Proteomics of Transformed Lymphocytes from a Family with Familial Pulmonary Arterial Hypertension

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Rationale: Not all family members with BMPR2 mutations develop pulmonary arterial hypertension (PAH), implying that additional modifier genes or proteins are necessary for full expression of the disease.

Objectives: To determine whether protein expression is altered in patients with familial PAH (FPAH) compared with obligate carriers and nondiseased control subjects.

Methods: Protein extracts from transformed blood lymphocytes from four patients with FPAH, three obligate carriers, and three married-in control subjects from one family with a known BMPR2 mutation (exon 3 T354G) were labeled with either Cy3 or Cy5. Cy3/5 pairs were separated by standard two-dimensional differential gel electrophoresis using a Cy2-labeled internal standard of all patient samples. Log volume ratios were analyzed using a linear mixed-effects model. Proteins were identified by matrix-assisted laser desorption ionization, time-of-flight mass spectrometry (MALDI-TOF MS) and tandem TOF/TOF MS/MS.

Measurements and Main Results: Hierarchical clustering, heat-map, and principal components analysis revealed marked changes in protein expression in patients with FPAH when compared with obligate carriers. Significant changes were apparent in expression of 16 proteins ($P < 0.05$) when affected patients were compared with obligates: nine showed a significant increase and seven showed a significant reduction.

Conclusions: A series of novel proteins with altered expression were found that could distinguish affected patients from obligate carriers and married-in controls in a single family with a BMPR2 mutation. These differences provide new information highlighting proteins that may be involved in the mechanism(s) that differentiates those individuals with a BMPR2 mutation who develop FPAH from those who do not.

Keywords: two-dimensional differential gel electrophoresis; obligate individuals without FPAH; catalytic activity; MALDI-TOF mass spectrometry

Pulmonary arterial hypertension (PAH), in both the idiopathic and familial forms, is a severe, frequently fatal condition typified by an increased pulmonary artery pressure and subsequent right heart failure. Until recently, there was little in the way of therapy beyond the use of vasodilators for these patients, save transplantation. However, the advent of prostacyclin analogs, PDE5 inhibitors, and endothelin receptor antagonists has increased the lifespan of patients with PAH and improved their quality of life such that the need for lung transplantation is delayed.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Although familial pulmonary arterial hypertension is associated with mutations in BMPR2, not all family members with the mutation develop the disease. Modifier genes and proteins may be necessary for development of the disease.

What This Study Adds to the Field

Proteomic techniques revealed divergent patterns of protein expression in patients with familial pulmonary arterial hypertension as compared with obligate individuals from the same family with no evidence of disease.

The development of familial PAH (FPAH) has been linked to heterozygous germline mutations in the bone morphogenic protein receptor 2 (BMPR2), a member of the transforming growth factor (TGF)- β superfamily (1–4). BMPs are synthesized and released from a variety of cell types, including pulmonary artery smooth muscle cells and endothelial cells, and induce formation of a heterodimeric complex between the constitutively active type II receptor kinase and the type I receptor (5), which subsequently activates downstream signaling pathways through Smad phosphorylation and/or mitogen-activated kinases (6). However, not all individuals with mutations in BMPR2 develop the disease and, to date, no specific marker or group of markers (modifiers) has been identified that would allow identification of individuals at risk for the development of either FPAH or the idiopathic form of the disease.

Recent studies suggest that serotonin (5-hydroxytryptamine [5-HT]) and its transporter (5-HTT) play a critical role in the pulmonary vascular smooth muscle hyperplasia and vascular remodeling associated with the development of PAH (7, 8). Further studies suggest a possible interplay between serotonin and BMPR2 (9–11). Other genes have also been suggested to contribute to the development of PAH: for example, somatic mutations of Bax (12), angiopoietin-1 (13), vascular endothelial growth factor (VEGF) (14), FLAP (5-lipoxygenase [5-LO] activating protein) (15), tenascin-C (16), procollagen and TGF- β (17), phosphorylated Smads (18), thromboxane, prostacyclin (19), plasminogen activator inhibitor (20), and von Willebrand factor (21). Mutations in Alk 1 have also been shown in hereditary hemorrhagic telangiectasia (22). Thus, it is likely that a number of genes and proteins are altered during the pathogenesis of PAH, but whether one specific protein or group of proteins can be implicated in the pathogenesis of all PAH is not certain.

The present study used proteomic techniques and transformed lymphocytes from individuals from one family with a mutation in BMPR2 (exon 3 T354G) to test the hypothesis that protein expression profiles are different in patients with

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FPAH compared with obligate individuals and married-in control subjects. It was considered that use of a single family would reduce intraindividual variation, leading to a more specific dataset. It is not known whether the modifier for clinical expression of BMPR2 is single or multiple, or whether it inhibits or promotes clinical expression, or both. To uncover changes in a vast array of proteins that may be involved in the pathogenesis of FPAH and to search for a potential marker or series of markers of this disease, young patients (<33 yr) affected with disease were compared with older individuals who do not exhibit disease (age, 55–78 yr). These two groups are the phenotypic extremes, selected intentionally to unmask any underlying difference(s). Our results suggest that the proteomic profile of affected patients differs from that of the family members with the mutation but without evidence of disease. Furthermore, our data demonstrate altered expression of proteins linked to several different intracellular functions. Additional studies demonstrate that the changes are not related to the difference in either age or gender between the two groups.

METHODS

Subjects

Ethylenediaminetetraacetic acid (EDTA) anticoagulated blood was collected from seven individuals within one Tennessee family with a known mutation in BMPR2 (exon 3 T354G). Four of these individuals had hemodynamic evidence of FPAH; three of these four were receiving continuous intravenous epoprostenol (age 10–32 yr; 2 females, 2 males; Table 1); the three unaffected individuals had no evidence of PAH (age, 55–78 yr; 2 females, 1 male). Blood was also obtained from three “married-in” individuals as control subjects (age, 33–38 yr; 2 females, 1 male). The study was approved by the institutional review board at Vanderbilt University Medical Center, and written, informed consent was obtained from all subjects included in the study. Unique identifiers to conceal identity were assigned to the samples before their receipt in the laboratory.

To establish that the age and gender of the family members did not contribute to our findings, we performed an additional experiment using control transformed lymphocytes obtained from the Fondation Jean Dausset–CEPH (<http://www.cephb.fr/>). The 11 samples used in this experiment were selected so that they were age and gender matched for each of the samples from the BMPR2 family and married-in control subjects under study.

TABLE 1. DEMOGRAPHICS OF PATIENTS WITH FAMILIAL PULMONARY ARTERIAL HYPERTENSION, OBLIGATES, AND MARRIED-IN CONTROL SUBJECTS

Age* (yr)	Gender	Mean Pulmonary Arterial Pressure (mm Hg)	PVR	Medication	Outcome
FPAH					
31	F	61	21	Flofan	Died, age 35
10	F	55	17	Flofan	Transplant, age 15
32	M	83	16	Flofan	Transplant, age 35
22	M	112	NA	None	Died, age 29
Obligates					
55	M				
58	F				
78	F				Dead
Married-in					
36	M				
33	F				
38	F				

Definition of abbreviations: F = female; FPAH = familial pulmonary arterial hypertension; M = male; PVR = pulmonary vascular resistance.

* Age at time of blood collection for transformation of lymphocytes.

Lymphocytes and Protein Isolation

Lymphocytes were transformed with Epstein-Barr virus (EBV), as previously described (23, 24), and grown in RPMI 1640 (Gibco, Grand Island, NY) containing 15% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 µg penicillin, and 100 µg streptomycin/ml. Before protein extraction, some of the cells from each family member were treated with the BMPR2/BMPR1A ligand BMP-4 (R&D Systems, Minneapolis, MN) for 4 hours. For protein extraction, the cells were washed three times to remove the serum and treated with lysis buffer containing 1% NP-40; 50 mM Tris, pH 7.4; 150 mM NaCl; 10 µg/ml aprotinin; 1 µg/ml leupeptin; 1 mM EDTA; 1 mM NaF; 0.25% sodium deoxycholate; 1 mM NaF; 1 mM orthovanadate; and 1 mM phenylmethanesulfonylfluoride (PMSF). The mixture was then sonicated and frozen at –70°C after protein concentration was assessed using a BCA protein kit (Pierce Co., Rockford, IL).

Two-Dimensional Differential Gel Electrophoresis and Imaging

The *N*-hydroxy succinimidyl ester forms of the Cy2, Cy3, and Cy5 dyes were used for prelabeling proteins under minimal stoichiometric conditions as previously described (25). Proteins were precipitated from the samples and labeled according to previously described methods (25) using 250-µg aliquots from each protein extract (patients with FPAH, obligate carriers, and married-in controls; ±BMP-4). Two-thirds of each sample was individually labeled with Cy3 or Cy5, whereas the remaining third of each sample was pooled and bulk-labeled with Cy2 to serve as a global internal standard on each gel. Individual samples were labeled with 200 pmol of either Cy3 or Cy5 (GE Healthcare, Piscataway, NJ) in an alternating fashion, such that all samples from any one group were not labeled with the same fluorochrome. The use of the pooled-sample internal standard on every gel allowed for direct comparison and quantification of individual protein signals between the Cy3- or Cy5-labeled samples relative to the signal for that protein from the Cy2 standard (rather than between three samples directly) (26, 27). Intragel ratios were then normalized between gels using the Cy2 standard. The labeling of each individual sample and its inclusion into the 10-gel matrix is shown in Table 2. Labeling of the second set of control-only samples was performed in a similar fashion using an independent six-gel set (data not shown).

Labeled protein extracts were separated by standard two-dimensional differential gel electrophoresis (2D-DIGE) using a manifold-equipped IPGphor First-Dimension Isoelectric Focusing unit (GE Healthcare) and 24-cm immobilized pH 4–7 gradient strips (GE Healthcare), followed by second-dimension 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using an Ettan DALT 12 unit (GE Healthcare). The estimated protein content applied to each gel was 500 µg. Isoelectrically focused samples were reduced and alkylated with dithiothreitol and iodoacetamide and equilibrated into a second-dimension loading buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris, pH 8.8) according to the manufacturer's protocol. Second-dimension SDS-PAGE gels were precast with low-fluorescence glass plates; one glass plate was presilanized (Bind-silane; GE Healthcare) to attach the polymerized gel to only one of the glass

TABLE 2. LABELING OF EACH INDIVIDUAL SAMPLE AND ITS INCLUSION INTO THE 10-GEL MATRIX

Gel	Cy3	Cy5	Cy2
1	A (F31)	O (F58)	mix std
2	A+ (F31)	C (F38)	mix std
3	O+ (F58)	A (F10)	mix std
4	O (M55)	C+ (F38)	mix std
5	C (M36)	A+ (F10)	mix std
6	C+ (M36)	O+ (M55)	mix std
7	A (M32)	A+ (M32)	mix std
8	C (F33)	C+ (F33)	mix std
9	O (F78)	O+ (F78)	mix std
10	A (M22)	A+ (M22)	mix std

Definition of abbreviations: A = affected; C = control (married in); mix std = standard comprised of aliquots of each sample; O = obligate.

Plus sign (+) indicates BMP-4 treatment.

Figure 1. Representative example of sensitivity and resolution from one two-dimensional differential gel electrophoresis (2D-DIGE) gel from the coordinated 10-gel series. (A) False color images are overlaid for comparative purposes only. Shown are the two individual samples coresolved on this gel, one of the obligates labeled with Cy3 and false-colored green, and one of the BMP4-treated control samples (labeled with Cy5 and false-colored red). Both were coresolved on this gel along with an equal aliquot of the Cy2-labeled 20-mix internal standard (false-colored blue), which was also present on the other nine gels (each with two

other samples from the 20-sample experiment). For each resolved protein, the abundance values are calculated relative to the internal standard signal within each gel, and then these ratios are normalized between gels to produce the abundance changes depicted in the graphs shown in Figure 5 (METHODS). (B) The Sypro Ruby post-stain for the same gel in A is shown. This image is essential to ensure accurate robotic protein excision, and demonstrates the high degree of correspondence between the DIGE and Sypro Ruby images.

plates. Specific Cy dye channels were imaged with a Typhoon 9400 variable mode imager (GE Healthcare), and the gels were stained with Sypro Ruby (Invitrogen) to ensure accurate automated protein spot excision as previously described (28).

2D-DIGE Analysis

Simultaneous comparison of protein abundance across all three sample groups was performed using DeCyder 2D version 6.5 software (GE Healthcare). The DeCyder differential in-gel analysis module generated ratios for each protein "spot" by comparing Cy3 and Cy5 signals to the Cy2 control signal (rather than between the Cy3- and Cy5-labeled individual samples directly). The DeCyder biological variation analysis module then matched all protein spot maps from the 10 gels, and separately normalized the differential in-gel analysis-generated Cy3: Cy2 and Cy5: Cy2 ratios relative to the Cy2 signals for each resolved feature. This enabled calculation of average abundance changes across all three to four samples within each group, and the application of multivariate (principal components analysis [PCA]) statistical analyses.

Proteins showing significant changes in expression were excised from the gels using an automated Spot Handling Workstation (GE Healthcare) and processed for in-gel trypsin digestion and mass spectrometry (MS). Matrix-assisted laser desorption ionization, time-of-flight (MALDI-TOF) MS was used to acquire the masses of the protonated molecules to within 20 ppm, and tandem TOF/TOF MS/MS was used to fragment selected ions to generate amino acid sequence information, both using a Voyager 4700 mass spectrometer (Applied

Biosystems, Foster City, CA). TOF/TOF fragmentation spectra were acquired in a data-dependent fashion based on the MALDI-TOF peptide mass map for each protein. Both types of mass spectral data were then collectively used to interrogate protein databases and generate statistically significant candidate identifications using GPS Explorer software (Applied Biosystems) running the MASCOT database search algorithm (Matrix Science, Boston, MA). Significant molecular weight search scores ($P < 0.05$), number of matched ions, number of matching ions with independent MS/MS matches, percentage of protein sequence coverage, and correlation of gel region with predicted molecular weight (MW) and isoelectric point (pI) were collectively considered for each protein identification.

Western Blot

Total cell protein from the four patients with FPAH and three obligate individuals was prepared as above. Ten micrograms of protein were loaded per lane and subjected to SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose membranes. The blots were probed with a monoclonal anti-Grb2 antibody (Sigma-Aldrich, St. Louis, MO), washed and incubated with a secondary antibody coupled to horseradish peroxidase. The blot was washed and reprobed with an antibody against total p42/44 (1:500; Santa Cruz Biotech, Santa Cruz, CA). The Western blots were developed using a Western Blot Chemiluminescence Detection Reagent (Perkin-Elmer, Life Science Products, Waltham, MA), and densitometric analysis was performed using image acquisition and analysis software (LabWorks; UVP, Upland, CA).

Figure 2. Dendrogram and heat map of protein expression from transformed lymphocytes from affected patients, obligate individuals, and married-in control subjects both with and without a 4-hour treatment with BMP-4. The dendrogram illustrates a distinct compartmentalization in protein expression patterns between the three groups. No differences are found between the samples with and without treatment with BMP-4. The differences in protein expression are clearly seen in the heat map. Expression patterns in selected proteins are visually presented as horizontal lines in an expression matrix using a relative scale ranging from -0.5 (green) to $+0.5$ (red).

Figure 3. Principal components analysis (PCA) of protein expression in transformed lymphocytes from affected patients with familial pulmonary arterial hypertension (FPAH), obligate individuals, and married-in control subjects. PCA reduces the complexity of a multidimensional analysis into two principal components, PC1 and PC2, which orthogonally divide the samples based on the two largest sources of variation in the dataset. Values within the circles of the PCA plots are within the 95th percentile confidence interval. All of the points from the affected patients with FPAH lie to the left of the perpendicular while those from the obligate individuals and married-in control subjects lie on the right, indicating a distinct expression pattern in the patients with FPAH versus the obligate individuals and the married-in control subjects. Affected = light green; affected + BMP-4 = dark green; obligates = light blue; obligates + BMP-4 = dark blue; married-in control subjects = pink; married-in control subjects + BMP-4 = red.

Statistics

A subset of 102 features exhibiting significant protein abundance changes between any of the six groups ($P < 0.05$, two-way analysis of variance [ANOVA] to account for BMP-4-specific changes) was subjected to multivariate analysis to assess the source of variation in the dataset. PCA reduces the complexity of a multidimensional analysis into two principal components, PC1 and PC2, which orthogonally divide the samples based on the two largest sources of variation in the dataset. The protein expression characteristics from all 102 features for each individual sample are represented by each data point in the PCA plots; values within the circles of the PCA plots are within the 95th percentile confidence interval. Hierarchical clustering (HC) was also performed on the dataset, with the global expression pattern of all significant features for each individual sample grouped in columns and displayed as an expression heat map. HC expression matrixes were calculated using Euclidean correlation and average linkage, with clustering of similar samples related by branched dendrograms.

Because two-way ANOVA revealed little difference in the 2D-DIGE data from BMP-4-treated and untreated samples from each individual, these data were combined and log volume ratios were analyzed using a linear mixed-effects model. This model included a "group" term (fixed) to evaluate expression differences between affected, obligate, and control individuals, and a "subject" term (random) to account for the multiple observations (gels) acquired for each subject. A separate mixed-effects model was fit to each selected protein. For proteins exhibiting significant group differences ($P < 0.05$), ANOVA and the Student's t test were used to evaluate global and pairwise differences between specific groups, respectively.

RESULTS

In this study, we compared protein expression patterns in transformed lymphocytes from four family members with a known BMPR2 mutation (exon 3, T354G, C118W, Bsp1286 I site) and FPAH to three obligates (individuals with the mutation but without evidence of the disease) from the same family, as well as from three married-in control subjects (for representative gel, see Figure 1). We performed HC analysis to assess changes in protein expression between the three groups. The dendrogram demonstrated ordering in a treelike structure based on pattern similarity between each of the samples. The first branch in the dendrogram showed a difference in clustering between the affected patients versus the obligate individuals and the married-in control subjects (Figure 2). A second level of clustering appeared between the obligate individuals versus the married-in control subjects. Samples treated with BMP-4 clustered with their relevant untreated sample, suggesting few discernible differences between these paired samples at the time point examined and that any differences between individuals are greater than the differences induced by 4 hours' exposure to BMP-4. Most noticeable, in the heat map, were the increases in protein expression (Figure 2, top) of the affected group and the decreases in protein expression (Figure 2, bottom) as compared with obligate individuals and married-in control subjects.

PCA was also used to assess the major sources of variation between samples. Each data point in the analysis represents a patient sample, and the variation within the same group of proteins used for HC analysis was distilled down to the two most significant components, PC1 and PC2, which are depicted on orthogonal axes. PC1, describing 40.7% of the variance, indicated a distinct expression pattern for the affected patients versus the obligate individuals and married-in control subjects. The data points for the affected samples localized in the left-hand quartiles, whereas the obligate individuals and control

Figure 4. Principal components analysis of protein expression from control transformed lymphocytes matched by age and sex to the individuals in Figure 3. No clustering of points is apparent, indicating that age and sex do not contribute to the different expression patterns seen in Figure 3.

TABLE 3. PROTEINS IDENTIFIED IN AFFECTED AND OBLIGATE INDIVIDUALS THAT VARIED IN EXPRESSION

Genbank ID	Protein	kD	pI	MOWSE (A, B, C, D)	Fold Expression	<i>t</i> Test	ANOVA
P62993	Growth factor receptor bound protein (Grb2)	25.3	5.9	242, 12, 7, 40%	1.4	0.002	0.001
P22061	Protein-L-isoaspartate (D-aspartate) O-methyltransferase	24.5	5.9	73, 7, 4, 29%	1.2	0.002	0.001
P12268	Inosine 5' monophosphate dehydrogenase	56	6.4	208, 17, 9, 34%	1.3	0.002	0.01
P08865	40S ribosomal protein SA	33	4.8	607, 13, 9, 40%	1.2	0.001	0.02
P33316	dUTP pyrophosphatase	19	8.1	153, 6, 6, 33%	1.7	0.002	0.02
Q04760	Glyoxalase I	21	5.2	119, 9, 4, 40%	3.2	0.001	0.02
O00151	PDZ and LIM domain protein 1 (elfin)	36	6.5	233, 8, 5, 27%	1.2	0.002	0.03
P68363	Tubulin- α	50.8	4.9	443, 16, 7, 46%	1.4	0.01	0.03
P08670	Vimentin	53.5	5.0	492, 31, 9, 69%	1.4	0.01	0.03
P60709	β -actin	42	5.3	483, 15, 11, 42%	-1.5	0.001	0.002
P09211	Glutathione S-transferase P	23	5.4	387, 9, 7, 27%	-1.8	0.001	0.002
P06733	α -enolase	47	7.0	580, 22, 13, 52%	-1.4	0.009	0.003
P09104	γ -enolase	47.5	4.9	206, 12, 7, 32%	-1.8	0.001	0.007
Q08623	Haloacid dehalogenase-like hydrolase domain-containing protein 1A (GS1 protein)	24	5.2	56, 6, 4, 19%	-2.6	0.001	0.008
P33241	Lymphocyte specific protein 1	37	4.7	190, 7, 3, 29%	-1.5	0.007	0.01
P30101	Protein disulfide isomerase A3 precursor	57	6.0	477, 26, 13, 42%	-1.2	0.01	0.05

Definition of abbreviations: ANOVA = analysis of variance; pI = isoelectric point.

MOWSE A, B, C and D are (A) combined MS and MS/MS search scores, (B) number of peptides matched, (C) number of peptides with MS/MS data, and (D) percentage of the amino acids accounted for by the matching peptides (coverage). Scores were calculated using the MASCOT version 1.9 database search algorithms.

subjects occupied the right-hand quartiles (Figure 3). PC2 accounted for an additional 16% of the variation (Figure 3). As was found in the HC analysis, samples treated with BMP-4 clustered with their relevant untreated sample.

One question that arose from these data was whether the different ages and gender of the individuals could contribute to the differences in the PCA distribution. Gender was unlikely to contribute because males and females were included in each group. To further examine the notion of age- and gender-related changes, we repeated the above study using transformed lymphocytes from 11 age- and gender-matched control subjects obtained from Fondation Jean Dausset-CEPH in an independent set of six DIGE gels. As shown in Figure 4, no obvious clustering was found except for the well-characterized Cy3/5 dye labeling bias (29, 30), which was minimal, but evident, in the absence of any biological change driving the variation. It is important to note that this potential bias was nullified in the control-obligate-affected experiment of Figure 3 by our labeling structure whereby the samples from each category were never labeled consistently with the same dye. Upon manual inspection of the DIGE analysis, we found evidence for only a few proteins showing altered expression with respect to gender, with less than five that fell within the 99th percentile confidence interval (Student's *t* test), and only one protein (haloacid dehalogenase-like hydrolase domain-containing protein 1A [GS1]) from the dataset in Table 2 displayed a statistically significant difference between males and females. However, this protein was also significantly reduced when the affected patients were compared with the younger married-in control subjects, suggesting that this is a chance finding. Overall, our data are consistent with the premise that the age and gender of the affected patients, obligate individuals, and married-in control subjects did not contribute to the discrete clustering of Figure 3.

After establishing that the variation between the individual samples described the different biological conditions (rather than technical or sex-/age-related biases), we manually selected a subset of significant features based on pairwise Student's *t* tests that were targeted for protein identification after in-gel

trypsin digestion and MS. Significant changes in protein expression between the affected patients and obligate individuals are shown in Table 3; graphical representations of several of the normalized DIGE results are shown in Figure 5. These graphs represent the relative expression levels of each protein across all 20 samples that have all been normalized to the Cy2-specific signal for that protein from the internal standard that is present on each gel.

Of the 16 proteins found to show altered expression when affected patients were compared with obligate individuals (Student's *t* test, $P < 0.05$), 9 showed a significant increase in expression and 7 showed a significant reduction. Proteins associated with cell movement (40S ribosomal protein SA, tubulin- α , and vimentin) were significantly higher than expected ($P < 0.05$ by Fisher's exact test) as assessed by WebGestalt (Vanderbilt University Medical Center, Nashville, TN) (31). In addition, six proteins were associated with binding (Grb2, dUTP pyrophosphatase, glyoxalase I, [inosine monophosphate] dehydrogenase 2, PDZ and LIM domain 1 [elfin], and vimentin), four have catalytic activity (protein-L-isoaspartate [D-aspartate] O-methyltransferase, dUTP pyrophosphatase, glyoxalase I, and [inosine monophosphate] dehydrogenase 2), and two play a role in signal transduction (Grb2 and 40S ribosomal protein SA).

Figure 6 shows a Western blot using a regrowth of the transformed lymphocytes and confirming increased basal Grb2 expression in the four FPAH cases compared with the three obligate individuals using protein from transformed lymphocytes (densitometric analysis of Grb2 related to total P42/44 expression, obligate samples = 1.07 ± 0.22 [mean $\pm$ SD], affected samples = 1.52 ± 0.40 ; these values were not significant [Student's *t* test] doubtless because of the small sample size; however, the increase was similar to that obtained by 2D-DIGE). As noted above, Figure 5 demonstrates that Grb2 protein (an adapter protein involved in signal transduction of several growth factors) is lower in the untreated obligate cells when compared with cells from the patients with FPAH either with or without BMP-4 treatment. Basal expression of Grb2 in the obligate cells was also increased in obligate cells stimulated with

Figure 5. Standardized log abundance of various protein expression data obtained from two-dimensional differential gel electrophoresis analysis. Affected = light green; affected + BMP-4 = dark green; obligates = light blue; obligates + BMP-4 = dark blue; married-in control subjects = pink; married-in control subjects + BMP-4 = red. Of the proteins identified, only Grb2 showed a trend toward a difference between obligate controls with and without treatment with BMP-4.

BMP-4. Despite *t* test analysis and manual inspection of the data from paired cells from affected patients, obligates, and control subjects, with and without treatment with BMP-4, this was the only BMP-4-induced difference in expression detected in this experiment.

Of the proteins with decreased expression, four have catalytic activity (protein disulfide isomerase family A, member 3; glutathione S-transferase; γ -enolase; and haloacid dehalogenase-like hydrolase domain containing 1A), three proteins are associated with binding (β -actin, γ -enolase, and lymphocyte-specific protein 1), and one is associated with signal transduction (lymphocyte-specific protein 1). Despite its name, lymphocyte-specific protein 1 is also expressed in endothelial cells (32).

Figure 6. Western blot showing Grb2 expression in unstimulated transformed lymphocytes from obligate individuals (lanes 1–3) and patients with familial pulmonary arterial hypertension (lanes 4–7). Total p42/44 is shown as a loading control.

Changes in protein expression between affected individuals and married-in control subjects are shown in Table 4. Of the 17 proteins identified that showed significant alterations in expression, 11 showed a significant increase and 6 showed a decrease. Of the 11 proteins showing an increase, there was a significant increase ($P < 0.05$, Fisher's exact test) in proteins associated with catalytic activity (caspase 3, peroxiredoxin 6, ubiquitin-conjugating enzyme E2N, proteasome subunit β type 9, triose-phosphate isomerase 1, and glyoxalase I). Four of the proteins showing a decrease were similarly altered when affected patients were compared with obligate carriers (marked with an asterisk in Table 4).

Comparison of protein expression between obligate individuals and married-in control subjects revealed significant differences in expression for only seven proteins (Table 5). Each of the four proteins that were significantly increased was also increased when the married-in control subjects were compared with the obligate individuals (Table 3). Of the three proteins exhibiting decreased expression, two were also decreased when the married-in control subjects were compared with affected patients (Table 4).

DISCUSSION

We used the global proteomic approach of 2D-DIGE/MS to examine transformed lymphocytes from members of a single

TABLE 4. PROTEINS IDENTIFIED IN AFFECTED INDIVIDUALS AND MARRIED-IN CONTROL SUBJECTS THAT VARIED IN EXPRESSION

Genbank ID	Protein	kDa	pI	MOWSE (A, B, C, D)	Fold Expression	t Test	ANOVA
P37837	Transaldolase	37.7	6.4	118, 13, 4, 32%	1.4	0.002	0.004
P07910	Heterogeneous nuclear ribonucleoprotein C1/C2	33.7	4.9	439, 18, 10, 50%	1.3	0.001	0.009
P55854	Small ubiquitin-related modifier 3 precursor	11.6	5.3	74, 4, 1, 36%	1.4	0.002	0.01
P30041	Peroxiredoxin 6	25	6.0	322, 13, 6, 55%	1.2	0.005	0.02
P61088	Ubiquitin-conjugating enzyme E2N	17	6.1	78, 4, 2, 35%	1.2	0.01	0.02
O43175	D-3-phosphoglycerate dehydrogenase	57.2	6.3	74, 10, 2, 21%	1.8	0.01	0.03
P28065	Proteasome subunit β type 9 precursor	23.3	4.9	194, 10, 5, 37%	1.3	0.004	0.03
P42574	Caspase-3 precursor	32.0	6.1	100, 9, 4, 29%	1.3	0.02	0.04
P62258	14-3-3 protein epsilon	29	4.6	263, 16, 7, 51%	1.2	0.01	0.05
P52566	Rho GDP-dissociation inhibitor 2	23	5.4	526, 12, 10, 58%	1.2	0.008	0.05
P60174	Triosephosphate isomerase	26.8	6.5	74, 9, 4, 35%	1.6	0.03	0.05
Q08623	Haloacid dehalogenase-like hydrolase domain-containing protein 1A (GS1 protein)*	24	5.2	56, 6, 4, 19%	-2.0	0.01	0.003
P60709	β -Actin*	42	5.3	335, 15, 8, 42%	-1.3	0.004	0.004
P13796	L-plastin	70.8	5.2	703, 28, 13, 44%	-1.3	0.001	0.007
P33241	Lymphocyte specific protein 1*	37	4.7	242, 11, 6, 33%	-1.9	0.004	0.03
P30153	Protein phosphatase 2, regulatory subunit A	65.8	4.9	620, 32, 13, 45%	-1.5	0.01	0.03
P09104	γ -Enolase*	47.5	4.9	319, 15, 7, 33%	-1.4	0.04	0.05

Definition of abbreviations: ANOVA = analysis of variance; GDP = guanine diphosphate; pI = isoelectric point.

MOWSE A, B, C and D are (A) combined MS and MS/MS search scores, (B) number of peptides matched, (C) number of peptides with MS/MS data, and (D) percentage of the amino acids accounted for by the matching peptides (coverage). Scores were calculated using the MASCOT version 1.9 database search algorithms.

* Also significantly reduced when affected patients compared with obligate control subjects.

family with a mutation in the *BMPR2* gene for alterations in protein expression. Multivariate statistical analyses (HC and PCA) indicated that protein expression patterns distinguished patients with FPAH from both obligate carriers and married-in control subjects. Age and gender had no effect on these findings. Comparison of data from patients with FPAH versus obligate carriers revealed significantly altered expression of 16 proteins; expression of 9 of these proteins was increased and 7 showed a reduction. Comparison of patients with FPAH with married-in control subjects revealed that expression of 11 proteins was significantly increased and expression in 6 was decreased. The 11 proteins showing an increase in expression in the affected patients versus control subjects differed from those that were up-regulated when the affected individuals were compared with the obligate carriers. Of the six that were decreased, four proteins were the same as those identified when the affected patients were compared with the obligate carriers. Comparison of obligate carriers with married-in control subjects revealed few significant changes in protein expression and, of the seven identified, only one was not found when the affected patients were compared with the control subjects. These data indicate that patients with FPAH exhibit significant differences in protein expression when compared with obligate carriers and

further differences in protein expression are apparent when compared with married-in control subjects.

Although several genes and proteins have been associated with the development of idiopathic PAH (IPAH), few studies have focused on patients with FPAH. For example, using microarray technology, altered expression of TGF- β receptor III, BMP-2, mitogen-activated protein kinase 5, receptor for activated protein kinase C (RACK)-1, apolipoprotein C-III, and the laminin receptor were found only in samples from patients with PAH (four with IPAH and two with FPAH) when compared with nondiseased control subjects (33). An IPAH library generated by suppression subtractive hybridization of lung tissue from four patients with IPAH and four control subjects identified a further 29 genes that may be involved in the pathogenesis of PAH (34). Few, if any, of the identified genes were identified in both studies. The contribution of interindividual variation to these studies is not known. Comparison of patients with FPAH with obligate carriers in a single family with a mutation in the *BMPR2* gene, while small in number, obviates much of the interindividual variation that is inherent in this type of study.

We chose to use transformed lymphocytes for the present studies for practical reasons: no other cells or cell lines are available. EBV infects, transforms, and allows us to grow large

TABLE 5. PROTEINS IDENTIFIED IN OBLIGATES AND MARRIED-IN CONTROL SUBJECTS THAT VARIED IN EXPRESSION

Genbank ID	Protein	kDa	pI	MOWSE (A, B, C, D)	Fold Expression	t test	ANOVA
P07910	Heterogeneous nuclear ribonucleoprotein C1/C2	33.7	4.9	439, 18, 10, 50%	1.2	0.001	0.002
P55854	Small ubiquitin-related modifier 3 precursor	11.6	5.3	74, 4, 1, 36%	1.3	0.01	0.01
P61088	Ubiquitin-conjugating enzyme E2N	17	6.1	78, 4, 2, 35%	1.2	0.01	0.02
P28065	Proteasome subunit β type 9 precursor	23.3	4.9	194, 10, 5, 37%	1.2	0.008	0.03
P13796	L-plastin	70.8	5.2	703, 28, 13, 44%	-1.3	0.004	0.007
P33241	Lymphocyte-specific protein 1	37	4.7	242, 11, 6, 33%	-1.4	0.03	0.03
P17742	Peptidylprolyl cis-isomerase A	18	7.8	86, 7, 2, 28%	-1.2	0.04	0.05

Definition of abbreviations: ANOVA = analysis of variance; pI = isoelectric point.

MOWSE A, B, C and D are (A) combined MS and MS/MS search scores, (B) number of peptides matched, (C) number of peptides with MS/MS data, and (D) percentage of the amino acids accounted for by the matching peptides (coverage). Scores were calculated using the MASCOT version 1.9 database search algorithms.

numbers of B lymphocytes. This has allowed accumulation of samples from patients with FPAH and obligate carriers from a single family with a known mutation during clinic visits over many years as well as samples from married-in control subjects. The collection has also allowed repeated use of these samples for various techniques, and replication of experiments. Although we acknowledge that use of cells from the pulmonary artery may have been a more appropriate choice, such cells were obviously not available from either the obligate carriers or married-in control subjects as they do not warrant biopsy or transplant. EBV-transformed B lymphocytes have recently been used with great effect for genetic testing and epidemiologic studies as well as to reveal additional mutations in the BMPR2 gene (35, 36). A proteomic study in EBV-transformed lymphocytes, while showing expected differences in expression of some proteins, has also revealed that many of the proteins are unaffected by the infection (37). That study did not report changes for any of the proteins of interest in our study. Furthermore, several articles suggest that alterations in the immune response may contribute to the pathogenesis of PAH (38, 39).

Analysis of the samples from affected and obligate individuals revealed several differences in protein expression, each protein with a variety of known functions. For example, six of the up-regulated proteins in the FPAH samples are associated with binding, three are associated with cell movement, two play a role in signal transduction, and one is up-regulated in response to stimuli (PDZ and LIM domain 1 [elfin]). Of those proteins showing reduced expression, four have catalytic and three have binding activity. Lymphocyte-specific protein 1 is involved in both stimulus response and signal transduction. Thus, a number of biological processes are likely altered in patients with FPAH.

We were a little surprised not to find altered expression of proteins currently known to play a role in the BMPR2/BMPR1A signaling pathways, but new participants in the pathway were recognized. For instance, our finding of an increase in Grb2 expression in the affected patients compared with obligates strongly suggests that this protein plays a role in activation of the BMPR2 signal transduction pathway. Grb2 is an adaptor protein that has been linked to activation of several growth factor receptors (e.g., epidermal growth factor and basic fibroblast growth factor) (40). In addition, our data demonstrate increased expression of the PDZ and LIM domain protein 1 (Elfin). LIM kinase is known to regulate actin dynamics by phosphorylating cofilin (41). It has also been shown to interact with the tail of the BMPR2 gene to down-regulate LIM kinase 1 activity and that stimulation with BMP-4 overcomes this down-regulation (42). Those authors suggested that deregulation of actin may play a role in the pathogenesis of PAH. The present study shows both an increase PDZ and LIM domain protein 1 and decreases in β -actin and lymphocyte-specific protein 1 (an intracellular F-actin binding protein) when affected patients are compared with obligate individuals. Since, thus far, all members of the PDZ/LIM family have been shown to associate with the actin cytoskeleton (43), our study perhaps confirms a role for LIM and actin in FPAH.

Comparison of the affected patients with married-in control subjects revealed further differences in protein profiles. Six of the proteins that were up-regulated had catalytic activity; a further four had binding activity, and two were response proteins. Of those exhibiting reduced expression, five had binding activity, two had catalytic activity, one had signal transduction activity, and three were part of protein. However, several of these differences are also found when samples from obligates are compared with control subjects, notably changes in ubiquitin-conjugating enzyme E2N, L-plastin, lymphocyte-specific protein 1, and proteasome subunit β type 9 precursor. These findings

perhaps suggest that all family members with a BMPR2 mutation exhibit differences in protein expression when compared with control subjects.

From the above, it is apparent that proteomic analysis reveals differences in protein expression that differ from the changes in mRNA identified by microarray. Both strategies have unique strengths and limitations. 2D-DIGE resolves thousands of proteins in a single run, and provides crucial molecular weight and isoelectric point information on intact proteins, although identification of some proteins may be hindered by their expression in various forms and the data may be skewed toward the more abundant proteins in the sample. Thus, 2D-DIGE profiling can examine a phenotype that might not be apparent by the use of microarrays. It should be remembered, however, that at present no proteomic technique is currently available that is as comprehensive as the microarray in which every gene in a given array is known. Against this one must set the disadvantage that microarray technology does not define whether differences in RNA expression lead to alterations in protein expression.

In summary, we have used 2D-DIGE/MS to examine the protein profiles and expression in EBV-transformed lymphocytes from a single family with FPAH with known BMPR2 mutation at exon 3 T354G. Comparison of the affected patients with obligate individuals revealed differences in patterns of protein expression that were highlighted by heat map, dendrograms, and principal components analyses. Identification of these proteins revealed significant increases in expression for nine proteins, whereas seven showed a significant reduction. Of the proteins showing significant changes in expression when affected patients were compared with obligates, our studies suggest that the adaptor protein Grb2 may play a role in signal transduction of the BMPR2 receptor. Alterations in protein expression were also revealed when affected patients were compared with married-in control subjects. These differences provide novel information highlighting proteins that may be linked to the mechanism(s) that defines why not all individuals with a BMPR2 mutation develop FPAH.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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