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RNA Modification Signature of Peripheral Blood as a Potential Diagnostic Marker for Pulmonary Hypertension

Liwen Zhang[1,2,3], **Yuzhu Li**[4], **Jian Wang**[4], **Cheng Hong**[4], **Wenju Lu**[4], **Yongcun Qu**[1,3], **Stephen M Black**[5], **Qi Chen**[6], **Tong Zhou**[7], **Wanzhu Jin**[1], **Enkui Duan**[1,8], **Haiyang Tang**[4,9,#], **Ying Zhang**[2,#]

[1]State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

[2]The Key Laboratory of Cell Proliferation and Regulation Biology, Ministry of Education, College of Life Sciences, Beijing Normal University, Beijing, 100875, China

[3]University of Chinese Academy of Sciences, Beijing, China

[4]State Key Laboratory of Respiratory Disease, National Clinical Research Center for Respiratory Disease, Guangdong Key Laboratory of Vascular Disease, Guangzhou Institute of Respiratory Health, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, 510120, China

[5]Center for Translational Science, Florida International University, Port St. Lucie, FL, USA

[6]Division of Biomedical Sciences, School of Medicine, University of California, Riverside, CA, 92521, USA

[7]Department of Physiology and Cell Biology, University of Nevada, Reno School of Medicine, Reno, Nevada 89557, USA

[8]Henan Key Laboratory of Fertility Protection and Aristogenesis, Luohe, Henan, 462000, China

[9]College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, China

Pulmonary hypertension (PH) is a hemodynamic disorder syndrome defined as a mean resting pulmonary artery pressure higher than 20 mmHg to 25 mmHg, which continues to cause high mortalities and low survival rates (1).Current clinical classification divides PH into five major groups, among which the precise diagnosis and classification of Group 1 (pulmonary arterial hypertension, PAH) caused by narrowed/thickened/stiff lung arteries and Group 3 due to lung disease remains an unmet challenge (2). Most of PH treatments are based on Group 1 studies, but specific therapy has no significant effects for Group 3 patients in clinic (3). Thus, developing easy, precise, and noninvasive biomarkers to identify Group 1 and 3 PH would be of significant clinical benefit to avoid delayed or inefficient treatment(4). Here, we report a new methodology that leverages high-throughput

#Corresponding authors: Haiyang Tang (tanghy2008@yahoo.com), Ying Zhang (yingzhanglab@gmail.com).
Author contributions:
Y.Z. and H.T. designed the project. Y.Z. and L.Z. developed and optimized the LC-MS/MS based RNA modification detection system. L.Z. performed the experiments with the help from Y.Q. under the supervision of Y.Z. and E.D.. Y.L., J.W., C.H., W.L. and H.T. recruited the human subjects and collected blood sample. T.Z. and W.J. performed the data analyses. Y.Z. and H.T. wrote the manuscript with input from Q.C., T.Z., and S.M.B.

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RNA modification profiling platform based on LC-MS/MS, that simultaneously identifies and quantifies multiple RNA modifications (5), to determine if it is possible to distinguish Group 1/3 PH patients from control individuals according to RNA modification signatures (PH patient recruitment following the updated guideline of the 6th Word Symposium for Pulmonary Hypertension (1)). As a result, we successfully developed a diagnostic signature composed of 7 RNA modifications (m^1A, m^1G, m^2G, m^5C, m^5U, Am, and Im), which efficiently classifies patients with Group 3 PH separate from both controls and patients with Group 1 PH in the discovery and validation cohorts.

The discovery cohort was composed of four controls, eight subjects with Group 1 PH, and three subjects with Group 3 PH (Figure 1A). Peripheral blood of these subjects was collected from the ulnar vein followed by RNA extraction. RNA was digested to ribonucleosides using benzonase nuclease, phosphodiesterase nuclease and alkaline phosphatase I. RNA modifications were detected and quantified using a high-throughput LC-MS/MS based approach we established (5). The data from LC-MS/MS were acquired by Xcalibur Workstation software and modified ribonucleoside concentrations were quantified using Xcalibur QuanBrowser. Using this approach, we efficiently identify and quantify 18 modified nucleosides, including ψ (psi), Am, Cm, Um, Gm, I, m^7G, m^2G, m^5U, m^5C, m^1A, m^2_2G, m^1G, ac^4C, m^5Um, m^3U, Im, and m^6A. Indeed, principal component analysis on the RNA modification profiles revealed a distinct pattern between the control and Group 3 PH subjects, with the Group 1 PH subjects largely falling in between (Figure 1B), suggesting there is significant potential discriminative power embedded in the RNA modification information. Further detailed analyses prioritized five upregulated (m^1A, m^1G, m^2G, m^5C, and m^5U) and two downregulated (Am and Im) RNA modification species in the Group 3 PH patients (Figure 1C) identifying an RNA modification signature.

We next computed an RNA modification index for each subject in the discovery cohort using the aforementioned RNA modification signature. This corresponded to a linear combination of the modifications within the signature:

$$I = \sum_{i=1}^{7} w_i (e_i - \mu_i)/\tau_i$$

Where I is the RNA modification index; w_i is the weight of modification i within the modification signature ($w_i = 1$ for m^1A, m^1G, m^2G, m^5C, and m^5U; $w_i = -1$ for Am and Im); e_i denotes the modification level of modification i; and μ_i and τ_i are the mean and standard deviation of the level of modification i across all the samples, respectively. As a result, we found that the RNA modification index was significantly higher in the Group 3 PH patients than in the controls and Group 1 PH patients; and the index of the Group 1 PH patients is significantly higher than that of the controls (t-test: $P < 0.05$) (Figure 1D).

To further assess the performance of the RNA modification signature, two independent validation cohorts (30 controls, 12 Group 1 PH subjects, and 18 Group 3 PH subjects in the first validation cohort [VAL1], which was based on fresh whole blood; 7 controls, 17 Group 1 PH subjects, and 10 Group 3 PH subjects in the second cohort [VAL2], which

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was based on EDTA-anticoagulant blood) were investigated (Figure 1A). Our results showed that, in both validation cohorts, the RNA modification index in the Group 3 and Group 1 PH patients was significantly higher than in the controls (*t*-test: $P < 0.05$). The index of Group 3 PH patients was also significantly higher than in the Group 1 PH patients in VAL1 and marginally higher in VAL2 (*t*-test: $P < 0.05$ in VAL1 and $P = 0.066$ in VAL2) (Figure 1E). The area under the receiver operating characteristic (ROC) curve (*AUC*) was 0.879 in VAL1 and 0.820 in VAL2 between the controls and PH patients, 0.917 in VAL1 and 0.957 in VAL2 between the controls and Group 3 PH patients, and 0.889 in VAL1 and 0.775 in VAL2 between the Group 3 PH patients and the other subjects (Figure 1F). These results suggest that our RNA modification index can distinguish Group 3 patients from Group 1 and controls in both validation cohorts. Moreover, we found that the RNA modification index in fresh blood performed better than that in EDTA-anticoagulant blood samples. Thus, our data support the conclusion that this RNA modification signature has a strong classification power for PH diagnosis, especially in fresh whole blood.

In summary, we have identified an RNA modification signature in peripheral blood, which efficiently distinguishes between controls and patients with Group 1/3 PH. Moreover, blood-based RNA modification signatures could be harnessed as a noninvasive biomarker for classifying other PH subgroups and potentially other diseases. Further, while whole-blood RNA levels have been reported to linked to disease diagnosis, our blood-based RNA modification signature, combined with RNA expression profiles, might be a powerful detection biomarker to facilitate precision medicine, which will require systematic studies in future investigations.

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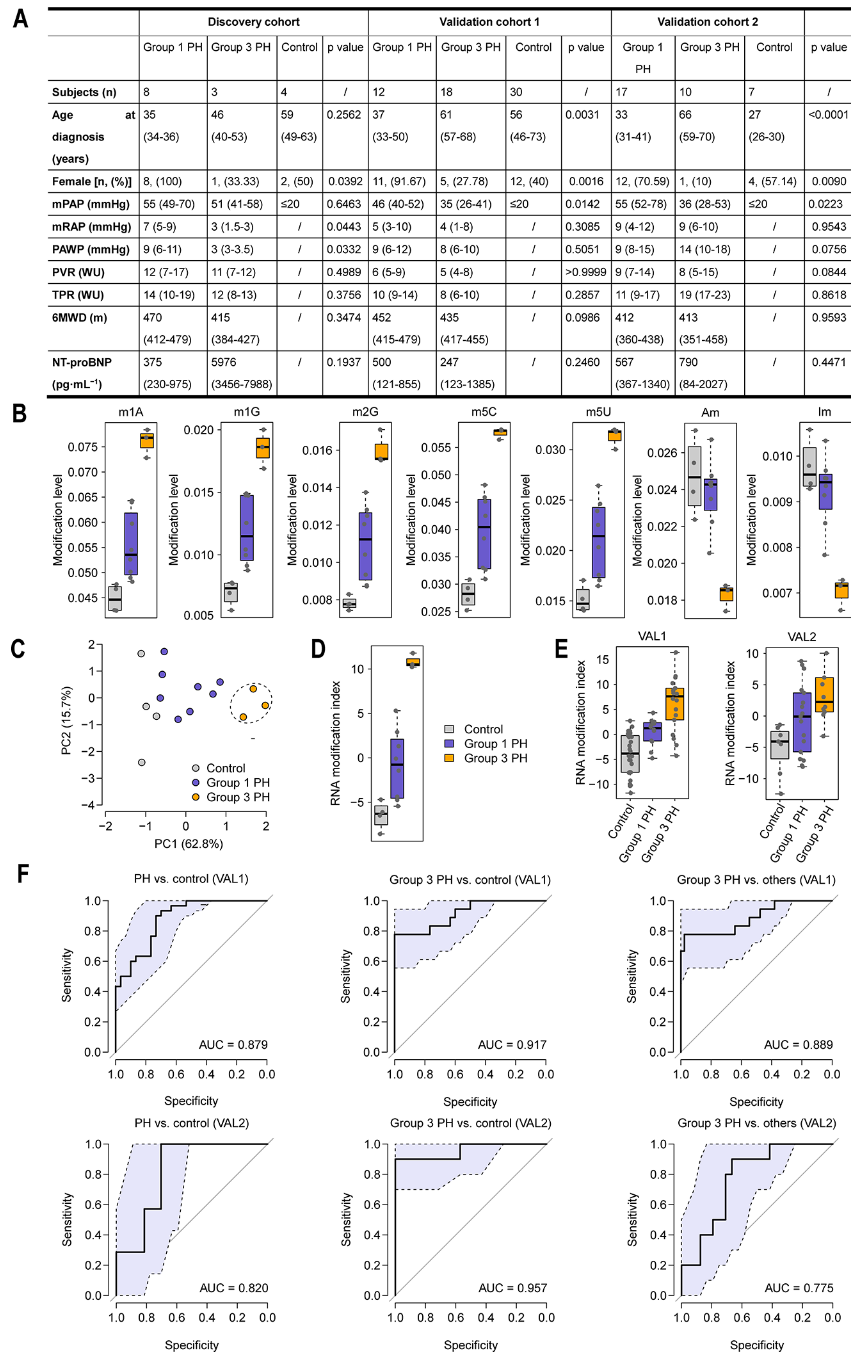

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	Discovery cohort				Validation cohort 1				Validation cohort 2			
	Group 1 PH	Group 3 PH	Control	p value	Group 1 PH	Group 3 PH	Control	p value	Group 1 PH	Group 3 PH	Control	p value
Subjects (n)	8	3	4	/	12	18	30	/	17	10	7	/
Age at diagnosis (years)	35 (34-36)	46 (40-53)	59 (49-63)	0.2562	37 (33-50)	61 (57-68)	56 (46-73)	0.0031	33 (31-41)	66 (59-70)	27 (26-30)	<0.0001
Female [n, (%)]	8, (100)	1, (33.33)	2, (50)	0.0392	11, (91.67)	5, (27.78)	12, (40)	0.0016	12, (70.59)	1, (10)	4, (57.14)	0.0090
mPAP (mmHg)	55 (49-70)	51 (41-58)	≤20	0.6463	46 (40-52)	35 (26-41)	≤20	0.0142	55 (52-78)	36 (28-53)	≤20	0.0223
mRAP (mmHg)	7 (5-9)	3 (1.5-3)	/	0.0443	5 (3-10)	4 (1-8)	/	0.3085	9 (4-12)	9 (6-10)	/	0.9543
PAWP (mmHg)	9 (6-11)	3 (3-3.5)	/	0.0332	9 (6-12)	8 (6-10)	/	0.5051	9 (8-15)	14 (10-18)	/	0.0756
PVR (WU)	12 (7-17)	11 (7-12)	/	0.4989	6 (5-9)	5 (4-8)	/	>0.9999	9 (7-14)	8 (5-15)	/	0.0844
TPR (WU)	14 (10-19)	12 (8-13)	/	0.3756	10 (9-14)	8 (6-10)	/	0.2857	11 (9-17)	19 (17-23)	/	0.8618
6MWD (m)	470 (412-479)	415 (384-427)	/	0.3474	452 (415-479)	435 (417-455)	/	0.0986	412 (360-438)	413 (351-458)	/	0.9593
NT-proBNP (pg·mL^{-1})	375 (230-975)	5976 (3456-7988)	/	0.1937	500 (121-855)	247 (123-1385)	/	0.2460	567 (367-1340)	790 (84-2027)	/	0.4471

Figure 1. The performance of the RNA modification signature in discovery and validation cohorts.

(A) Patient characteristics of the discovery and validation cohorts. Values are expressed as n, median (interquartile range) or n (%), unless otherwise stated. mPAP mmHg: Mean pulmonary arterial pressure mmHg; mRAP mmHg: Mean right atrial pressure mmHg; PAWP mmHg: Pulmonary arterial wedge pressure mmHg; PVR wood: Pulmonary vascular resistance Wood units; TPR wood: Total pulmonary resistance Wood units; 6MWD: 6-min walk distance; NT-pro BNP: N-terminal pro-brain natriuretic peptide. The t-test was performed for statistical analysis, except age at diagnosis were analyzed by ANOVA test

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and Chi-Squared test was used for gender comparison. (B) Principal component analysis on the RNA modification data. PC1: the first principal component; PC2: the second principal component. Each dot represents one human subject in the discovery cohort. (C) The RNA modification signature. (D) Comparison of the RNA modification index among the control, Group 1 PH, and Group 3 PH subjects in the discovery cohort. Mean ± standard deviation: −6.48±1.60 for control, −0.83±3.90 for Group 1 PH, and 10.84±0.84 for Group 3 PH. (E) Comparison of the RNA modification index among the control, Group 1 PH, and Group 3 PH subjects in two validation cohorts. Mean ± standard deviation: −3.90±4.16 for control, 0.48±2.72 for Group 1 PH, and 6.19±5.41 for Group 3 PH in VAL1 and −5.22±4.09 for control, −0.43±5.71 for Group 1 PH, and 4.39±6.41 for Group 3 PH in VAL2. (F) The ROC curve of the RNA modification index in distinguishing between the PH and control subjects, between the Group 3 PH and control subjects, and between the Group 3 PH patients and other subjects (controls and Group 1 subjects) in the validation cohorts. The light blue area indicates the confidence interval of the ROC curve. VAL1: the first validation cohort; VAL2: the second validation cohort.