Online Data Supplement

Identifying microRNAs targeting Wnt/β-catenin pathway in end-stage idiopathic pulmonary arterial hypertension

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Journal of Molecular Medicine 2016

Supplementary Methods

RNA isolation

Snap frozen RNA*later* archived human lung tissue samples were used for the miRNA and mRNA studies. For miRNA microarray screening, total RNA, including miRNAs, was isolated with RNeasy kit (QIAGEN Hilden, Germany) from lung homogenate according to the manufacturer's protocol. RNA concentration, purity and RNA integrity number (RIN) of microarray assay were determined on a Bioanalyzer (Agilent, Santa Clara CA). Only samples with RNA integrity number (RIN) ≥7.0 were acceptable for microarray analysis. The RNA for miRNA and mRNA qRT-PCR validation was isolated by a miRNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. RNA concentration and quality were measured using a NanoDrop 2000 spectrophotometer (Thermo, Wilmington, DE, USA).

miRNA microarray

Isolated RNA samples were hybridized to a Human miRNA Microarray V3 kit (G4872A, Agilent Technologies) platform (which contains 2,006 human miRNAs from the Sanger database, Release 19.0) according to the manufacturer's instructions in the JHMI Microarray Core Facility. Briefly, total RNA (100

ng) was first dephosphorylated with 15 units of calf intestine alkaline phosphatase at 37°C for 30min and followed by end-labeling with pCp-Cy3 (Agilent Technologies) and T4 RNA ligase (Agilent Technologies) at 16°C for 2h. Samples were then dried by SpeedVac for 2h, mixed with 10x blocking agent and 2x Hi-RPM hybridization buffer (Agilent Technologies), and hybridized at 55°C with rotation at 20 rpm in an Agilent G2545A microRNA hybridization oven for 20h. Finally, microarrays were washed and scanned using an Agilent scanner controlled by Agilent Scan Control 7.0 software. Data were acquired using Agilent Feature Extraction 9.5.3.1 software for miRNA microarray, generating a summarized GeneView signal intensity file for each sample by combining intensities of miRNA replicate probes and background subtraction.

The data from this study have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE67597. Data quality control was carried out by principal component analysis (PCA) and box-whisker plot. One outlier from the IPAH group was excluded. Quantile normalization was applied to the dataset to minimize potential technical variation and obtain an equal distribution of probe signal intensities. The microarray signals with minimal values were thresholded to 0.001, followed by log2 transformation. The miRNAs with a mean log2-transformed signal intensity less than -2 either in control or IPAH group were removed before statistical analysis. Expression differences were compared using one-way ANOVA between two groups. Data quality control, transformation and analysis were performed using Partek Genomics Suite 6.6 (Partek Incorporated, St Louis, MO). Data were annotated and visualized with Spotfire DecisionSite (TIBCO Software, Boston, MA).

miRNA qRT-PCR validation

To validate the microarray data, the expression levels of miRNAs were analyzed by qRT-PCR. miRNA-specific complementary DNA (cDNA) of each candidate miRNA was synthesized from 10ng of total RNA using the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. Real-time PCR was carried out by Applied Biosystems 7300 Real-Time PCR System using TaqMan miRNA Assays and TaqMan Universal Master Mix II, no UNG (all from Applied Biosystems, Foster City, CA). Assays used for validation are listed in Table S2. Expression levels of miRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method, with U6 snRNA as endogenous control. We excluded reactions with a Ct value greater than 35 in the results and all samples were tested in triplicate using 96-well plates.

LNA-ISH

In order to gain cellular resolution of miRNA expression, we performed *in situ* hybridization experiments in human IPAH and control lung tissues using DIG-labeled locked-nucleic-acid (LNA)-modified DNA oligonucleotide probes specifically recognize mature miRNA species and do not hybridize with precursor miRNAs. We focused our efforts on the two dysregulated miRNAs (*miR-199b-5p* and *miR-656*) that displayed strong positive correlation with features of vascular remodeling. Locked nucleic acid - In situ hybridization was performed on formalin-fixed paraffin embedded (FFPE) tissue samples according to the manufacturer's protocol. Unstained slides were cut from tissue blocks using RNA precautions. An 8 minute proteinase-K treatment at 37°C (Dako, Denmark) was performed on the slides prior to hybridization. An Illumina hybridizer was used instead of a Dako hybridizer for the *in situ* hybridization step. Levamisole (Dako) was used to inhibit endogenous alkaline phosphatase. Counterstain was performed with 50% eosin (Richard-Allen, Kalamazoo, MI). Double digoxigenin (DIG)-labeled miRCURY LNA detection probes for *miR-199b-5p*, *miR-656* and U6 snRNA (Exiqon A/S, Denmark) were used.

Selection of miRNA target genes in Wnt/ β-catenin pathway for qRT-PCR validation

Wnt ligand and Wnt receptor are big families, we chose *WNT7A* and *WNT16* because they are putative targets of *miR-656*, while *FZD5* and *FZD10* are their receptors, respectively. *WNT5A* and *FZD4* were selected since both were targeted by most (5 out of 6) of the validated miRNAs.

Gene expression analysis

Expression level of microRNA target genes were detected by TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). cDNA was synthesized from 1μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR was carried out by Applied Biosystems 7300 Real-Time PCR System using TaqMan Gene Expression Assays and TaqMan Gene Expression Master Mix (all from Applied Biosystems, Foster City, CA). Expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as endogenous control. We excluded reactions with a Ct value greater than 35 in the results and all samples were tested in triplicate using 96-well plates.

Data presentation and statistical analyses

Data are presented as absolute numbers, percentages, mean (± standard deviation, SD) or median and quartile. Parametric distribution was explored by Shapiro-Wilk test. For microarray analysis, the filtering criteria of fold change (FC, increase or decrease) ≥ 1.5 and *p*-value <0.05 were employed. An unpaired t-test or Mann-Whitney U test was applied to the comparisons of relative miRNA or mRNA expression levels between control subjects and IPAH patients, and a *p*-value of less than 0.05 was considered significant.

Supplementary Results

Table S1. Top canonical pathways generated from target genes (N=4,664) of 21 dysregulated miRNAs identified by microarray screening in IPAH patients. Pathways are ranked by their *p*-value.

Ingenuity Canonical Pathways	*p*-value	Number of target genes/Number of total genes in the pathway (%)	Number of Publications
Molecular Mechanisms of Cancer	5.3E-19	155/365 (42.5)	785
HGF Signaling	2.0E-14	59/105 (56.2)	214
PTEN Signaling	1.9E-10	57/118 (48.3)	538
TGF-β Signaling	2.4E-10	46/87 (52.9)	564
Wnt/β-catenin Signaling	4.2E-10	73/169 (43.2)	407
Cardiac Hypertrophy Signaling	7.9E-10	89/223 (39.9)	110

Table S2. Summary of miRNA qPCR assays

miRNA name	Assay ID
let-7a-5p	**000377**
miR-26b-5p	**000407**
miR-27b	**000409**
miR-199a-3p	**002304**
miR-199b-5p	**000500**
miR-205-5p	**000509**
miR-656-3p	**001510**

Table S3. 73 Wnt/β-catenin signaling pathway genes targeted by the 6 out of 11 dysregulated miRNAs

Gene symbol	Type	Location	*let-7a-5p*	*miR-26b-5p*	*miR-27b-3p*	*miR-199a-3p*	*miR-199b-5p*	*miR-656*
ACVR1	kinase	Plasma Membrane						
ACVR1B	kinase	Plasma Membrane	1				1	
ACVR1C	kinase	Plasma Membrane	1	1	1			

ACVR2A	kinase	Plasma Membrane	1		1	1	1	
ACVR2B	kinase	Plasma Membrane	1	1	1		1	
AKT2	kinase	Cytoplasm	1					
APC	enzyme	Nucleus			1			
APC2	enzyme	Cytoplasm	1					
AXIN2	other	Cytoplasm						
BMPR2	kinase	Plasma Membrane						
CCND1	transcription regulator	Nucleus	1					
CD44	enzyme	Plasma Membrane				1		
CDH2	other	Plasma Membrane		1				
CDH5	other	Plasma Membrane			1			
CREBBP	transcription regulator	Nucleus		1				
CSNK1D	kinase	Cytoplasm	1		1			
CSNK1G1	kinase	Cytoplasm		1	1			
CSNK1G3	kinase	Cytoplasm						
CSNK2A1	kinase	Cytoplasm						
CSNK2A2	kinase	Cytoplasm						
DKK2	other	Extracellular Space			1			
DKK3	cytokine	Extracellular Space	1					
DVL2	other	Cytoplasm			1			
DVL3	other	Cytoplasm	1					
FRAT1	other	Cytoplasm		1				
FRZB	other	Extracellular Space						
FZD3	G-protein coupled receptor	Plasma Membrane	1		1			
FZD4	G-protein coupled receptor	Plasma Membrane	1		1		1	
FZD5	G-protein coupled receptor	Plasma Membrane						
FZD6	G-protein coupled receptor	Plasma Membrane					1	
FZD7	G-protein coupled receptor	Plasma Membrane			1			
FZD8	G-protein coupled receptor	Plasma Membrane						
GJA1	transporter	Plasma Membrane						
GSK3B	kinase	Nucleus		1	1		1	

JUN	transcription regulator	Nucleus						
KREMEN1	other	Plasma Membrane	1					
LEF1	transcription regulator	Nucleus		1				
LRP1	transmembrane receptor	Plasma Membrane						
LRP5	transmembrane receptor	Plasma Membrane						
LRP6	transmembrane receptor	Plasma Membrane			1			
MYC	transcription regulator	Nucleus	1					
NLK	kinase	Nucleus	1	1	1	1	1	
NR5A2	ligand-dependent nuclear receptor	Nucleus			1			
PPARD	ligand-dependent nuclear receptor	Nucleus						
PPM1L	phosphatase	Cytoplasm					1	
PPP2R2A	phosphatase	Cytoplasm	1					
PPP2R3A	phosphatase	Nucleus		1				
PPP2R5A	phosphatase	Cytoplasm		1				
PPP2R5E	phosphatase	Cytoplasm				1		
RARA	ligand-dependent nuclear receptor	Nucleus			1			
RUVBL2	transcription regulator	Nucleus			1			
SFRP1	transmembrane receptor	Plasma Membrane			1			
SFRP4	transmembrane receptor	Plasma Membrane						
SOX4	transcription regulator	Nucleus			1			
SOX6	transcription regulator	Nucleus	1					
SOX7	transcription regulator	Nucleus		1				
SOX8	transcription regulator	Nucleus					1	
SOX11	transcription regulator	Nucleus	1	1			1	
SOX13	transcription regulator	Nucleus			1			
SOX17	transcription regulator	Nucleus			1			
TCF4	transcription regulator	Nucleus						
TCF7L2	transcription regulator	Nucleus		1				

TGFB2	growth factor	Extracellular Space					1	
TGFBR1	kinase	Plasma Membrane	1		1			
TGFBR2	kinase	Plasma Membrane		1				
TGFBR3	kinase	Plasma Membrane	1		1			
TP53	transcription regulator	Nucleus	1					
WNT1	cytokine	Extracellular Space	1					
WNT2	cytokine	Extracellular Space						
WNT10B	other	Extracellular Space					1	
WNT3A	cytokine	Extracellular Space			1			
WNT5A	cytokine	Extracellular Space		1				
WNT7B	other	Extracellular Space					1	

Table S4. Selection of miRNAs for validation by qRT-PCR. The 7 miRNAs selected for qRT-PCR validation are highlighted in bold.

miRNA name	*p*-value	FC in microarray	# Targets in Wnt/β-catenin	# Targets in Cancer	# Targets in HGF	# Targets in PTEN	# Targets in TGF-β	# Targets in Cardiac hypertrophy	TGF-β	Inflammatory	Hypoxia
let-7a-5p	0.047	1.501	27	43	14	18	11	27	Yes	Yes	Yes
miR-1278	0.046	-1.671	9	0	0	0	0	0			
miR-197-3p	0.046	-1.617	27	13	1	3	4	5			
miR-199a-3p	0.015	2.790	28	4	3	0	2	3	Yes	Yes	
miR-199b-5p	0.046	2.621	22	22	10	9	8	8			Yes
miR-205-5p	0.005	5.754	26	17	8	6	6	5	Yes		Yes
miR-26b-5p	0.048	2.432	28	33	15	8	11	24	Yes		Yes
miR-27b-3p	0.045	1.654	39	57	20	22	17	29	Yes	Yes	Yes
miR-548b-3p	0.016	-1.883	16	2	0	0	1	1			
miR-656	0.030	-2.383	24	0	0	0	0	0	Yes		
miR-99a-5p	0.026	2.807	5	5	1	4	1	3			

Table S5. Summary of qRT-PCR validation results for Wnt/β-catenin signaling pathway genes

Gene symbol	Description	Function	Fold change	*p*-value
WIF1	WNT inhibitory factor 1	Wnt inhibitor	4.384	<0.001
WNT5A	Wingless-type MMTV integration site family, member 5A	Wnt ligand	1.514	0.230
WNT7A	Wingless-type MMTV integration site family, member 7A	Wnt ligand	1.611	0.195
WNT16	Wingless-type MMTV integration site family, member 16	Wnt ligand	Not detectable	Not detectable
FZD4	Frizzled family receptor 4	Wnt receptor	2.342	0.031
FZD5	Frizzled family receptor 5	Wnt receptor	1.823	0.068
FZD10	Frizzled family receptor 10	Wnt receptor	14.595	0.003
GSK3B	Glycogen synthase kinase 3 beta	β-catenin inhibitor complex component	0.783	0.819
AXIN2	Axin 2	β-catenin inhibitor complex component	2.316	0.017
APC	Adenomatous polyposis coli	β-catenin inhibitor complex component	1.346	0.243
AKT1	V-akt murine thymoma viral oncogene homolog 1	GSK3B phosphorylation inhibitor	1.486	0.163
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	Wnt/β-catenin pathway core gene	2.317	<0.001
CTNNBIP1	Catenin, beta interacting protein 1	CTNNB1 and TCF family combining inhibitor	1.199	0.246
HDAC1	Histone deacetylase 1	Downstream target inhibitor	1.294	0.080
CCND1	Cyclin D1	Downstream target	5.639	<0.001
VEGFA	Vascular endothelial growth factor A	Downstream target	3.815	0.001

Table S6. Number of validated gene targets of 6 miRNAs from miRWalk

miRNA name	Number of gene targets
let-7a-5p	308
miR-26b-5p	84
miR-27b-3p	82
miR-199b-5p	48
miR-199a-3p	41
miR-656	1

Supplementary Figures

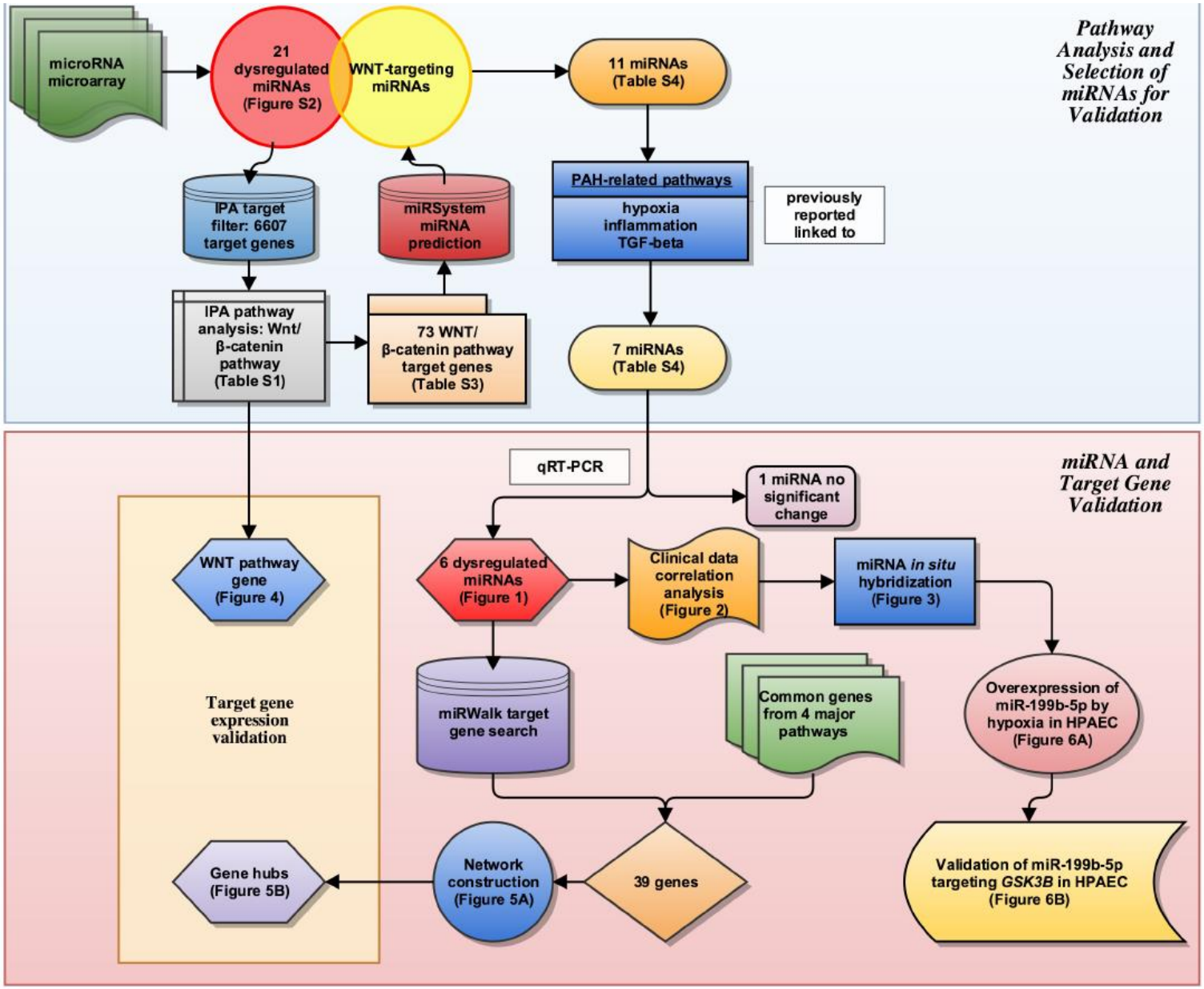

Figure S1. Study design and work flow.

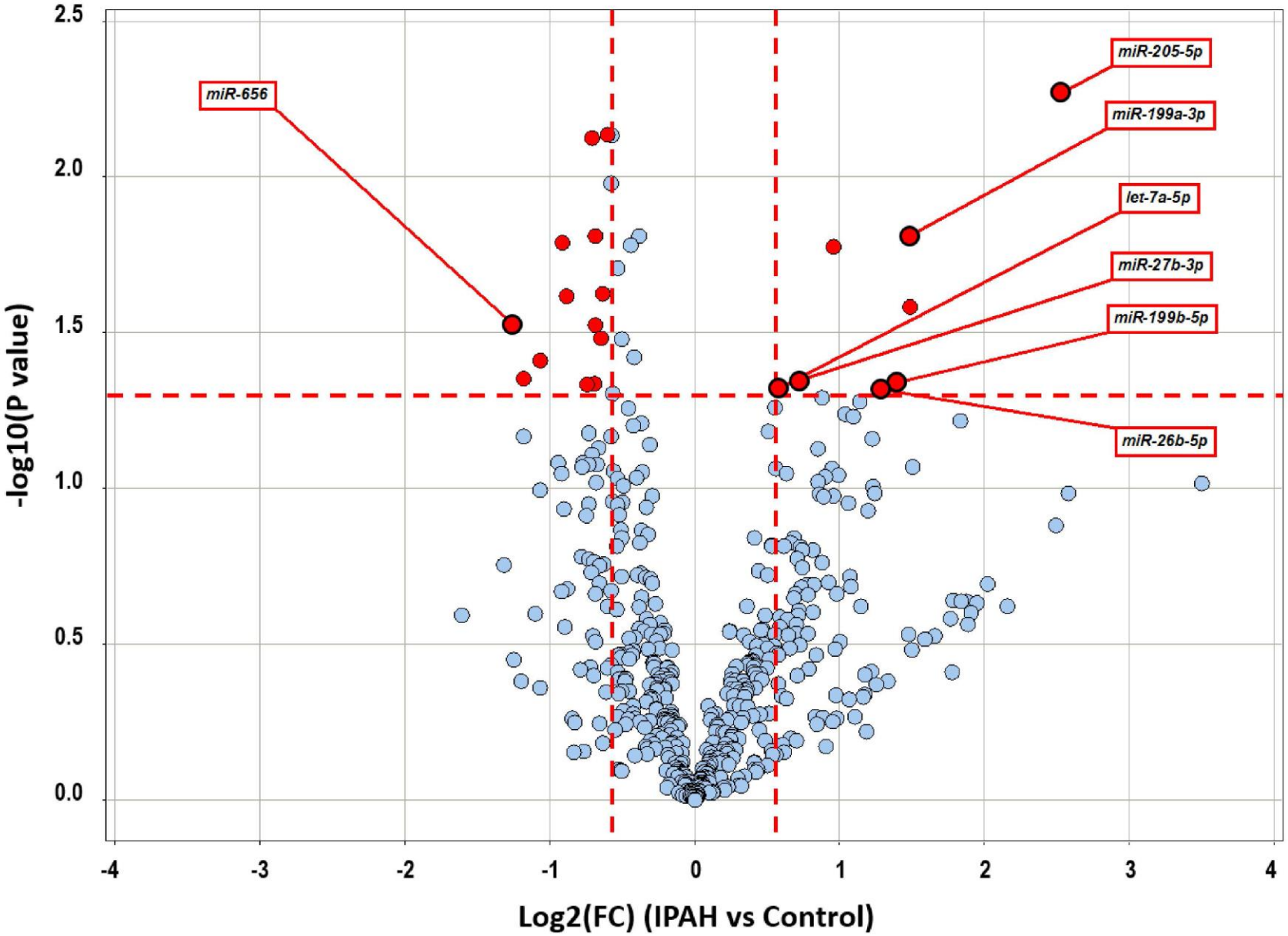

Figure S2. miRNA expression profiling in lung tissue of IPAH patients and controls (volcano plot). Each dot represents one probe set. The miRNAs with: 1) FC ≥ 1.5 in either direction (vertical lines), and 2) *p*-value < 0.05 (horizontal line) are highlighted in red.

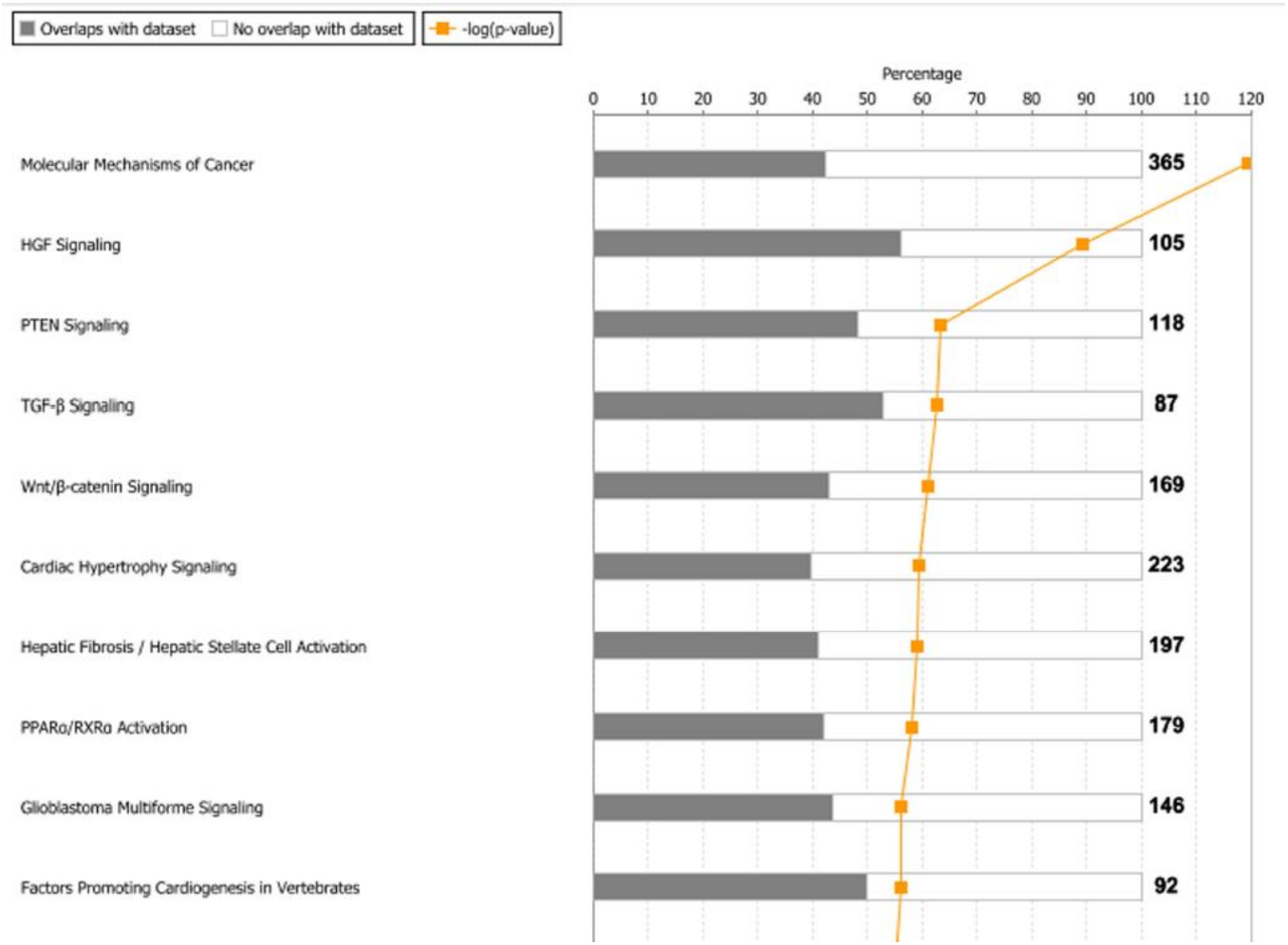

Figure S3. Pathway analysis by IPA shows Molecular Mechanisms of Cancer, HGF Signaling, PTEN Signaling, TGF-β Signaling, Wnt/β-catenin Signaling and Cardiac Hypertrophy Signaling, Hepatic Fibrosis/Hepatic Stellate Cell Activation, PPARα/RXRα Activation, Glioblastoma Multiforme Signaling and Factors Promoting Cardiogenesis in Vertebrates are top canonical pathways the target genes of 21 dysregulated miRNAs involved in.

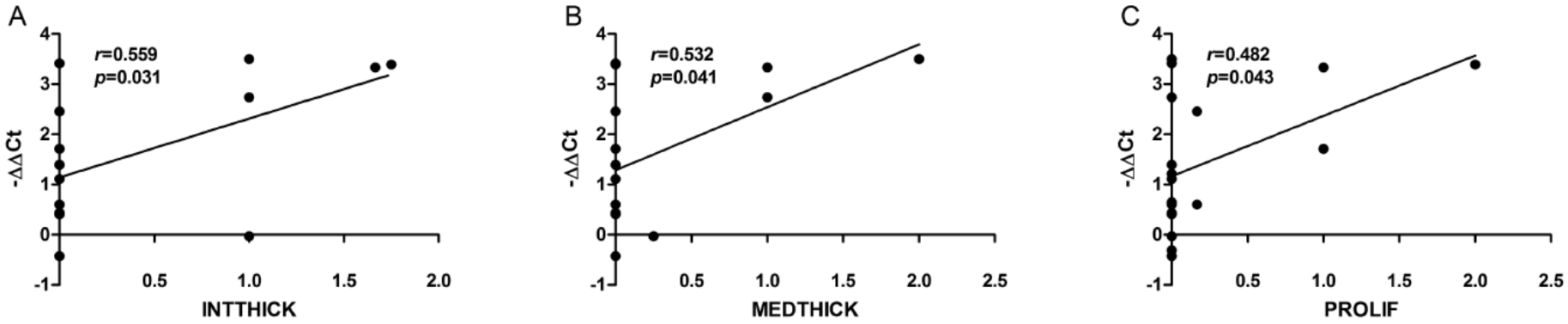

Figure S4. Correlations of expression level of *miR-656* with (A) intima thickening score (INTTHICK) (N=15, r=0.539, *p*=0.031), (B) media thickening score (MEDTHICK) (N=15, r=0.532, *p*=0.041) and (C) proliferated score (PROLIF) (N=18, r=0.482, *p*=0.043).

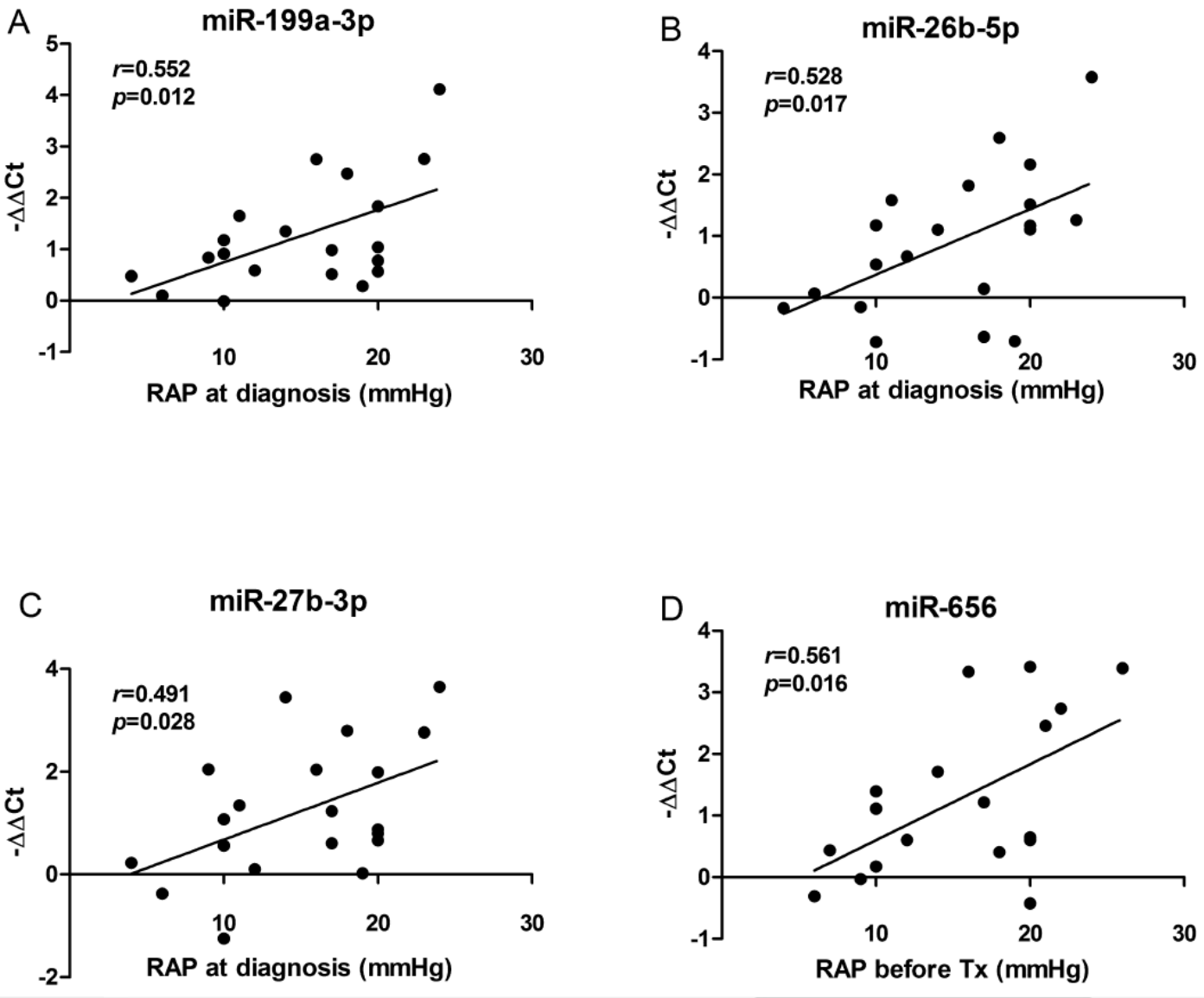

Figure S5. Correlations of right atrial pressure (RAP) at diagnosis with expression levels of (A) *miR-199a-3p* (N=20, *r*=0.552, *p*=0.012), (B) *miR-26b-5p* (N=20, *r*=0.528, *p*=0.017), (C) *miR-27b-3p* (N=20, *r*=0.491, *p*=0.028); (D) Correlation of RAP before transplantation with expression level of *miR-656* (N=18, *r*=0.561, *p*=0.016).

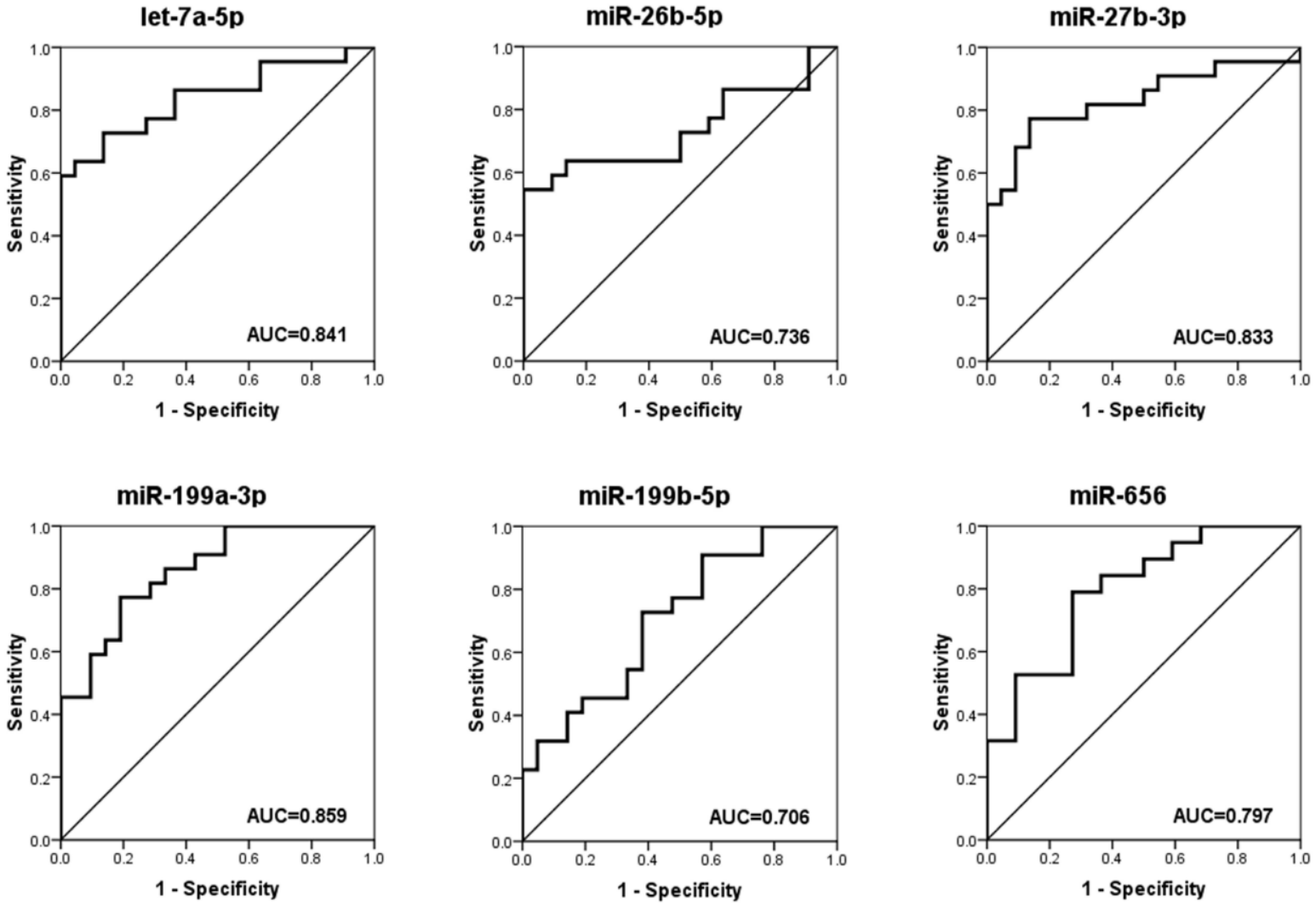

Figure S6. Receiver operating characteristic (ROC) curves showing the relationship between tissue levels of *let-7a-5p*, *miR-26-5p*, *miR-27b-3p*, *miR-199a-3p*, *miR-199b-5p*, *miR-656* and the diagnosis of IPAH.

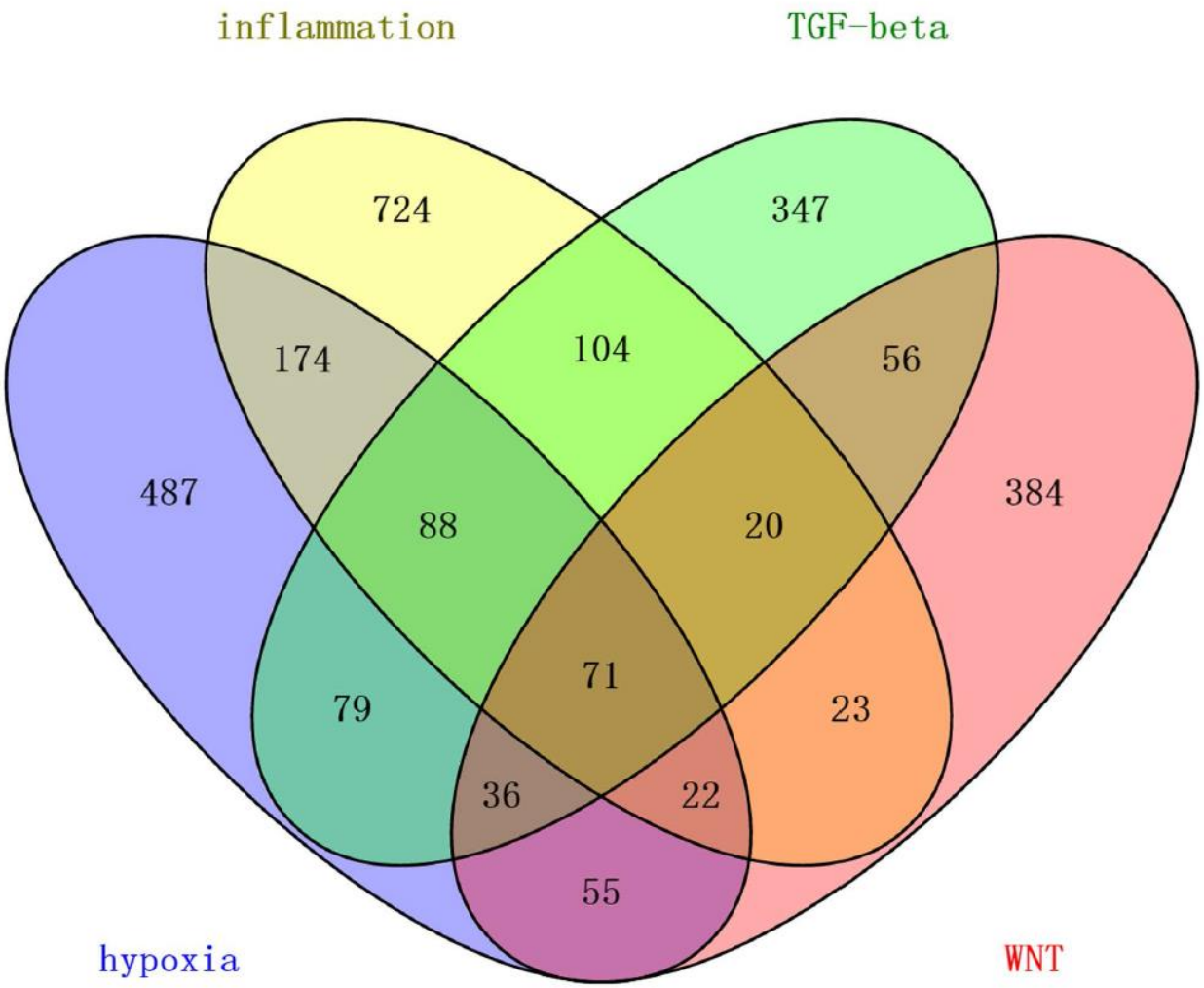

Figure S7. Common genes in 4 pathways associated with PAH. A total number of 71 genes are common genes in hypoxia, inflammation, TGF-β and Wnt pathways.

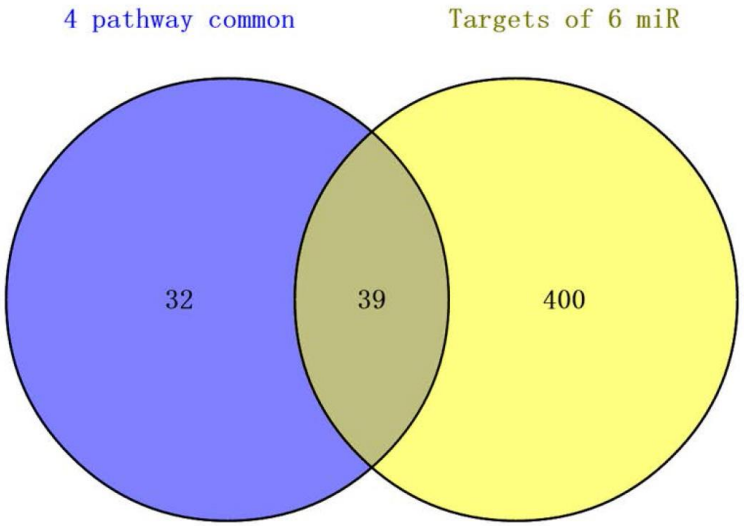

Figure S8. Confirmation of the genes for network construction. Venn diagram shows 39 genes are validated miRNA targets which involved in 4 pathways.

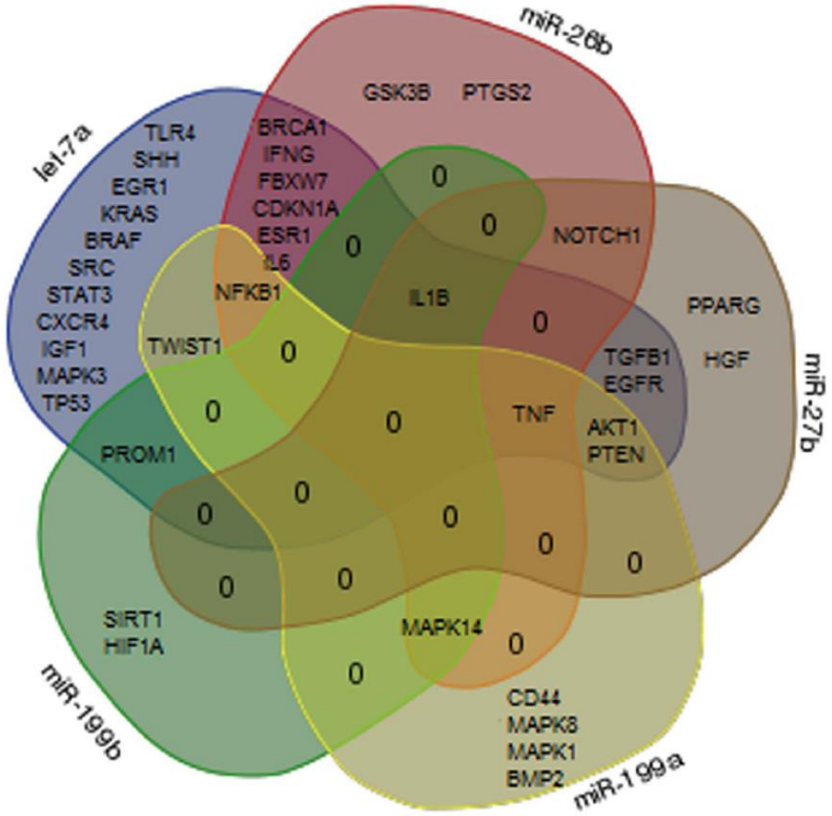

Figure S9. Venn diagram showing target gene distribution of each miRNA. No target gene of *miR-656* is involved in 4 pathways.

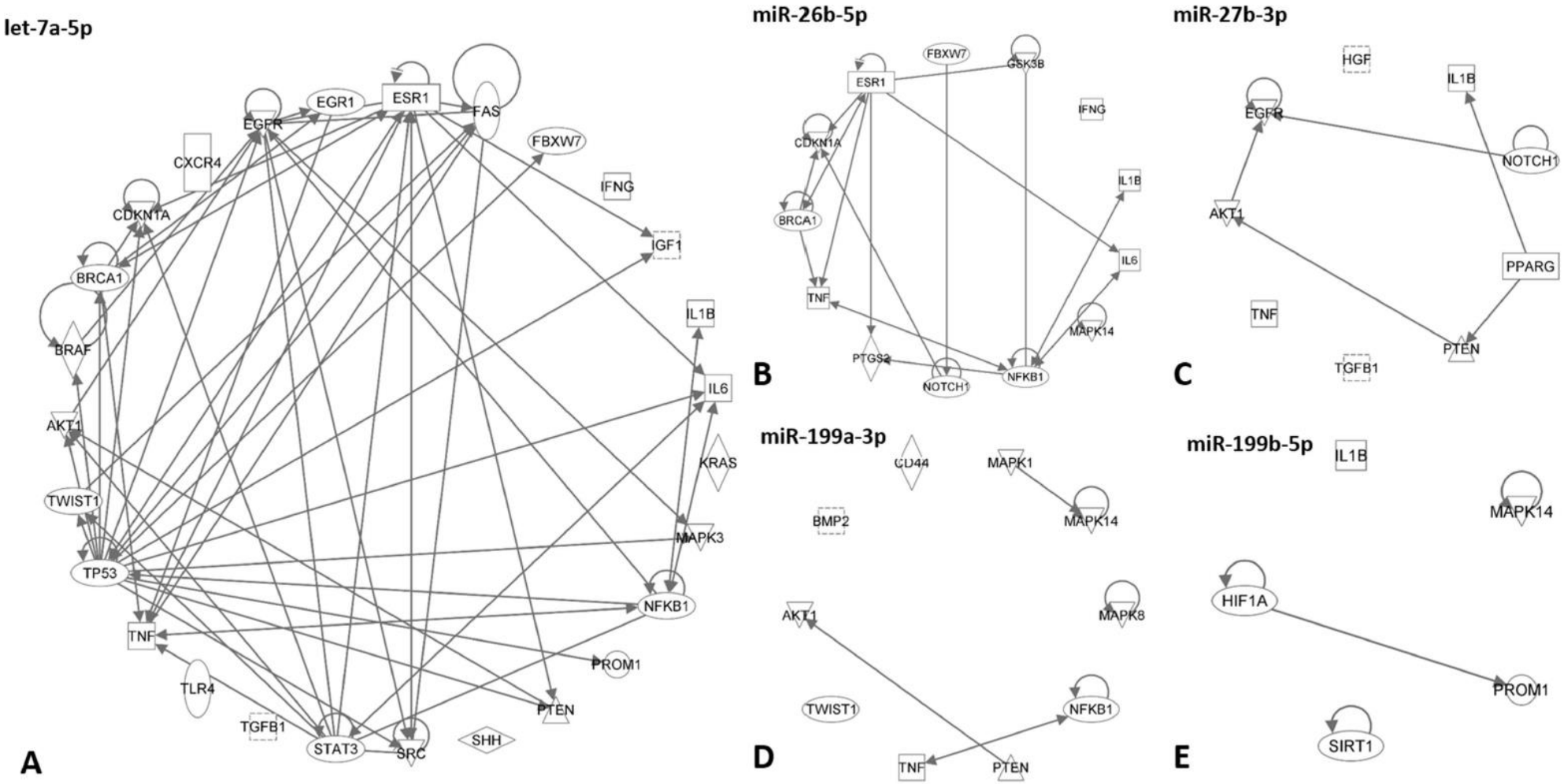

Figure S10. Gene-gene interaction network showing experimentally observed direct interactions between gene targets of: A) *let-7a-5p*; B) *miR-26b-5p*; C) *miR-27b-3p*; D) *miR-199a-3p*; E) *miR-199b-5p*. All genes are involved in all four pathways: hypoxia, inflammation, TGF-β and Wnt.

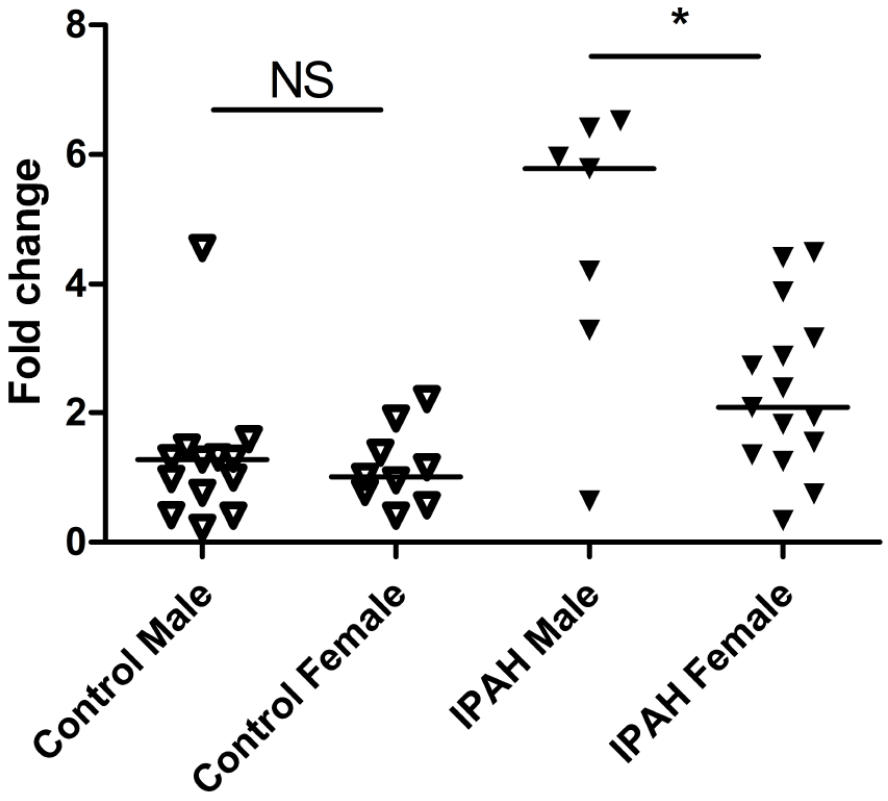

Figure S11. ESR1 expression levels in different groups and genders. The expression level of ESR1 in male IPAH patients is significantly higher than that in female patients. But there is no significant difference between male controls and female controls.