most reliable potential biomarker, and its expression was further evaluated in a large number of patients by immunohistochemistry.

Immunohistochemical staining of AGR2 in lung adenocarcinoma

To examine the expression of AGR2 protein of lung adenocarcinoma, immunohistochemical staining was performed with a monoclonal antibody specific for AGR2, which did not react with

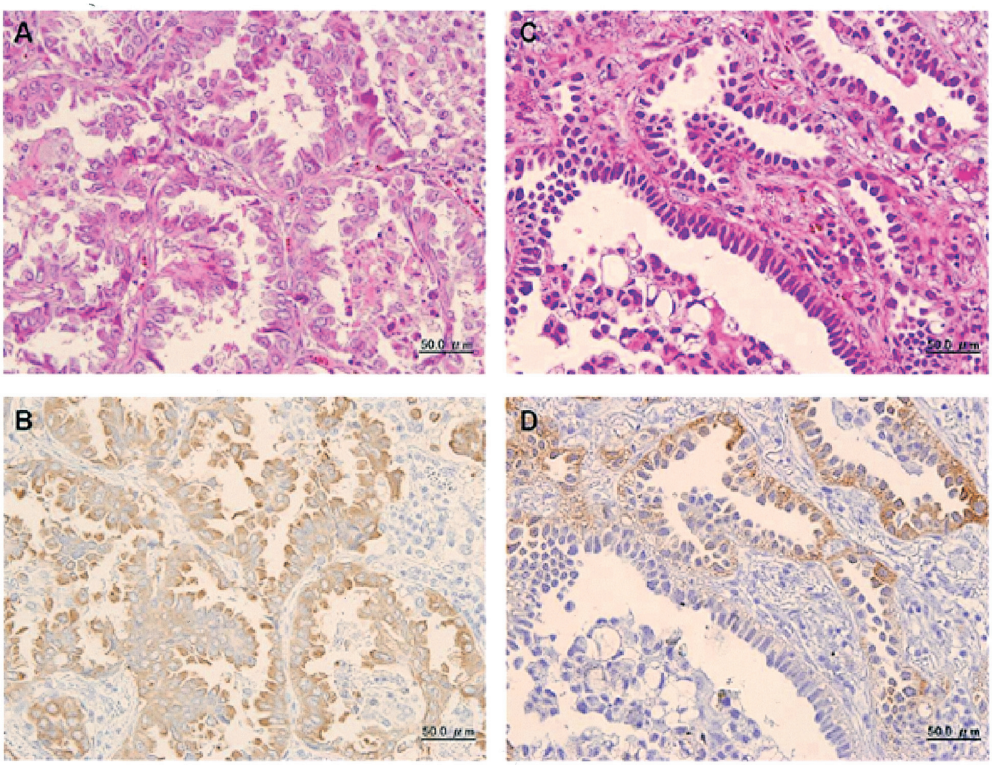

Figure 2. Immunohistochemical detection of AGR2 (B and D) and HE stain (A and C) in human lung adenocarcinoma. B, Strong positive staining of AGR2. D, Weak to moderately stained AGR2. AGR2, anterior gradient homolog 2.

AGR3 in samples from 268 patients who underwent surgical resection. Representative staining for AGR2 is shown in Figure 2. In the normal lung tissue, immunohistochemical staining for AGR2 displayed a weak granular cytoplasmic appearance in the bronchial epithelial cells, type 2 alveolar epithelial cells, and secretory epithelial cells of the bronchial gland. By contrast, strongly positive granular cytoplasmic staining for AGR2 was observed in the primary lung adenocarcinoma polarized toward the surface of the papillae, with a prevalent papillary pattern and the less granular pattern, and lacking specific polarization with a prevalent glandular pattern. Staining showed a heterogeneous pattern and great variation from tumor to tumor in the proportion of cancer cells, ranging from no staining to strongly positive staining. AGR2 was mainly positive in the cytoplasm of cancer cells. From 268 cases examined, AGR2 was strongly positive in 196 cases (73.1%; score +2), weakly to moderately positive in 56 cases (20.9%; +1), and negative in 16 cases (6.0%; 0).

Correlation between AGR2 expression and clinical outcomes of patients with lung adenocarcinoma

Table 2. Association between AGR2 staining grade and clinicopathological parameters

	No. of patients				p value
	Total	Negative	Positive (1+)	Positive (2+)	
Age					
<65	114	7	20	87	0.509
≧65	154	9	36	109	
Gender					
male	155	12	30	113	0.308
female	113	4	26	83	
Smoking					
nonsmoker	101	5	21	75	0.856
smoker	167	11	35	121	
Differentiation					
well	77	7	23	47	0.085*
moderate	117	6	20	91	
poor	74	3	13	58	
pT factor					
T1	144	9	30	105	0.979
T2-4	124	7	26	91	
pN factor					
N0	186	11	38	137	0.972
N1-3	82	5	18	59	
pStage					
Ⅰ	157	10	31	116	0.989*
Ⅱ	26	1	7	18	
Ⅲ	68	4	14	50	
Ⅳ	17	1	4	12	

Fisher's exact test; *Chi square test; pT factor, pathological T factor; pN factor, pathological N factor; pStage, pathological stage; and AGR2, anterior gradient homolog 2.

We evaluated the correlation between AGR2 expression and clinicopathological outcomes using a chi squared test. As shown in Table 2, intensities of AGR2 expression (score 0, +1, +2) were not significantly associated with age (<65, ≧65; p=0.509), gender (male, female; p=0.308), smoking history (nonsmoker, smoker; p=0.856), histological differentiation (well, moderate, poor differentiation; p=0.085), pT factor (pT1, pT2-4; p=0.979), pN factor (pN0, pN1-3; p=0.972), or disease stage (Ⅰ, Ⅱ, Ⅲ, Ⅳ; p=0.989). To evaluate associations between cancer prognosis and intensity of AGR2 expression (score 0, +1 versus +2), apart from the established prognostic factors of pT status, pN status, histological differentiation, and disease stage, univariate survival analysis was performed with disease-specific survival curves according to the Kaplan-Meier method and differences in survival were assessed with the log-rank test (Fig. 3). From these results, negative AGR2 expression was found to be significantly associated with poor prognosis compared with the strongly positive expression (p=0.007). The decrease in intensity of AGR2 expression was associated with the poorer survival; however, statistical significance was not reached between negative and weak to moderately positive AGR2 expression, and between weak to moderately positive and strongly positive AGR2 expression. In Cox multivariate analysis, AGR2 expression changes were not significant (data not shown).

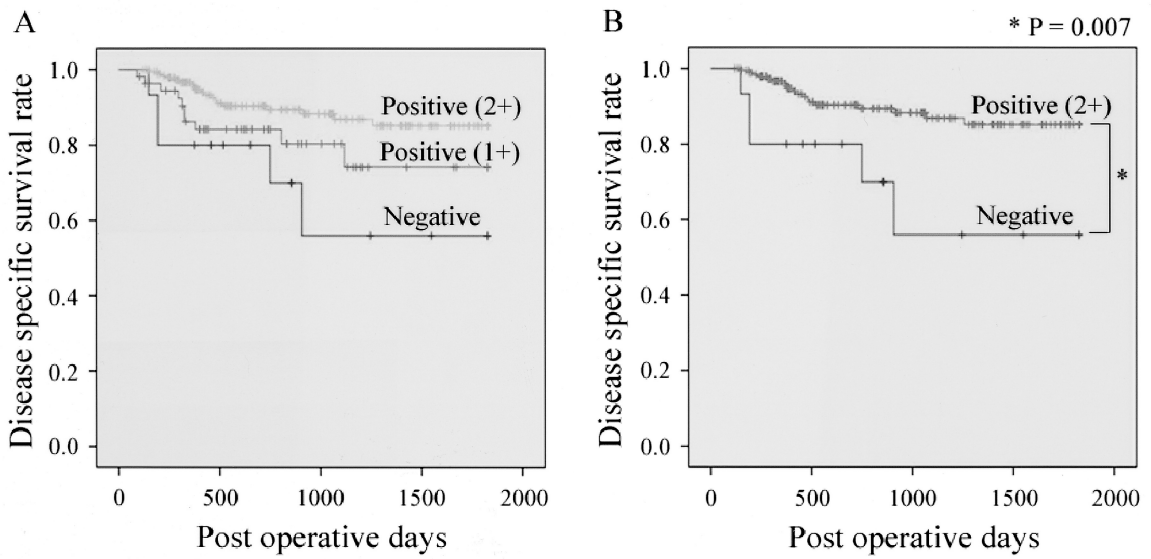

Figure 3. Kaplan-Meier curves for disease-specific survival. A, Survival curves for AGR2 positive (2+), positive (1+), and negative expression in human lung adenocarcinoma. B, Survival curves for AGR2 positive (2+) and negative expression. AGR2, anterior gradient homolog 2.

Discussion

In proteomic analyses of clinical oncology, the main purpose is the selection and validation of proteins that are potential biomarkers, which are identified and quantified by proteomic techniques, including LC-MS/MS coupled with iTRAQ technology. The ultimate purpose is the clarification of pathological functions of proteins related to carcinogenesis, and the development of diagnostic procedures and new therapeutic strategies. Many proteomics studies have been performed to discover new markers for lung cancer using human materials such as cancer tissue, plasma, and pleural effusion of lung cancer patients; however, no new reliable biomarker has been applied in the clinical setting. Liquid chromatography-tandem mass spectrometry makes it

possible to analyze and identify a great number of proteins and peptides concurrently, and the iTRAQ technology allows multiplexed relative quantitative proteome analysis under the same experimental conditions and, with a combination with LC-MS/MS techniques, has increased the analytical accuracy of results[4-6,8-10].

Here, we were able to identify several new proteins that were strongly and frequently overexpressed in human lung adenocarcinoma compared with the adjacent normal lung tissue, which were evaluated as potential biomarkers. To the best of our knowledge, this is the first experimental evidence of significant overexpression of AGR2, RRBP1, COPA, TXNDC5, CKAP4, ANP32A, and NONO based on the proteome analysis. Furthermore, significantly high overexpression of AGR2 detected by both proteome and immunohistochemical techniques indicated that this protein is a potential biomarker of human adenocarcinoma. A large number of human lung adenocarcinoma samples (252/268) proved to be AGR2 positive.

AGR2, also known as hAG-2[11] and Gob-4[12], is the human ortholog of the secreted *X. laevis* Anterior Gradient protein (XAG-2). In the *X. laevis* embryo, XAG-2 protein is highly expressed in the mucus-secreting cement gland and induces cement gland differentiation, ectodermal patterning, and expression of neural marker genes in a fibroblast growth factor-dependent manner[13-16]. The function of XAG-2 in adult *X. laevis* is largely unknown. The AGR2 protein consists of 175 amino acids with a molecular weight of approximately 20000 Da, and is widely expressed in human tissues that contain mucus-secreting cells[11]. It was suggested, based on Basic Local Alignment Search Tool Analysis[17], that the AGR2 may represent a novel member of the protein disulfide isomerase family involved in protein maturation in the endoplasmic reticulum[18]; however, the function of the AGR2 in humans remains largely unclear. Subsequent studies have found that expression of AGR2 is elevated in human adenocarcinoma of the breast[14], esophagus[19], pancreas[20], prostate[21], and NSCLC[22]. There are also suggestions that AGR2 is a p53 inhibitor[19], promotes tumor growth, cell migration, and transformation *in vitro*[20,23], and increases the rate of metastasis *in vivo*, across a range of cancers[24]. However, the significance of AGR2 overexpression in cancer biology has not been clearly revealed and its clinical usefulness as a novel biomarker and therapeutic target has only recently been proposed. With regards to NSCLC, it has been reported that the AGR2 is strongly upregulated in lung adenocarcinoma cell lines, indicating that it is a novel candidate oncogene in lung cancer[25]. It has been also observed that AGR2 is commonly overexpressed in NSCLC tissue and that its expression is associated with the negative residual status of patients; however, its prognostic value could not be demonstrated[22].

In the present study, negative AGR2 expression was associated with poor prognosis of lung cancer. There are three reports on the association between AGR2 expression and prognosis of breast cancer[26-28]. In the study of Fritzsche et al, expression of AGR2 in breast cancer cells was associated with significantly longer survival of 155 breast carcinoma patients treated with various adjuvant therapies[27]. In another study, in 351 breast cancer patients treated by adjuvant hormonal therapy, upregulation of AGR2 was associated with a poor prognosis in patients with ERα-positive breast cancers after treatment with anti-estrogen therapy[28]. Barraclough et al have shown that the presence of AGR2 in the primary tumor is a possible prognostic indicator of poor outcome in 315 patients suffering from operable (stage Ⅰ and Ⅱ) breast cancer[26]. Furthermore, in prostate cancer, it was reported that elevated AGR2 was

significantly associated with poor patient survival[29].

In conclusion, in this study, AGR2 was evaluated as a potential biomarker of lung adenocarcinoma based on combined proteomic and immunohistochemical analyses of a large number of patients. Its negative tumor tissue expression was associated with poor disease-specific survival. Further studies are required to clarify the biological significance of AGR2 overexpression in adenocarcinoma and, ultimately, to evaluate its usefulness as a novel diagnostic or prognostic biomarker and therapeutic target in clinical practice.

Acknowledgements

We thank Azusa Inagaki and Kaori Touma for their technical assistance, and Yukiko Iura for her help during the preparation of this manuscript. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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