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ORIGINAL ARTICLE

Possible association between a polymorphism of *EPAS1* gene and persistent pulmonary hypertension of the newborn: a case-control study

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Abstract

Objective: To explore possible genes related to the development of persistent pulmonary hypertension of the newborn (PPHN).

Methods: The authors identified 285 single nucleotide polymorphisms (SNPs) of 11 candidate genes (*BMPR2*, *EPAS1*, *PDE3A*, *VEGFA*, *ENG*, *NOTCH3*, *SOD3*, *CPS1*, *ABCA3*, *ACVRL1*, and *SMAD9*), using an Illumina Asian Screening Array-24 v1.0 BeadChip Array. The FastLmmC and R package was used for statistical analyses. The chi-square test and Cochran-Armitage trend test were used to compare the allele and genotype frequencies between the groups and to test the genetic models, respectively.

Results: A total of 45 PPHN infants and 294 control subjects were analyzed. The most common cause of PPHN was meconium aspiration syndrome. Among the 285 SNPs, 17 SNPs from 6 candidate genes (*BMPR2*, *EPAS1*, *PDE3A*, *VEGFA*, *ENG*, and *NOTCH3*) were significantly associated with PPHN ($P < 0.05$). After using the Bonferroni correction ($P < 0.00018$), only the rs17034984 SNP located in intron 1 of the *EPAS1* gene remained significantly different between the PPHN and control subjects ($P = 0.00014$). The frequency of the TC/TT genotype of rs17034984 in the gene with the dominant model was significant in the patients with PPHN (OR = 5.38, 95% CI: 2.15–13.49). The T allele frequency of rs17034984 in the gene showed a significant difference compared with the control subjects (OR = 4.89, 95% CI: 2.03–11.82).

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Conclusions: The present study suggests that the rs17034984 variant of *EPAS1* gene is associated with PPHN.

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Introduction

Persistent pulmonary hypertension of the newborn (PPHN) is the most serious complication of several neonatal respiratory diseases, leading to adverse outcomes such as asphyxia, chronic lung disease, neurodevelopmental sequelae, and death, despite the use of modern therapeutic modalities for treating PPHN such as high-frequency oscillatory ventilation, inhaled nitric oxide, and extracorporeal membrane oxygenation.^{1,2} Although the primary etiologies of PPHN are various factors such as chronic hypoxia, meconium aspiration syndrome, birth asphyxia, sepsis, infection, and respiratory distress syndrome,^{1,3,4} a genetic contribution to PPHN has been increasingly recognized. Previous studies have shown that SNPs in the *CRHR1* (rs4458044), *CPS1* (rs41272673, rs4399666, rs2287599, rs192759073, rs1047883, and rs2229589), *EDN1* (rs2070699), and *NOTCH3* (rs1044008) genes are susceptibility loci associated with PPHN.^{5–9} It has also recently been reported that mutations in a few genes such as *TLL3* and *ITGAM*¹⁰ were associated with PPHN. *TBX4* variant has also been found in relation to neonatal and pediatric pulmonary hypertension.¹¹

In this study, the authors aimed to determine any associations between SNPs of previous PPHN candidate genes and to perform a validation of any genetic associations found to elucidate a possible biological involvement of the protein(s) encoded from the gene(s) in the etiology of PPHN.

Materials and methods

Study population

The authors recruited 45 patients with PPHN who were hospitalized at Hat Yai hospital, one of the biggest hospitals in Southern Thailand where the prevalence of PPHN is relatively high, from 2.6 to 2.8 per 1000 live births, and constituting ~30% of the all-cause mortality rate.^{1,12} Retrospective data of PPHN infants discharged from the hospital between January 2013 and March 2019, born to mothers with a gestational age of ≥ 35 weeks and birth weight of ≥ 2000 g, were retrieved from the hospital computer data system. The authors recruited 20 patients from the retrospective data. The authors also recruited additional PPHN neonates who were hospitalized at the hospital between April 2019 and November 2019 (Figure 1). The authors excluded patients with congenital structural cyanotic heart diseases and other congenital disorders such as congenital diaphragmatic hernia and birth defects due to chromosomal disorders. For a healthy control group, the authors recruited 59 neonates who were born at the hospital between April 2019 and November 2019 and 235 adults who participated in the Thai SNP database project of Ramathibodi Hospital, Bangkok, Thailand. A summary of subjects involving in the

study is shown in Figure 1 and baseline characteristics are presented in supplementary Table 1. This study was approved by the Research Ethics Committee of the Hat Yai Hospital (EC No. 24/2562).

Study definitions

The diagnostic criteria for PPHN were presentation shortly after birth with refractory hypoxemia plus one of the three following conditions: 1) documented pulmonary hypertension, as defined by echocardiographic evidence of elevated pulmonary pressure (right to left or bidirectional shunt) and/or 2) a pre-to-postductal partial pressure of oxygen gradient equal to or greater than 20 mm Hg, and/or 3) a pulse oximetry oxygen saturation (SpO₂) gradient equal to or greater than 10%.¹³

Genomic DNA extraction

Genomic DNA was extracted from a 200 μ L sample of peripheral blood using a Qiagen DNA Mini kit (Qiagen, Hilden, Germany). The quantity and quality of the DNA samples were evaluated using an Eppendorf BioPhotometer plus (Eppendorf AG, Hamburg, Germany) and automated electrophoresis (Agilent 2200 TapeStation system, Santa Clara, California, United States).

Genotyping and quality control

A total of 659,184 single nucleotide polymorphism (SNPs) probes specific to several regions across the genome were fabricated on an Illumina Asian Screening Array-24 v1.0 BeadChip Array. SNP genotyping using an Illumina Infinium HTS Assay platform (Illumina, San Diego, California) was carried out and 612,128 (93%) SNPs were successfully genotyped. For quality control, the authors applied a low call rate $< 98\%$, a Hardy–Weinberg equilibrium calculations p -value $< 1 \times 10^{-8}$, and minor allele frequency (MAF) of each SNP < 0.05 to exclude data with low quality. After the quality control exclusion, 498,160 autosomal SNPs were left for the genome-wide association study (GWAS) analysis. The authors selected the SNPs in the genes related to PPHN and pulmonary arterial hypertension (PAH). Finally, 285 SNPs of 11 candidate genes (*BMP2R*, *EPAS1*, *PDE3A*, *VEGFA*, *ENG*, *NOTCH3*, *SOD3*, *CPS1*, *ABCA3*, *ACVRL1*, and *SMAD9*) were analyzed.

Immunohistochemistry (IHC) detection of HIF-2 α in umbilicus and lung tissues

For the validation study, the authors evaluated the expression of hypoxia-inducible factor 2-alpha (HIF-2 α), a protein encoded from the *EPAS1* gene, in the pulmonary vascular endothelium of lung tissues obtained from deceased PPHN

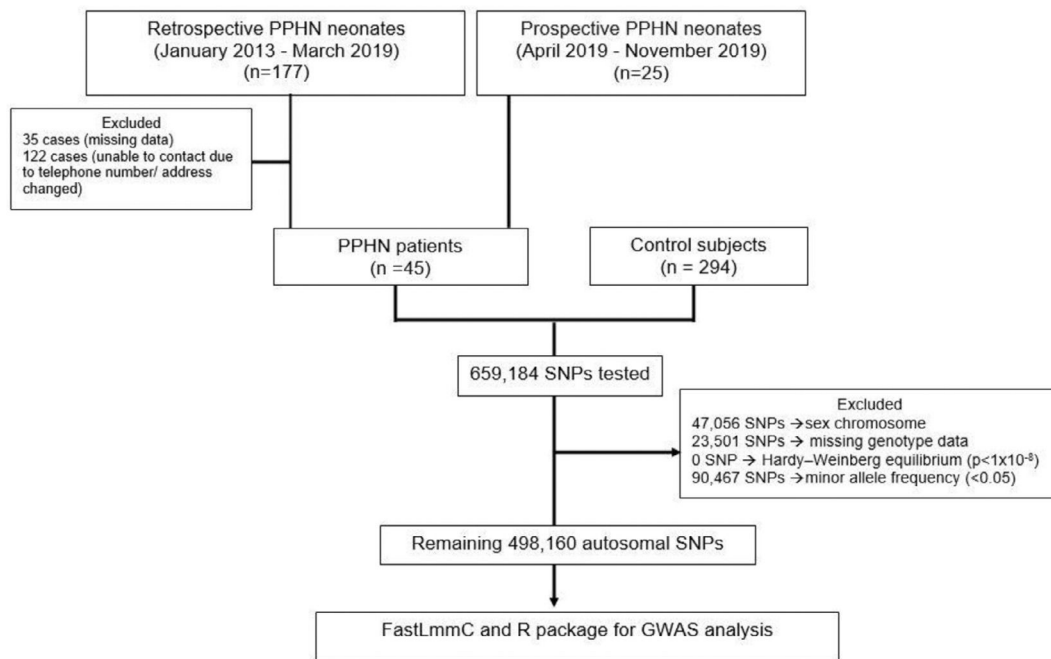


Figure 1 A flow chart illustrating subject selection and study workflow.

patients ($n = 3$) compared to normal adult lung tissue ($n = 3$). In addition, the authors carried out IHC staining of HIF-2 α in the umbilicuses of 8 healthy newborns to evaluate the expression of the protein in the umbilical artery and vein. Paraffin-embedded umbilical and lung tissues were stained with hematoxylin and eosin to examine histopathology of the tissues. Then, IHC using anti-HIF-2 α antibody (ab199, Abcam, UK) with dilution 1:100 v/v was performed on VENTANA BenchMark ULTRA platform (Ventana Medical Systems Inc., USA) to examine for HIF-2 α expression.

Statistical analysis

The authors used FastLmmC (v2.02.20121124) and the R package for GWAS (v. 3.3.3) for statistical analysis. Regional plots were generated using LocusZoom v.0.4.8. The allele and genotype frequencies of the candidate genes were calculated by the genotypes of the subjects. The chi-square test was used to compare the allele and genotype frequencies between the groups. When comparing the genotype frequency of each significant SNP between groups, the authors used the Cochran-Armitage trend test between all genotypes. Odds ratio (OR) and 95% confidence intervals (CI) were calculated to indicate the degree of association between groups.

Results

Association of SNPs in previously reported candidate genes and PPHN/PAH

The authors analyzed 285 SNPs in 11 candidate genes, including *BMP2*, *EPAS1*, *PDE3A*, *VEGFA*, *ENG*, *NOTCH3*, *SOD3*, *CPS1*, *ABCA3*, *ACVRL1*, and *SMAD9*, that were found to be associated with the pathogenesis of PPHN/PAH in

previous studies. A p -value of < 0.05 was considered significant. The authors found that 17 SNPs in 6 candidate genes: *BMP2*, *EPAS1*, *PDE3A*, *VEGFA*, *ENG*, and *NOTCH3*, were significantly associated with PPHN (Table 1). Of these, rs17034984 in the *EPAS1* gene was the only one to remain significant after the Bonferroni correction ($P < 0.00018$).

Genotype distribution, allele frequency, and fit inheritance model of significant SNPs in candidate genes ($P < 0.05$) associated with PPHN/PAH

The genotype and allele frequencies of candidate gene polymorphisms are summarized in Supplementary Table 2. Various models of inheritance (codominant, dominant, and recessive) were tested and genetic effects were calculated for each genotype compared to the reference genotype in each model. The authors found that the frequency of the TC/TT genotype of rs17034984 in the *EPAS1* gene with the dominant model was significant in the patients with PPHN (OR = 5.38, 95% CI: 2.15–13.49). In addition, the allele frequency of the minor allele T of rs17034984 in the *EPAS1* gene showed a significant difference compared with the control subjects (OR = 4.89, 95% CI: 2.03–11.82).

HIF-2 α immunohistochemistry staining of the vascular endothelium of umbilicus and lung tissues

HIF-2 α protein was expressed in lung tissues from 2 infants with PPHN. Localization of HIF-2 α was seen in the nuclei of endothelial cells of the pulmonary arteries (Figure 2A). However, there was one infant with PPHN in whom HIF-2 α protein expression was not detected (Figure 2B). The HIF-2 α protein was also expressed in the endothelial cells of the umbilical arteries and veins from the 8 normal newborns (Figure 2C). The HIF-2 α protein was not expressed in normal adult lung tissues and endothelial cells of the pulmonary

Table 1 Candidate genes related to the pathogenesis of PPHN (17 SNPs from 6 candidate genes).

Gene	Gene name	Chromosome position	Total SNPs tested	Significant SNPs	Position	p-value
<i>BMPR2</i>	<i>Bone morphogenetic protein receptor type2</i>	2q33.1–33.2	14	rs79171078	Intron	0.037
<i>EPAS1</i>	<i>Endothelial PAS domain protein 1</i>	2p21	41	rs17034984	Intron	0.00014*
				rs77238140	Intron	0.008
				rs2121267	Intron	0.008
				rs6726454	Intron	0.014
				rs4953344	Intron	0.021
				rs2346176	Intron	0.024
				rs1374749	Intron	0.032
				rs1868089	Intron	0.038
				rs6758592	Intron	0.038
				rs4953345	Intron	0.045
<i>PDE3A</i>	<i>Phosphodiesterase 3A</i>	12p12.2	132	rs10770682	Intron	0.013
				rs57238599	3' untranslated region UTR	0.002
				rs6487091	Intron	0.042
<i>VEGFA</i>	<i>Vascular endothelial growth factor A</i>	6p21.1	25	rs3025012	Intron	0.021
<i>ENG</i>	<i>Endoglin</i>	9q34.11	9	rs34116890	Intron	0.018
<i>NOTCH3</i>	<i>NOTCH receptor 3</i>	19p13.12	17	rs10404382	Intron	0.043

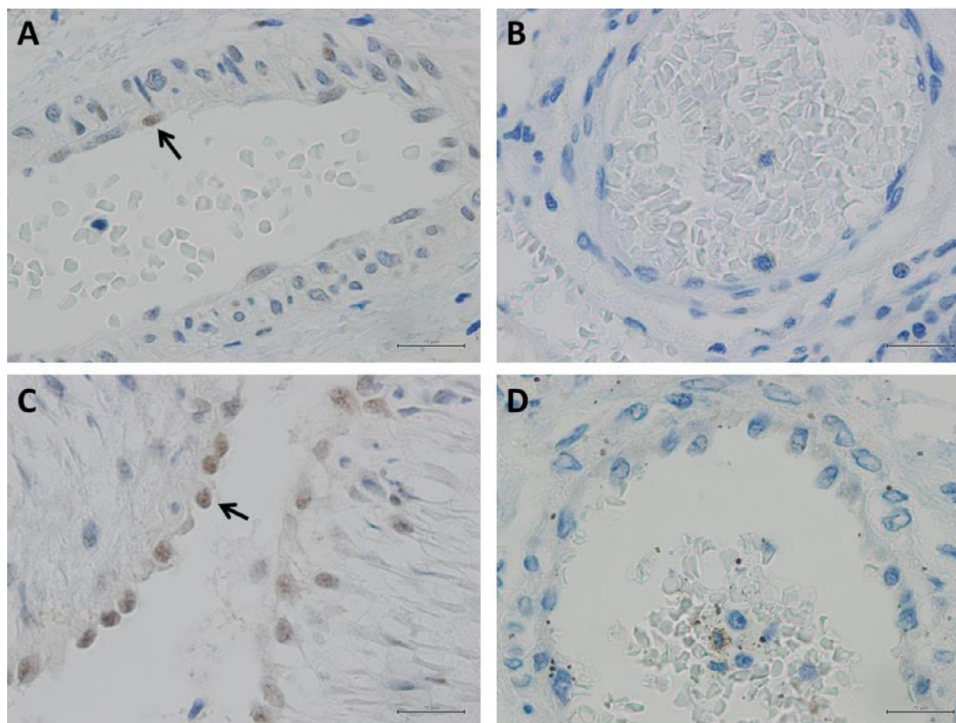


Figure 2 A, Positive HIF-2 α was detected in the nuclei of few endothelial cells (arrow) of bronchial vessels in PPHN patients; B, Endothelial cells of bronchial arterioles stained negatively for HIF-2 α ; C, Positive HIF-2 α was detected in the nuclei of many endothelial cells (arrow) of umbilical cord vessels in healthy newborns; D, Immunostaining of HIF-2 α stained negatively in adult bronchial vessels. Scale bar = 20 μ m.

Table 2 Expression of HIF-2 α protein in different tissues.

Tissue	Level of HIF-2 α protein	
	Manual scoring	IHC staining intensity normalized by nucleus (image analysis)
PPHN lung (case 1)	1	0.009
PPHN lung (case 2)	1	0.010
PPHN lung (case 3)	0	0.001
Normal adult lung (n = 3)	0	0.002
Umbilical cord (case 1)	2	0.026
Umbilical cord (case 2)	2	0.038
Umbilical cord (case 3)	2	0.036
Umbilical cord (case 4)	2	0.033
Umbilical cord (case 5)	2	0.029
Umbilical cord (case 6)	2	0.085
Umbilical cord (case 7)	2	0.042
Umbilical cord (case 8)	2	0.039

Note: intensity of staining: 0, no staining; 1, weak; 2, moderate; and 3, strong.

vessels (Figure 2D). Determination of protein expression was scored by two pathologists (intensity of staining: 0, no staining; 1, weak; 2, moderate; and 3, strong). In addition, semi-quantitative analysis of the IHC images was performed using ImageJ Fiji software (version 2.1.0/1.53C), following an integrated protocol.¹⁴ Expression of HIF-2 α in different tissues were shown in Table 2.

Discussion

PPHN is the most serious respiratory disorder in Thailand and has a high mortality rate. Its high prevalence in the study's institution led the authors to hypothesize whether genetic factors play an important role in the development of PPHN. Using SNP analysis, the authors found that rs17034984 in the *EPAS1* gene showed a significant association with PPHN in the studied population. In addition, the authors found that the T allele of rs17034984 in the *EPAS1* gene is a possible predisposing factor for PPHN and the TC/TT genotype with the dominant model could be a predictor of PPHN.

The rs17034984 SNP is located in intron 1 of the *EPAS1* gene. The *EPAS1* gene, located on the short arm of chromosome 2 (2p21), encodes endothelial PAS domain-containing protein 1, which is also known as hypoxia-inducible factor 2 alpha (HIF-2 α) protein.¹⁵ This protein is a subunit of the HIF protein complex, which plays a crucial role in the body's ability to adapt to changing oxygen levels. HIF-2 α is a transcription factor involved in the induction of oxygen-regulated genes. It regulates erythropoiesis and physiological responses to hypoxia through the HIFs signaling pathway.¹⁶ In hypoxic conditions, the HIFs protein is translocated to the nucleus to bind the hypoxic response element (HRE) of target gene promoters and subsequently initiate gene transcription. When oxygen levels rise, HIFs are hydroxylated by prolyl hydroxylase domain proteins (PHD), leading to interaction with other proteins, and with the addition to

ubiquitin results in HIFs degradation.¹⁵ Previous studies reported that the *EPAS1* gene polymorphisms were associated with adaptation to hypobaric hypoxia in the Tibetan population.^{17,18} In addition, HIF-2 α regulates the vascular endothelial growth factor (VEGF) expression and seems to be implicated in the development of blood vessels and the tubular system of the lung. An animal model study also showed that the gain-of-function of HIF-2 α was related to the development of pulmonary vascular remodeling.¹⁹ Another study found that chronic hypoxia could induce HIF-2 α stability, leading to increased arginase expression and dysregulating normal vascular nitric oxide homeostasis.²⁰ In addition, endothelial HIF-2 α was a major mediator in the development of hypoxic-induced pulmonary hypertension, and its functions could induce pulmonary hypertension through an increased expression of endothelin-1, encoded from the *EDN1* gene and a concomitant decrease in vasodilatory apelin receptor signaling.²¹ Gene network analysis showed that *EPAS1* gene is co-expressed with *EDN1* gene. The Gene network of 11 candidate genes in the present study (*BMPR2*, *EPAS1*, *PDE3A*, *VEGFA*, *ENG*, *NOTCH3*, *SOD3*, *CPS1*, *ABCA3*, *ACVRL1*, and *SMAD9*) and other genes that had been shown to be associated with PPHN is shown in Supplementary Figure 1.

In the present study, 10 SNPs of the *EPAS1* gene showed a significant difference between patients with PPHN and the controls (Table 1); however, rs17034984 was the only SNP that remained associated with PPHN after the Bonferroni correction. The T allele on rs17034984 was a risk factor for PPHN and the TC/TT genotype with the dominant model was significantly higher in patients with PPHN. However, to date, there have been few studies regarding HIF-2 α in neonates. One study reported that HIF-2 α was highly expressed in the lungs of neonates with a congenital diaphragmatic hernia and was possibly correlated with PPHN in neonates.²² Taking these findings together, the authors believe that a genetic variant of the *EPAS1* gene is possibly associated with PPHN.

To demonstrate that expression of the HIF-2 α protein (i.e., expression of the *EPAS1* gene) in pulmonary vascular endothelial cells is involved in PPHN pathophysiology, the authors carried out IHC staining in pulmonary vascular endothelial cells and other tissues. The authors found that HIF-2 α protein was expressed in the nucleus of pulmonary vascular endothelial cells of some PPHN patients, whereas there was no HIF-2 α protein expression in normal adult lung tissues. Additionally, the authors observed HIF-2 α protein expression in fetal umbilical vascular endothelial cells, possibly as a result of exposure to a hypoxic environment during physiological development. During *in utero* development, expression of both HIF-1 α and HIF-2 α are relatively high in the fetal lungs due to fetal exposure to a hypoxic environment.^{23–25} HIF-1 α expression is predominant in respiratory epithelial cells, while HIF-2 α is predominant in vascular endothelial cells and type II pneumocytes.²⁶ Due to a limited number of PPHN lung samples, the authors could not perform statistical analysis to make a solid conclusion that HIF-2 α was significantly higher expressed in neonatal PPHN lungs. However, the present study's results suggest that more studies are needed to further explicate the role(s) of HIF-2 α in the pathophysiology of PPHN.

There are some limitations to the present study. First, the number of neonates with PPHN recruited into this study was relatively small. Second, the number of SNP probes

fabricated onto the microarray might not cover all SNPs associated with the pathogenesis of PPHN. Third, although the present study carried out a preliminary validation study, using IHC staining to detect HIF-2 α protein expression in lung tissues from neonates with PPHN, the authors could not compare the study's findings with normal lung tissues from neonates because autopsies of deceased neonates were not routinely performed in the study's center. Instead, the authors carried out IHC staining to detect HIF-2 α protein expression in normal adult lung tissues. Concerning potential bias in adult control subject selection due to unknown medical history in their neonatal period, this concern was deemed minimal. Since the prevalence of PPHN is ~3 per 1000 livebirth, which means the vast majority of individuals do not have PPHN.

In conclusion, in this study, the authors explored novel candidate SNPs that might be involved in the pathophysiology of PPHN. The authors' analysis revealed that rs17034984 in the *EPAS1* gene is a possible genetic susceptibility locus associated with PPHN. In combination with IHC staining to detect HIF-2 α protein expression, the authors found that HIF-2 α , encoded by the *EPAS1* gene, might be a factor associated with PPHN via pulmonary vascular remodeling related to different oxygen levels in fetuses. However, further studies are needed to confirm the authors' findings and to further explore the pathophysiological mechanism(s) underlying this association.

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Conflicts of interest

The authors declare no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at <https://doi.org/10.1016/j.jpmed.2021.09.003>.

References

1. Nakwan N, Jain S, Kumar K, Hosono S, Hammoud M, Elsayed YY, et al. An Asian multicenter retrospective study on persistent pulmonary hypertension of the newborn: incidence, etiology, diagnosis, treatment and outcome. *J Matern Fetal Neonatal Med.* 2020;33:2032–7.
2. Jain A, McNamara PJ. Persistent pulmonary hypertension of the newborn: advances in diagnosis and treatment. *Semin Fetal Neonat Med.* 2015;20:262–71.
3. Rocha G, Baptista MJ, Guimarães H. Persistent pulmonary hypertension of non-cardiac causes in a neonatal intensive care unit. *Pulm Med.* 2012;2012:818–971.
4. Steurer MA, Jelliffe-Pawlowski LL, Baer RJ, Partridge JC, Rogers EE, Keller RL. Persistent pulmonary hypertension of the newborn in late preterm and term infants in California. *Pediatrics.* 2017;139:e20161165.
5. Pearson DL, Dawling S, Walsh WF, Haines JL, Christman BW, Bazyk A, et al. Neonatal pulmonary hypertension—urea-cycle intermediates, nitric oxide production, and carbamoyl-phosphate synthetase function. *N Engl J Med.* 2001;344:1832–8.
6. Byers HM, Dagle JM, Klein JM, Ryckman KK, McDonald EL, Murray JC. Variations in *CRHR1* are associated with persistent pulmonary hypertension of the newborn. *Pediatr Res.* 2012;71:162–7.
7. Mei M, Cheng G, Sun B, Yang L, Wang H, Sun J, et al. *EDN1* gene variant is associated with neonatal persistent pulmonary hypertension. *Sci Rep.* 2016;6:29877.
8. Kaluarachchi DC, Smith CJ, Klein JM, Murray JC, Dagle JM, Ryckman KK. Polymorphisms in urea cycle enzyme genes are associated with persistent pulmonary hypertension of the newborn. *Pediatr Res.* 2018;83:142–7.
9. Liu X, Mei M, Chen X, Lu Y, Dong X, Hu L, et al. Identification of genetic factors underlying persistent pulmonary hypertension of newborns in a cohort of Chinese neonates. *Respir Res.* 2019;20:174.
10. Wang M, Zhuang D, Mei M, Ma H, Li Z, He F, et al. Frequent mutation of hypoxia-related genes in persistent pulmonary hypertension of the newborn. *Respir Res.* 2020;21:53.
11. Galambos C, Mullen MP, Shieh JT, Schwerk N, Kiehl MJ, Ullmann N, et al. Phenotype characterisation of *TBX4* mutation and deletion carriers with neonatal and paediatric pulmonary hypertension. *Eur Respir J.* 2019;54:1801965.
12. Nakwan N, Pithaklimnuwong S. Acute kidney injury and pneumothorax are risk factors for mortality in persistent pulmonary hypertension of the newborn in Thai neonates. *J Matern Fetal Neonatal Med.* 2016;29:1741–6.
13. Nakwan N. The practical challenges of diagnosis and treatment options in persistent pulmonary hypertension of the newborn: a developing country's perspective. *Am J Perinatol.* 2018;35:1366–75.
14. Crowe AR, Yue W. Semi-quantitative determination of protein expression using immunohistochemistry staining and analysis: an integrated protocol. *Bio-protocol.* 2019;9:e3465.
15. Reference SNP (rs) Report: rs17034984. [Cited 2020 August 19]. Available from: <https://www.ncbi.nlm.nih.gov/snp/rs17034984>
16. Shimoda LA, Semenza GL. HIF and the lung: role of hypoxia-inducible factor in pulmonary development and disease. *Am J Respir Crit Care Med.* 2011;183:152–6.
17. Beall CM, Cavalleri GL, Deng L, Elston RC, Gao Y, Knight J, et al. Natural selection on *EPAS1* (HIF2 α) associated with low hemoglobin concentration in Tibetan highlanders. *Proc Natl Acad Sci U S A.* 2010;107:11459–64.
18. Simonson TS, Yang Y, Huff CD, un H, Qin G, Witherspoon DJ, et al. Genetic evidence for high-altitude adaptation in Tibet. *Science.* 2010;329:72–5.
19. Newman JH, Holt TN, Cogan JD, Womack B, Phillips 3rd JA, Li C, et al. Increased prevalence of *EPAS1* variant in cattle with high-altitude pulmonary hypertension. *Nat Commun.* 2015;6:6863.
20. Cowburn AS, Crosby A, Macias D, Branco C, Colaço RD, Southwood M, et al. HIF2 α -arginase axis is essential for the

- development of pulmonary hypertension. *Proc Natl Acad Sci USA*. 2016;113:8801–6.
21. Kapitsinou PP, Rajendran G, Astleford L, Michael M, Schonfeld MP, Fields T, et al. The endothelial prolyl-4-hydroxylase domain 2/hypoxia-inducible factor 2 axis regulates pulmonary artery pressure in mice. *Mol Cell Biol*. 2016;36:1584–94.
 22. Huang Y, Boerema-de Munck A, Buscop-van Kempen M, de Krijger R, Tibboel D, Rottier RJ. Hypoxia inducible factor 2 α (HIF2 α /EPAS1) is associated with development of pulmonary hypertension in severe congenital diaphragmatic hernia patients. *Pulm Circ*. 2018;8:2045894018783734.
 23. Groenman F, Rutter M, Caniggia I, Tibboel D, Post M. Hypoxia-inducible factors in the first trimester human lung. *J Histochem Cytochem*. 2007;55:355–63.
 24. Grover TR, Asikainen TM, Kinsella JP, Abman SH, White CW. Hypoxia-inducible factors HIF-1 α and HIF-2 α are decreased in an experimental model of severe respiratory distress syndrome in preterm lambs. *Am J Physiol Lung Cell Mol Physiol*. 2007;292:L1345–51.
 25. Asikainen TM, Ahmad A, Schneider BK, White CW. Effect of preterm birth on hypoxia-inducible factors and vascular endothelial growth factor in primate lungs. *Pediatr Pulmonol*. 2005;40:538–46.
 26. Huang Y, Kempen MB, Munck AB, Swagemakers S, Driegen S, Mahavadi P, et al. Hypoxia-inducible factor 2 α plays a critical role in the formation of alveoli and surfactant. *Am J Respir Cell Mol Biol*. 2012;46:224–32.