CLINICAL RESEARCH ARTICLE

Dual-specificity phosphatase (*DUSP*) genetic variants predict pulmonary hypertension in patients with bronchopulmonary dysplasia

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BACKGROUND: Pulmonary hypertension (PH) in patients with bronchopulmonary dysplasia (BPD) results from vasoconstriction and/or vascular remodeling, which can be regulated by mitogen-activated protein kinases (MAPKs). MAPKs are deactivated by dual-specificity phosphatases (DUSPs). We hypothesized that single-nucleotide polymorphisms (SNPs) in *DUSP* genes could be used to predict PH in BPD.

METHODS: Preterm infants diagnosed with BPD ($n = 188$) were studied. PH was defined by echocardiographic criteria. Genomic DNA isolated from patient blood samples was analyzed for 31 SNPs in *DUSP* genes. Clinical characteristics and minor allele frequencies were compared between BPD-PH (cases) and BPD-without PH (control) groups. Biomarker models to predict PH in BPD using clinical and SNP data were tested by calculations of area under the ROC curve.

RESULTS: In our BPD cohort, 32% ($n = 61$) had PH. Of the *DUSP* SNPs evaluated, *DUSP1* SNP rs322351 was less common, and *DUSP5* SNPs rs1042606 and rs3793892 were more common in cases than in controls. The best fit biomarker model combines clinical and *DUSP* genetic data with an area under the ROC curve of 0.76.

CONCLUSION: We identified three *DUSP* SNPs as potential BPD-PH biomarkers. Combining clinical and *DUSP* genetic data yields the most robust predictor for PH in BPD.

INTRODUCTION

Bronchopulmonary dysplasia (BPD is a chronic lung disease most commonly seen in preterm infants who require prolonged, positive pressure ventilation and supplemental oxygen. Recent studies have shown that preterm infants who go on to develop BPD have disrupted alveolar and vascular growth, as demonstrated by persistent hypoplasia of lung microvasculature and alveolar simplification.[1,2] The resultant decrease in vascular surface area reduces the lung's capacity for gas exchange and affects perfusion throughout the pulmonary vascular network. Subsequent hypoxia-induced vasoconstriction, elevated pulmonary vascular resistance, and remodeling with intimal hyperplasia of small pulmonary arteries have been implicated in the pathogenesis of BPD-associated pulmonary hypertension (PH).[1] Approximately 25–40% of preterm infants with BPD will develop PH and there is an increased risk of mortality with the diagnosis of PH in BPD patients.[3] Previously identified clinical risk factors for PH in BPD include low birth weight, necrotizing enterocolitis, retinopathy of prematurity, and patent ductus arteriosus.[3,4]

Mitogen-activated protein kinases (MAPKs) play an important role in regulating gene expression, cell proliferation and differentiation, apoptosis, and the synthesis of pro-inflammatory cytokines in response to extracellular stimulation.[5,6] MAPKs are activated by phosphorylation of the threonine and tyrosine residues in the Thr-Xaa-Tyr motif in the activation loop domain and inactivated by dephosphorylation catalyzed by dual-specificity phosphatases (DUSPs). Members of the typical DUSP subfamily contain an N-terminal MAPK-binding motif and are critical for the termination of the MAPK cascades.[7] Thus they are also referred to as MAP kinase phosphatases (MKPs). These typical DUSPs exhibit differences in expression patterns, subcellular localization, and substrate preferences.

Studies from patients with pulmonary arterial hypertension have observed dysregulated pulmonary artery smooth muscle cell growth that is dependent on both p38 MAPK and c-Jun N-terminal kinase (JNK).[8] Inhibiting p38 MAPK in mice with right ventricular (RV) failure resulted in improved RV function and inhibition of RV fibrosis.[9] We have previously shown that *DUSP1 (MKP-1)* knockout mice exposed to chronic hypoxia had greater thickening of their pulmonary arteries as well as evidence of RV hypertrophy compared to wild-type (WT) mice.[10] Similarly, DUSP (MKP-1) was found to inhibit hypoxic proliferation of human pulmonary arterial smooth muscle cells.[11] The role of the DUSP family in BPD-PH, however, remains unknown. Therefore, we postulate that the development of PH in BPD may be influenced by the regulatory actions of the *DUSP* family of genes. In the present study, we

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hypothesized that mutations in the *DUSP* gene family are involved in the pathogenesis of BPD-PH and therefore that single-nucleotide polymorphisms (SNPs) within the *DUSP* gene family will be differentially distributed in cohorts of BPD patients with and without PH. We also sought to determine whether SNPs in the *DUSP* family of genes could be combined with clinical data to serve as a useful biomarker to predict which BPD patients will develop PH.

METHODS
The Institutional Review Board at Nationwide Children's Hospital approved this study. Preterm infants diagnosed with BPD were enrolled from the neonatal intensive care unit at Nationwide Children's Hospital (level 4, all referral) after September 1, 2009. Diagnostic criteria for BPD were determined according to the NICHD consensus statement as a supplemental oxygen requirement at 28 days of life.[12] Consistent with the physiologic definition of BPD established by Walsh et al. in 2003,[13] in order to standardize and reduce the variation in observed rates of BPD, Nationwide Children's Hospital NICU maintains oxygen saturation goals of 90–95% for patients <36 weeks postmenstrual age (PMA) and >94% for patients >36 weeks PMA. Enrollment, clinical data abstraction, and specimen collection were completed through the Ohio Perinatal Research Network and Perinatal Research Repository at The Research Institute at Nationwide Children's Hospital, Columbus, OH. BPD patients with congenital heart disease, congenital diaphragmatic hernia, and lung hypoplasia were excluded from this study. We did not exclude BPD patients with isolated atrial septal defect, ventricular septal defect, or patent ductus arteriosus. Clinical characteristics were obtained from systematic medical chart review. Both postnatal steroids prior to admission and steroids during admission, as well as caffeine, were all given systemically.

Pulmonary hypertension
PH was diagnosed after 28 days of life by echocardiogram. Evidence of elevated pulmonary arterial pressure on echocardiogram included intraventricular septal flattening, right ventricular hypertrophy and/or dilation, tricuspid regurgitant jet velocity >3 m/s, and/or pulmonary regurgitation.[3,14–17] From a BPD study cohort of 188 preterm infants with BPD, 61 (32%) of these patients were diagnosed with PH based on these echocardiographic parameters and were therefore considered cases. Controls were defined as patients with BPD who did not have PH by these echocardiographic criteria. Some patients in this study have been enrolled previously in research studies.[14,17–19]

Single-nucleotide polymorphisms
As described in previous studies,[17,18] patient blood samples were collected after parent consent and study enrollment. Blood samples were collected and stored by the Perinatal Research Repository at the Abigail Wexner Research Institute at Nationwide Children's Hospital. DNA was isolated from blood leucocytes and SNPs were genotyped by Agena MassArray (Agena, San Diego, CA). For each loci, the SNP (minor allele) was defined as the allele with a minor allele frequency (MAF) <0.5. SNPs were selected for their putative functionality, which was determined by SNP location within the gene loci and potential for altering protein structure and expression as determined from the NCBI/SNP database. PubMed literature searches were conducted to focus on *DUSP* genes that had known associations with cardiopulmonary disease. This selection process yielded a total of 31 SNPs on 9 out of the 12 *DUSP* family of genes, which included typical *DUSP* family members: *DUSP1*, *DUSP2*, *DUSP4*, *DUSP5*, *DUSP6*, *DUSP7*, *DUSP9*, *DUSP10*, and *DUSP16*. Each of these nine typical *DUSP* family members have a MAPK-binding motif that interacts with the common domain of MAPKs.[7]

Statistics
This was a case–control study. Clinical characteristics were compared using chi-squared tests for categorical variables and Student's *t* test for continuous variables. Statistical significance was considered at $p < 0.05$. This study was designed to test a specific hypothesis and only candidate *DUSP* family genes were included in the study. Given the limitation of study sample size, less conservative values are also of interest and may be hypothesis generating. Therefore, no correction was made for multiple testing. Calculated MAF for cases and controls were compared using chi-squared test with one degree of freedom, similar to previous studies.[17,18,20] Univariate and multivariate logistic regression was employed to assess clinical and genetic predictors of PH in BPD. Chi-square, *t* test, and logistic regression analyses were completed with STATA/IC 12.0 (STATA Corp., College Station, TX, USA). Biomarker analyses utilizing the area under the curve (AUC) of receiver operating characteristic (ROC) curves were completed using the GraphPad Prism 7 Software (La Jolla, CA). Clinical and SNP AUC models were constructed after analysis by *t* test revealed six possible early clinical predictors of PH in BPD (birth weight, gestational age, small for gestational age (SGA), surfactant, postnatal steroids prior to admission, and mechanical ventilation). However, since birth weight, gestational age, and SGA are interdependent variables, we chose to exclude gestational age and SGA and include only four possible early clinical predictors. Similarly, analysis by *t* test revealed three possible genetic predictors (rs322351, rs1042606, rs3793892). These AUC models were based on data resubstitution method, which uses the same data to construct and evaluate the model. Although data resubstitution can underestimate the classifier error, it has less variability than other methods, such as cross-validation, especially for small sample sizes.[21]

In order to better evaluate the data in the absence of an external validation cohort, we also completed the cross-validation analysis. Cross-validation has the advantage of producing a less biased result; however, for small sample sizes such as the present study, cross-validation has greater variability than resubstitution. Both methods of prediction have been shown to be comparable in terms of ranking accuracy.[21] Cross-validation was achieved by 100 splits of all clinical and SNP data into 2/3 training and 1/3 test sets. Each iteration of training/test set split divided the data into a different 2/3 training and 1/3 test set. For each split, the training data were used to identify the top three DUSP SNPs and the top four clinical covariates based on statistical significance from univariable associations. Logistic regression models were built using the top three SNPs, the top four clinical variables, and the top three SNPs and top four clinical variables combined. The AUC was evaluated on test data, averaged across test data sets, and presented as median and interquartile range (IQR).[22,23]

RESULTS
Clinical characteristics for the entire cohort, BPD-PH patients (cases), and BPD without PH patients (controls) are shown in Table 1. We found that birth weight and gestational age were lower in cases than in controls (Table 1). There was also a greater rate of SGA in cases than in the controls (Table 1). We did not find any sex or race differences. There was a trend toward a greater rate of African Americans with BPD-PH compared to BPD-without PH. To further explore this, a chi-squared analysis comparing African American and Caucasian groups was conducted and found no significant difference ($p = 0.34$) between cases and controls. APGARs were similar between the two groups. Although rates of respiratory distress syndrome were similar among cases and controls, we found that surfactant was given less frequently in cases than in controls (Table 1). Postnatal steroids prior to admission were more common in cases than in controls. More

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Table 1.	Clinical characteristics of the BPD cohort			
	BPD-without PH, $N = 127$	BPD-PH, $N = 61$	Total, $N = 188$	p Value
Birth history				
Birth weight (g)	972 ± 414	806 ± 343	918 ± 399	0.004*
Gestational age (weeks)	$27^0 \pm 2^5$	$26^2 \pm 2^4$	$26^5 \pm 2^5$	0.041*
SGA (<10th %tile)	9 (7%)	12 (20%)	21 (11%)	0.010*
LGA (>90th %tile)	10 (8)	2 (3%)	12 (6%)	0.228
Sex (male)	83 (65%)	35 (57%)	118 (63%)	0.289
Race				0.579
Other	5 (4%)	3 (5%)	8 (4%)	
Asian	2 (2%)	0 (0%)	2 (1%)	
African American	33 (26%)	20 (33%)	53 (28%)	
Caucasian	87 (69%)	38 (62%)	125 (66%)	
APGAR, 1 min[a]	4 ± 2	4 ± 2	4 ± 2	0.563
APGAR, 5 min[b]	6 ± 2	6 ± 2	6 ± 2	0.494
RDS	119 (95%)	60 (98%)	179 (96%)	0.288
Surfactant	80 (63%)	29 (48%)	109 (58%)	0.044*
Postnatal steroids prior to admission[c]	14 (12%)	20 (33%)	34 (19%)	0.001*
Transported intubated/vent[a]	68 (54%)	42 (69%)	110 (59%)	0.060
Referral NICU admit age (days)	31 ± 48	38 ± 49	33 ± 48	0.365
Respiratory support				
nCPAP[b]	94 (76%)	49 (80%)	143 (77%)	0.490
Mechanical ventilation[b]	90 (73%)	56 (92%)	146 (79%)	0.003*
>30% oxygen at 36 weeks PMA	111 (87%)	58 (95%)	169 (90%)	0.102
Co-morbidities				
Pneumonia	28 (22%)	17 (28%)	45 (24%)	0.414
Any IVH[a]	27 (22%)	7 (12%)	34 (18%)	0.093
Any ROP	30 (24%)	11 (18%)	41 (22%)	0.385
Sepsis	7 (6%)	6 (10%)	13 (7%)	0.274
NEC-medical[a]	26 (21%)	7 (11%)	33 (18%)	0.118
NEC-surgical[a]	11 (9%)	6 (10%)	17 (9%)	0.818
NEC-perf	8 (6%)	6 (10%)	14 (7%)	0.387
Hospital medications/interventions				
Steroids during admission[a]	68 (58%)	45 (75%)	113 (64%)	0.027*
Caffeine[b]	90 (73%)	44 (72%)	134 (72%)	0.949
iNO[b]	11 (9%)	19 (31%)	30 (16%)	<0.001*
Central line	93 (73%)	44 (72%)	137 (73%)	0.874
Hospital discharge information				
NICU discharge oxygen	93 (73%)	52 (85%)	145 (77%)	0.066
Mortality	3 (2%)	4 (7%)	7 (4%)	0.155

Pearson chi^2 (1) for count data N (%), Student's t test for continuous variables, mean ± sd

SGA small for gestational age, LGA large for gestational age, BPD bronchopulmonary dysplasia, PH pulmonary hypertension, NICU neonatal intensive care unit, RDS respiratory distress syndrome, nCPAP nasal continuous positive airway pressure, PMA postmenstrual age, IVH intraventricular hemorrhage, ROP retinopathy of prematurity, NEC necrotizing enterocolitis, iNO inhaled nitric oxide

*Statistical significance, $p < 0.05$

[a]Missing data for APGAR 1 min, NEC-medical, NEC-surgical, any IVH, steroids during admission, transported intubated/vent; BPD $N = 125$, BPD-PH $N = 61$

[b]Missing data for APGAR 5 min, caffeine, iNO, mechanical ventilation, nCPAP; BPD $N = 124$, BPD-PH $N = 61$

[c]Postnatal steroids prior to admission, BPD $N = 117$, BPD-PH $N = 60$

We evaluated 31 SNPs in 9 out of the 12 typical DUSP genes that are known to interact with MAPKs[7] and found that SNPs in DUSP1 and DUSP5 were significantly different between cases and controls (Table 2). These SNPs remained significantly different even after exclusion of BPD patients requiring <30% oxygen at 36 weeks PMA. DUSP1 SNP rs322351 had MAF that was lower in cases than in controls. Conversely, two SNPs in DUSP5, rs1042606 and rs3793892, had MAF that were greater in cases than in controls.

We next used logistic regression analysis to examine various dependent variables in the probability of developing PH in BPD (Table 3). For the clinical variables, we found that birth weight <700 g, postnatal steroids prior to admission, steroids during admission, total endotracheal tube ventilation >70 days, mechanical ventilation, iNO therapy, and no surfactant, all increased probability of developing PH. After adjusting for all other clinical variables in the table, only postnatal steroids prior to admission increased probability of developing PH (odds ratio 4.7 [1.76, 12.59], $p = 0.002$). Therefore, if a BPD patient was exposed to postnatal steroids prior to admission, the odds of PH in BPD were 4.7 times greater than if the BPD patient had not been exposed to treatment. When examining the three DUSP SNPs that were significantly different between groups, independent logistic regression of each DUSP SNP resulted in significant predictive modeling of PH in BPD. DUSP1 SNP rs322351 was associated with a 41% decrease in the odds of developing PH in BPD ($p = 0.020$), and this finding persisted even after adjustment for the other two DUSP5 SNPs ($p = 0.023$) (Table 3). Both DUSP5 SNPs rs1042606 and rs3793892 were associated with an approximately 1.6 times greater risk of developing PH in BPD ($p = 0.036$ and $p = 0.042$, respectively); however, this finding did not persist after adjustment for the other two DUSP SNPs (Table 3).

We next calculated AUC of the ROC curve for several clinical and SNP models, keeping in mind that a perfect biomarker is 100% sensitive and 100% specific, resulting in an AUC of 1. For the clinical models (Table 4), we included early significant clinical predictors as identified by Tables 1 and 3: birth weight <700 g, postnatal preadmission steroids, mechanical ventilation, and no surfactant. When these clinical predictors were analyzed separately, birth weight <700 g had the largest AUC for the ROC curve, although it was only 0.612 (Table 4). We next examined various combinations of these clinical factors using ROC curves, and the largest AUC for PH in BPD was 0.729 when birth weight <700 g +postnatal preadmission steroids+mechanical ventilation+no surfactant were analyzed together. For the genetic data, the ROC curve with the largest AUC was for the DUSP1 WT rs322351, which was modeled as a 0–2 variable dependent on the number of WT alleles present at the rs322351 locus (Table 3). We found that combining the SNPs improved the AUC of the ROC curve slightly, and there was little difference in the AUC of the ROC curve when combining all three SNPs or two of the three SNPs. The best AUC of the ROC curve for genetic data (DUSP1 SNP rs322351+DUSP5 SNP rs1042606) was only 0.632.

We were interested to know whether we could improve the AUC of the ROC curve by including both clinical and SNP data. Figure 1 shows the ROC curves of the best AUC (0.729) from the combined clinical data (from Table 4) and the best AUC (0.632) from the combined SNP data (from Table 4). When we combined these clinical and SNP data together, we found a small increase in the AUC of the ROC curve to 0.755 (Fig. 1). We compared each of the AUCs generated in Fig. 1 and found that the clinical+SNP AUC of 0.755 was significantly greater than the SNP AUC of 0.632 ($p = 0.035$). Cross-validation analysis found a lower median AUC for all groups (clinical median AUC 0.657 [IQR 0.603–0.704], SNP median AUC 0.543 [IQR 0.512–0.584], clinical+SNP median AUC 0.642 [IQR 0.589–0.701]), the clinical median AUC was slightly greater than the clinical+SNP median AUC, and there continued to be a difference between SNP and clinical+SNP median AUC.

cases were treated with mechanical ventilation than were controls. We found no differences in the use of nasal continuous positive airway pressure (nCPAP), >30% oxygen at 36 weeks PMA, or in the common co-morbidities of prematurity between cases and controls. For hospital medications, we found that steroid treatment given during hospital admission occurred more frequently in cases than in controls. As expected, cases of BPD-PH received more inhaled nitric oxide (iNO) treatment than BPD-without PH controls. We found no differences in rates of infection or use of a central line between cases and controls.

Table 2. Single-nucleotide polymorphisms in *DUSP* genes

SNP	Gene	Chromosome; functional consequence	Major allele > minor allele	BPD MAF, $N = 127$	BPD-PH MAF, $N = 61$	*p* Value
rs881150	*DUSP1*	5; upstream variant 2KB	T>A	0.217	0.246	0.524
rs881152	*DUSP1*	5; upstream variant 2KB	G>A	0.217	0.246	0.524
rs322351	*DUSP1*	5; downstream variant 500B	C>T	0.444	0.320	0.021*
rs322380	*DUSP1*	5; intron variant	G>A	0.295	0.377	0.112
rs322382	*DUSP1*	5; upstream variant 2KB	C>T	0.339	0.426	0.098
rs3805476	*DUSP1*	5; downstream variant 500B	G>A	0.157	0.156	0.965
rs2969489	*DUSP2*	2; upstream variant 2KB	G>A	0.484	0.566	0.140
rs1724121	*DUSP2*	2; downstream variant 500B	A>G	0.335	0.287	0.352
rs567436	*DUSP4*	8; intron variant	A>T	0.283	0.238	0.349
rs569209	*DUSP4*	8; intron variant	G>A	0.283	0.238	0.349
rs474824	*DUSP4*	8; intron variant	T>C	0.406	0.467	0.257
rs1042606	*DUSP5*	10; utr variant 3 prime	C>A	0.335	0.443	0.042*
rs1889568	*DUSP5*	10; intron variant	A>G	0.319	0.418	0.059
rs2282239	*DUSP5*	10; intron variant	A>G	0.323	0.418	0.071
rs3793892	*DUSP5*	10; intron variant	T>G	0.315	0.418	0.049*
rs915216	*DUSP5*	10; downstream variant 500B	T>C	0.335	0.434	0.060
rs10744	*DUSP6*	12; utr variant 3 prime	A>T	0.327	0.295	0.536
rs704073	*DUSP6*	12; utr variant 3 prime	C>T	0.323	0.295	0.587
rs704076	*DUSP6*	12; intron variant	G>T	0.303	0.279	0.626
rs769700	*DUSP6*	12; intron variant	T>C	0.307	0.279	0.573
rs704074	*DUSP6*	12; utr variant 3 prime	A>G	0.327	0.295	0.536
rs4687607	*DUSP7*	3; intron variant	G>A	0.226	0.221	0.916
rs3821841	*DUSP7*	3; nc transcript variant	C>T	0.091	0.049	0.159
rs9851576	*DUSP7*	3; upstream variant 2KB	A>G	0.224	0.221	0.946
rs7883986	*DUSP9*	X; intron variant	C>T	0.303	0.246	0.249
rs4243539	*DUSP9*	X; upstream variant 2KB	T>C	0.476	0.467	0.868
rs7882888	*DUSP9*	X; intron variant	C>A	0.146	0.175	0.465
rs10746413	*DUSP10*	1; intron variant	G>C	0.409	0.418	0.874
rs10779454	*DUSP10*	1; intron variant	C>T	0.362	0.402	0.460
rs4255620	*DUSP16*	12; intron variant	C>G	0.461	0.418	0.437
rs4763833	*DUSP16*	12; intron variant	T>C	0.429	0.508	0.150

DUSP1 ($n = 6$), *DUSP2* ($n = 2$), *DUSP4* ($n = 3$), *DUSP5* ($n = 5$), *DUSP6* ($n = 5$), *DUSP7* ($n = 3$), *DUSP9* ($n = 3$), *DUSP10* ($n = 2$), and *DUSP16* ($n = 2$) were studied (31 total SNPs)
BPD bronchopulmonary dysplasia, *DUSP* dual-specificity phosphatase, *MAF* minor allele frequency, *PH* pulmonary hypertension, *SNP* single-nucleotide polymorphism
*Statistical significance, $p < 0.05$

DISCUSSION

In this biomarker study of clinical factors and *DUSP* loci for the prediction of PH in BPD, we have several novel findings: (1) BPD-PH patients had a lower birth weight and gestational age and a greater percentage of SGA than in BPD-without PH controls, (2) BPD-PH patients received less surfactant, more postnatal pre-admission steroids, and more mechanical ventilation than did BPD-without PH controls, (3) BPD-PH patients received more steroids during admission and iNO treatment than did BPD-without PH controls, (4) *DUSP1* SNP rs322351 was less common, and *DUSP5* SNPs rs1042606 and rs3793892 were more common in cases than in controls, (5) the overall best fit biomarker combines clinical and *DUSP* genetic data with an area under the ROC curve of 0.76, which is significantly greater than DUSP genetic data alone area under the ROC curve of 0.63.

Previous clinical studies by our group did not detect a difference in SGA between BPD-PH and BPD-without PH controls,[14,17,18] while in the present study we recruited our largest BPD cohort to date of 188 patients, thus improving power, and

were able detect a greater percentage of SGA in cases than in controls. SGA is indicative of an early and poorly developed vascular network in these premature neonates, thus contributing to both their lung disease (BPD) and PH. Consistent with previous clinical studies, we observed lower birth weight and gestational age.[14] Similar distribution of sex and race were observed. The largest minority group in our cohort, African American, had a trend toward greater rates of BPD-PH compared to BPD-without PH. Since SNP distribution can be dependent on racial background, the observation that the African American group trended toward more severe disease (BPD-PH) warrants future study. In our cohort, BPD-PH patients received less surfactant, a novel finding, indicating that in the premature neonate, early surfactant administration prior to nCPAP may be an important preventative strategy against PH in BPD. We also found greater steroid treatment in BPD-PH patients than in BPD-without PH patients, with similar distribution of BPD severity among groups. This is consistent with our study in 2014, where we found a trend toward greater postnatal steroid use in BPD-PH compared to BPD-without

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Table 3. Clinical and genetic predictors of PH in BPD cohort

	Unadjusted OR [95% CI]	p Value	Adjusted OR [95% CI]	p Value
Potential clinical predictors of PH				
Birth weight <700 g	2.87 [1.51, 5.46]	0.001*	2.53 [0.94, 6.81]	0.065
Gestational age <25 weeks	1.70 [0.87, 3.31]	0.121	0.63 [0.23, 1.77]	0.385
Postnatal steroids prior to admission[a]	3.68 [1.70, 7.98]	0.001*	4.70 [1.76, 12.59]	0.002*
Steroids during admission[b]	2.16 [1.08, 4.31]	0.029*	0.65 [0.26, 1.62]	0.360
Total ETT vent >70 days	2.68 [1.27, 5.67]	0.01*	1.97 [0.76, 5.09]	0.163
Mechanical ventilation[c]	4.23 [1.56, 11.46]	0.001*	3.22 [0.81, 12.86]	0.055
iNO[c]	4.65 [2.04, 10.58]	<0.001*	2.63 [0.99, 6.97]	0.051
Surfactant	0.53 [0.29, 0.99]	0.046*	0.47 [0.22, 1.02]	0.055
Potential genetic predictors of PH				
DUSP1 SNP rs322351	0.586 [0.369, 0.930]	0.020*	0.582 [0.364, 0.929]	0.023*
DUSP5 SNP rs1042606	1.638 [1.027, 2.612]	0.036*	1.201 [0.268, 5.381]	0.811
DUSP5 SNP rs3793892	1.623 [1.012, 2.602]	0.042*	1.371 [0.300, 6.262]	0.684

Odds ratios measured separately by logistic regression presented as unadjusted and adjusted OR and p value. N = 188
BPD bronchopulmonary dysplasia, CI confidence interval, DUSP dual-specificity phosphatase, ETT endotracheal tube, iNO inhaled nitric oxide, OR odds ratio, PH pulmonary hypertension, SNP single-nucleotide polymorphism
*Statistical significance, p < 0.05
[a]Postnatal steroids prior to admission, BPD N = 117, BPD-PH N = 60
[b]Steroids during admission, BPD N = 125, BPD-PH N = 61
[c]Mechanical ventilation, BPD N = 124, BPD-PH N = 61

Table 4. Area under the ROC curve for clinical and SNP data, respectively

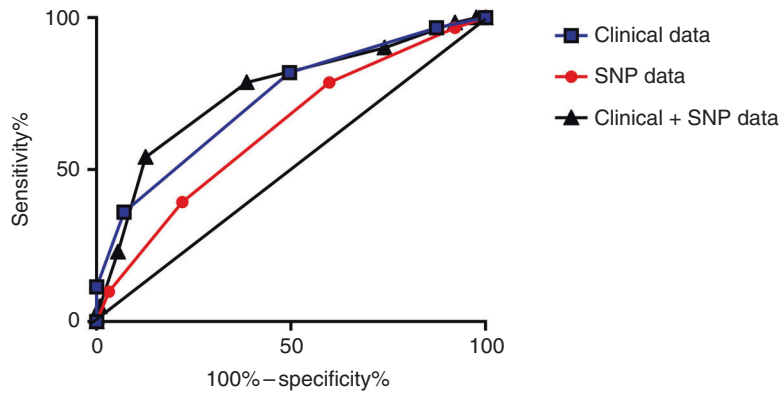|	AUC
Clinical models	
Birth weight <700 g (0–1)	0.612
Postnatal, preadmission steroids (0–1)	0.607
Mechanical vent (0–1)	0.596
No surfactant (0–1)	0.577
Birth weight <700 g+postnatal, preadmission steroids (0–2)	0.656
Birth weight <700 g+mechanical ventilation (0–2)	0.667
Birth weight <700 g+no surf (0–2)	0.647
Birth weight <700 g+postnatal, preadmission steroids +mechanical ventilation (0–3)	0.701
Birth weight <700 g+postnatal, preadmission steroids +mechanical ventilation+no surfactant (0–4)	0.729[a]
SNP models	
DUSP1 WT rs322351 (0–2)	0.599
DUSP5 SNP rs1042606 (0–2)	0.574
DUSP5 SNP rs3793892 (0–2)	0.572
All 3 DUSP loci (0–6)	0.623
DUSP1 WT rs322351+DUSP5 rs1042606 (0–4)	0.632[b]
DUSP1 WT rs322351+DUSP5 rs3793892 (0–4)	0.630

AUC calculated for BPD cohort (BPD alone, n = 127 and BPD-PH, n = 61)
AUC area under the curve, BPD bronchopulmonary dysplasia, ROC receiver operating characteristic, DUSP dual-specificity phosphatase, PH pulmonary hypertension, SNP single-nucleotide polymorphism, WT wild type
[a]Best fit clinical model: birth weight <700 g+postnatal, preadmission steroids+mechanical ventilation+no surf (0–4 variable) with AUC 0.729
[b]Best fit SNP model: additive DUSP1 rs322351+DUSP5 rs1042606 (0–4 variable) with AUC 0.632

patients.[18] The time course of steroid treatment and diagnosis of BPD and/or PH requires further study, since 75% of BPD-PH patients received steroids during admission and this could in part explain why severe BPD (>30% oxygen at 36 weeks PMA) was not statistically greater in BPD-PH compared to that in BPD-without PH patients. Inflammatory processes are downregulated by steroids by way of DUSP1 (MKP-1).[24] Therefore, treatment with steroids, or perhaps more specifically targeting overexpression of DUSP1 could potentially ameliorate PH in BPD, however, this has yet to be studied. Mechanical ventilation use was greater in BPD-PH patients than BPD-without PH patients, consistent with previous studies.[14,17,25] As expected, iNO treatment was greater in BPD-PH patients, and its role as a potent vasodilator[26,27] likely improved PH but did not prevent PH in BPD.

We found that DUSP1 SNP rs322351 was less common, and DUSP5 SNPs rs1042606 and rs3793892 were more common, in cases than in controls. Both DUSP1 (MKP-1) and DUSP5 are typical DUSPs with a MAPK-binding motif[7] and are also a part of a subset group of DUSPs that are inducible nuclear phosphatases.[28] A difference between DUSP1 (MKP-1) and DUSP5 is that DUSP5 is MAPK extracellular signal-regulated kinases 1/2 (ERK1/2) specific, whereas DUSP1 (MKP-1) has phosphatase activity toward both ERK1/2 and the stress-activated kinases p38 and JNK.[28–32] However, only DUSP1 (MKP-1) can act as both a phosphatase of MAPK and a MAPK substrate. MAPK ERK-mediated phosphorylation of DUSP1 (MKP-1) can lead to ubiquitination/protein degradation or increased protein stability, depending on the DUSP1 (MKP-1) phosphorylation site.[28,33,34] Similar to DUSP1 (MKP-1), DUSP5 protein can be stabilized by MAPK ERK, thus reinforcing respective phosphatase activities in a positive feedback loop.[28] Future studies could determine whether DUSP1 SNP rs322351, DUSP5 SNP rs1042606, and/or DUSP5 SNP rs3793892 modify DUSP protein function to contribute to the pathophysiology of PH in BPD. MAPKs phosphorylate and activate numerous downstream targets, including transcription factors and protein kinases that play a key role in fundamental cellular processes that are influenced by inflammation, including cellular survival,

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CLINICAL + SNP MODELS	AUC	SE	95% CI	p-value
Clinical data: birthweight <700 g + post-natal, pre-admission steroids + mechanical ventilation + no surfactant (0–4)	0.729	0.040	(0.651, 0.807)	<0.0001
SNP data: DUSP1 WT rs322351+DUSP5 SNP rs1042606 (0–4)	0.632	0.043	(0.547, 0.716)	0.004
Clinical + SNP data: (0–8)	0.755*	0.039	(0.678, 0.832)	<0.0001

Fig. 1 The best fit biomarker model to predict pulmonary hypertension (PH) in bronchopulmonary dysplasia (BPD) combines clinical and single-nucleotide polymorphism (SNP) data. Area under the curve (AUC) calculated for BPD cohort (BPD alone, $n = 127$ and BPD-PH, $n = 61$). Combining the best fit independent clinical biomarker model (birth weight <700 g+postnatal, pre-admission steroids+mechanical ventilation+no surfactant) with the best fit independent SNP biomarker model (*DUSP1* WT rs322351+*DUSP5* SNP rs1042606) yields a 0–8 variable with an AUC 0.755. *AUC Clinical+SNP data different from AUC SNP data ($p = 0.035$)

proliferation, and apoptosis.[35] We speculate that the effect of MAPK is to promote cellular proliferation of the pulmonary vascular wall, contributing to PH, while DUSP1/5, by negatively regulating MAPKs, would prevent PH.

Human studies of DUSP1 (MKP-1) have shown that the antiproliferative effect of sildenafil on pulmonary artery smooth muscle cells is mediated by the upregulation of DUSP1 (MKP-1).[36] These findings suggest that DUSP1 (MKP-1) may be protective against hallmark features in the progression of PH in patients with BPD. MAPK ERK1/2-mediated cell proliferation has been specifically implicated in the pathophysiology of PH,[37,38] and thus DUSP5, with its characteristic MAPK ERK1/2 specificity, is a promising therapeutic target for MAPK ERK1/2-mediated PH. Future clinical studies evaluating MAPK ERK1/2, JNK, and p38 profile in our BPD cohort with and without PH could determine whether a MAPK ERK1/2-specific inhibitor, such as DUSP5, might serve as a targeted and effective therapy for BPD-PH patients.

This study was limited by small cohort size. BPD is considered a rare disease by NIH, and we have expanded our single-center cohort to include as many BPD patients as possible. However, the relatively small sample size limited the power of this study to detect potentially important differences. We have immediate plans to replicate this study in a larger and independent BPD cohort. Another major limitation has been the difficulty of diagnosing PH in neonates and infants; however, bedside echocardiography is now the standard of care and serves as a useful and less-invasive tool to reliably diagnose neonatal PH.[39]

Taken together, these clinical and *DUSP* SNP data produce the best area under the ROC curve to date for BPD-PH (0.76). The most predictive clinical biomarkers, birth weight <700 g, postnatal preadmission steroids, mechanical ventilation, and no surfactant, also contribute to the pathophysiology of PH. We found that *DUSP1* and *DUSP5* SNPs are predictive of PH in BPD. Future studies are needed to combine and add biomarkers to further improve the AUC in order to create a clinically applicable biomarker panel for BPD-PH.

AUTHOR CONTRIBUTIONS
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