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Identification of Zip8-Correlated Hub Genes in Pulmonary Hypertension By Informatic Analysis

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Abstract

Background. Pulmonary hypertension (PH) is a syndrome characterized by marked remodeling of the pulmonary vasculature and increased pulmonary vascular resistance, ultimately leading to right heart failure and even death. The localization of Zrt/Irt-like Protein 8 (ZIP8, a metal ion transporter, encoded by SLC39A8) was abundantly in microvascualature endothelium and its pivotal role in lung has been demonstrated. However, the role of Zip8 in the progression of PH remains unclear.

Methods. Bioinformatics analysis was employed to identify the expression of SLC39A8 expression, all differentially expressed genes (DEGs) between PH and NC from the Biotechnology Gene Expression Omnibus (NCBI GEO) database. Gene set enrichment analysis (GSEA) was performed to analyze the enriched signaling pathways. Hub genes were identified by cytohubba analysis in Cytoscape. Reverse transcriptase-polymerase chain reaction was used to validate the expression of SLC39A8 and its correlated genes in PH (SU5416/Hypoxia) mice.

Results. The expression of SLC39A8 expression was downregulated in PH patients, and this variation was validated in PH (SU5416/Hypoxia) mice lung tissue. SLC39A8-correlated differentially expressed genes (DEGs) were mainly enriched in the metabolic pathways. Seven hub genes of SLC39A8-correlated metabolic DEGs were identified, and the expression of these genes were analyzed in PH patients and controls, and further validated in PH mice. Finally, four genes expression were downregulated in PH mice, such as Fasn, Nsdhl, Acat2, and Acly. Of the 4 genes, Fasn and Acly are the key fatty acids synthesis enzymes, Nsdhl is involved in cholesterol synthesis, while Acat2 is involved in cholesterol metabolic transformation. These results provide novel insight into the role of Zip8 in PH.

Introduction

Pulmonary hypertension (PH) is a syndrome characterized by marked remodeling of the pulmonary vasculature and increased pulmonary vascular resistance and pressure , which ultimately leads to right heart failure and even death[1]. PH pathogenesis is multifactorial and is presented as an aberrantly elevated pulmonary artery pressure (PAP) and a persistent increase in pulmonary vascular resistance and vascular remodeling[2]. There are several pathological features of the disorder, such as remodeling of the distal pulmonary vasculature, and infiltration of inflammatory cells, extension of the pulmonary artery smooth muscle cells (PASMC) into typically nonmuscularized vessels[1].

Currently, drugs mediates improvement of vascular relaxation and inhibition of cell proliferation has shown favorable results; but the disease is progressive and the long term results are far from ideal. The newer drugs are urgently needed to improve the survival and exercise tolerance. SLC39A8 encodes a zinc transporter ZIP8, a member of ZIPs, whose expression was found to be highest in kidney, lung, and testis[3], and relatively more abundantly in endothelium[4]. The important role of ZIP8 in the lung has been demonstrated in several studies, for example, loss of ZIP8 expression was associated with impaired renewal capacity of type 2 alveolar epithelial cells (AEC2s) and enhanced lung fibrosis[5], increased ZIP8 expression in lung epithelial cells was associated with protective role against TNF-induced cytotoxicity[6], and increased ZIP8 expression in lung was associated with re-organization of filamentous actin[7]. These data demonstrated the important role of ZIP8 in lung. There were also other researches that suggested that the expression of ZIP12 (another member of ZIPs) was induced in the vasculature in human patients and rat models of PH in vivo [8-11], which was at least partially responsible for hypoxia-induced PH in both human and rats[8]. Based on the above research background, we reasoned that ZIP8, hereafter referred as SLC39A8, also plays a vital role in the progression of PH. In this study, we analyzed SLC39A8 expression in PH patients and mice, and explored the role of SLC39A8 in the progression of PH and the potential mechanism involved.

Materials & Methods

Data Collection and Processing

Using the keywords “pulmonary hypertension” and “Homo sapiens”, 7 datasets were screened out. The original data were obtained from the National Center for Biotechnology Gene Expression Omnibus (NCBI GEO) database. All the downloaded files were processed using the

R package (version 4.2.1), and the data were normalized, calibarated and log2-transformed. Of these datasets, only those containing the expression of SLC39A8 PH patients and normal control (NC) samples were selected, such as GSE24988 (62 PH and 22 NC), GSE113439 (15 PH and 11 NC), GSE117261 (58 PH and 25 NC) and GSE15197 (18 PH and 13 NC). Considering the small number of specimens in each dataset, the 4 datasets were merged (153 PH and 71 NC), in the following called the merged dataset, and followed by batch normalization using "sva" and "limma" R package to eliminate the batch effect.

Identifying Differentially Expressed Genes

Differential expression analysis was performed between PH lung tissue and normal tissue using Limma package in R language. Genes were considered to be differential expression genes (DEGs) only with an adjusted $P<0.05$. Results were visualized using "volcano" and "heatmap" plots constructed using "ggplot2".

Mouse model of PH/Animal Experiment

8-10-week-old C57BL/6J male mice were purchased from SPF (Beijing) Biotechnology Co., Ltd.. Animals were randomized into two groups, kept at 20–25℃ under a 12 h light–dark cycle and obtained food and water freely. To induce the mice PH model (n=10), received a single weekly subcutaneous injection of SU5416 (Su, 20 mg/kg body weight, suspended in carboxymethylcellulose solution). Then mice were housed in a hypoxic environment (10% O_2, Hx) for 4 weeks. Carboxymethylcellulose solution consists of four major components including 0.5% (wt/vol) carboxymethylcellulose sodium, 0.9% (wt/vol) sodium chloride, 0.4% (vol/vol) polysorbate 80, and 0.9% (vol/vol) benzyl alcohol in deionized water. Control mice (n=10) received vehicle instead of SU5416 and were subjected to normoxic conditions. A mean pulmonary arterial pressure (mPAP) of ≥25 mmHg indicated successful induction of PH, and researchers who tested mPAP was blinded to animal groups.

All animals survived until the end of the experiment. This study did not require euthanasia. At the end of the treatment, all mice were anaesthetized with pentobarbital sodium (30mg/kg, i.p.) before sacrificed, then lung tissue samples were collected for the subsequent experiments. All experimental protocols were approved by Ethics Committee of Xinxiang Medical University (XYLL 20230062) and administrated strictly following Guidelines of the Laboratory Animal Center of Henan Province, Xinxiang Medical University.

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from lung tissues and quantified by a Trizol reagent (Invitrogen, Waltham, MA), according to the manufacturer's instructions. The concentration of RNA was determined with Nanodrop 1000 (Thermo Scientific, Wilmington, DE), and 1 μg of RNA from each sample was reverse transcribed (QuantiTect Reverse Transcription Kit, Qiagen), and PCR was performed in technical triplicate for each sample by using a thermal cycler (GeneAmp PCR system 2400; PerkinElmer, Fremont, CA). Primer sequences used for the target genes analyzed are listed in Table 1.

Gene set Enrichment Analysis (GSEA) of DEGs

Gene set enrichment analysis (GSEA) was performed using GSEA/MsigDB (http://www.broadingstitute.org/gsea/msigdb/index.jsp) to analyze the DEGs. C2.cp.all.v2022.1.Hs.symbols.gmt [All Canonical Pathways] (3050) was selected as the reference gene set. Gene sets with |NES|>1 P.adj < 0.05, and FDR (qvalue) < 0.25 were considered significantly enriched. Enrichment of gene sets was ranked according to normalized enrichment score (NES).

Protein-Protein Interaction (PPI) Network Analysis and the Hub Genes identified

PPI network was constructed by Cytoscape software, In this study, STRING (version 11, http://www.webgestalt.org/) was used to analyze the PPI of DEGs, and an interaction with a combined score of > 0.4 was considered statistically significant. Then, the PPI network was constructed and visualized using the Cytoscape software (v3.9.1)[12], which is an open source software platform for visualizing complex networks and integrating these with any type of attribute data. Hub genes were identified using cytoscape plugin Cytohubba, and three different algorithms such as MCC, Degree and Closeness were selected.

Statistical Analysis

Statistical analyses were performed using SPSS 18.0, GraphPad Prism 9.0, and R 3.5.1. Wilcoxon rank sum test and Welch t' test were used to compare the difference of gene expression between two PH and NC. Pearson correlation analysis was used to examine the relationship between the expression of SLC39A8 and other genes. A Student's t-test was used to compare the difference in SLC39A8 and its related genes expression in the lung between PH mice and control mice. Data were expressed as mean ± S.E.M.

Results

SLC39A8 was lowly expressed in PH

As shown in Figure 1A, volcano plot showed a total of 5228 DEGs (padj<0.05) were identified from the merged datasets, of which 3031 were downregulated and 2197 were upregulated. The heatmap of DEGs in the pooled dataset showed hierarchical clustering of altered transcription in two groups (Figure 1B), which may facilitate to identify the function of unknown transcripts or the unknown function of known transcripts by collecting similar expression patterns. Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways from the GSEA analysis of all DEGs were shown in Figure 1C, the top 5 pathways such as "Arrhythmogenic Right Ventricular Cardiomyophthy Arvc"[13], "Ecm Receptor Interaction"[14, 15], "Wnt Signaling Pathway" [16, 17], "Hypertrophic Cardiomypathy Hcm"[18, 19], "Focal Adhesion"[20-22], "Dilated Cardiomyopathy"[23, 24] are all associated with PH. On the other hand, 3 of the top 5 enriched Reactome pathways from the GSEA analysis (Figure 1D) such as "Degradation of the Extracellular Matrix"[25, 26], "Cilium Assembly", "Extracellular Matrix Organization"[25] and "Signaling By Tgfb Family Members"[27] are also involved in the pathogenesis of PH. We next analyzed the expression of SLC39A8 in lung tissue between PH and NC. As shown in Figure 1E, the expression of SLC39A8 decreased in lung tissue of PH patients (Figure 1E). To further validate this variation of SLC39A8 in PH, we examined the expression of Slc39a8 in a mice model of PH induced by SU5416/Hypoxia by quantitative RT-

PCR. Likewise, Slc39a8 expression in lung tissue of PH mice was noticeably reduced (Figure 1F).

Identifying SLC39A8-correlated DEGs

To further explore potential molecular mechanisms of SLC39A8 in PH, we performed correlation analysis among all DEGs and SLC39A8 using Pearson correlation coefficients. Then 6083 SLC39A8-correlatted were identified when considered p.adj<0.05.

According to the results of correlation analysis, 2851 DEGs are negative correlated with SLC39A8, and 3232 DEGs are positive correlated with SLC39A8. As shown in Figure 2A, top 20 positive-correlated DEGs and top 20 negatively-correlated DEGs were displayed in heat maps of Pearson correlations. Then, the top 5 positively SLC39A8-correlated DEGs were presented in Figure 2B, such as lysosomal associated membrane protein 3 (LAMP3), phosphatase and actin regulator-1 (PHACTR1), methionine synthase reductase (MTRR), methionine synthase reductase ATP-binding cassette transporter A3 (ABCA3) and sciellin (SCEL). And top 5 negatively associated genes were also presented in Figure 2C, such as clusterin (CLU), Transmembrane protein family (TMEM45A), eyes absent homolog 2 (EYA2), Musashi2 (MSI2) and Serpin Peptidase Inhibitor, Clade F 1 (SERPINF1). Among them, CLU[28, 29] and ABCA3[30, 31] have been shown to be associated with PH, which indirectly suggests SLC39A8 may play a pivotal role in the progression of PH.

GSEA analysis of SLC39A8-correlated DEGs

After identified SLC39A8-correlated genes, we further performed gene set enrichment analysis (GSEA) using gene set collections from the MsigDB (https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp). ClusterProfiler[4.4.4] was used for GSEA analysis, and the c2.cp.all.v2022.1.Hs.symbols.gmt [All Canonical Pathways](3050) was selected as the reference gene set. P.adj<0.05 and false discovery rate (FDR, qvalue) <0.25 were used to indicate significant enrichent score. These enriched pathways were consisted of Wikipathways, Reactome and KEGG. As shown in Figure 3A, The SLC39A8-correlated genes were enriched in “Cholesterol Metabolism With Bloch and Kandutschrussell pathways”, “Cholesterol Biosynthesis Pathway”, “Cholesterol Synthesis Disorders”, “Nrf2 pathway” and “Mevalonate Arm of Cholesterol Biosynthesis Pathway” in [WikiPathways] based on Normalized Enrichment Score (NES). Among the top 5 WikiPathways enriched in the SLC39A8-correlated DEGs, 4 were clustered into a group “cholesterol metabolism”, which was the most significant and have been demonstrated associated with PH[32-34]. Then the top3 pathways were displayed in Figure 3B, respectively. According to the results of GSEA enrichment analysis of Reactome pathways, the SLC39A8-correlated DEGs were enriched in, “Antigen Processing Cross Presentation”, “Cholesterol Biosynthesis”, “innate Immune System” and “Regulation Of Cholesterol Biosynthesis By Srebp Srebf”. Among the top 5 Reactome pathways, 2 were clustered into a “cholesterol metabolism” group. Furthermore, GSEA enrichment analysis of KEGG pathways reavealed that the pathways enriched by SLC39A8-correlated DEGs included “Terpenoid Backbone Biosynthesis”, “Steroid Biosynthesis”, “Neuroactive Ligand Receptor Interaction”, “Focal Adhesion” and “Wnt Signaling Pathway”. Of these pathways, “Neutrophil

Degranulation" and "innate Immune System" [35], Wnt signaling pathway [16, 17], "Steroid Biosynthesis"[36] have already been linked to PH. Based on these results of GSEA analysis, we concluded that the role of SLC39A8 in the progression of PH may attributed to cholesterol and/or steroid metabolism.

Identifying key SLC39A8-correlated metabolic DEGS between PH and NC

Accumulating evidence has indicated that PAH is associated with metabolic dysfunction[37-40]. And a previous study has identified extracted 1,660 human genes assigned to 86 metabolic pathways from the KEGG database[41]. Based on these findings, we subsequently identified SLC39A8-correlated metabolic DEGs between the PH and NC, and constructed the PPI network of DEGs. As shown in Figure 4A, a venn diagram revealed 202 common genes in three groups, including 1600 transcripts of metabolic genes, 6083 SLC39A8-correlated DEGs, and 5228 DEGs between PH and NC. The SLC39A8-correlated metabolic DEGs showed a significant cluster of interactions and networks. And the PPIs with combined scores greater than 0.4 were obtained for constructing networks by using the STRING database (Figure 4B). Hub genes were identified by the Cytohubba plugin in Cytoscape (https://string-db.org/). The top 15 hub genes were identified using three different algorithms closeness, degree and MCC as shown in Figure 4 C-E. A Venn diagram (Figure 4F) revealed 7 common genes in the three groups, indicating that they represented the key SLC39A8-correlated metabolic DEGs between PH and NC.

Validation of key SLC39A8-correlated metabolic DEGs in a mice model Using RT-PCR Analysis

To further elucidate the relationship between SLC39A8 and the 7 key genes. We presented the expression of these genes in PH patients and controls in the merged dataset and performed correlation analysis between these genes. As shown in Figure 5 A, the expression of 6 genes were low in PH patients, such as cholesterol acyl-transferase 2 (ACAT2), NAD(P)-dependent steroid dehydrogenase-like (NSDHL), farnesyl diphosphate synthase (FDPS), fatty acid synthase (FASN), ATP-citrate lyase (ACLY) and Acetyl-CoA synthetase 2 (ACSS2). While the expression of FDFT1 did not differ between PH patients and NC group. It is possible that the actual differences were small and no significance when differential analysis is statistically different with a high p value, which attributed to different statistical methods. Meanwhile, correlation between SLC39A8 and these genes were shown in Figure 5B. Interestingly, the expression of these genes was positively correlated with SLC39A8. Finally, we identified 6 genes which are most likely correlated with SLC39A8, such as ACAT2, NSDHL, FDPS, FASN, ACLY and ACSS2.

In order to further verify the results of bioinformatic analysis, we examined the expression of these genes in PH mice and control mice. RT-PCR was used to validate the 6 key genes (NSDHL, ACAT2, ACLY, and FASN) expression in PH mice and control mice. As shown in Figure 5C, the expression of Acat2, Nsdhl, Fasn and Acly were significantly low in PH mice, which were consistent with the results of bioinformatics analysis. However, the expression of Fdps and Acss2 did not differ between the two groups (data are not shown).

Discussion

Pulmonary hypertension (PH) is a fatal rare disease that characterized by pulmonary vascular remodeling, involving pulmonary artery endothelial cells, smooth muscle cells, and fibroblasts[42, 43]. ZIP8 is a recently identified membrane transporter of essential and toxic divalent metals. The important role of ZIP8 in the lung has been demonstrated, however, the role of ZIP8 in PH and the mechanism involved are unknown. In recent years, the rapid evolution in high-throughput sequencing technologies has provided a new perspective for PH research. The use of transcriptome technology to reveal the role of gene expression has long been appreciated. In this study, we performed DEGs analysis between PH patients and controls, and found SLC39A8 expression was significantly reduced in PH patients. Furthermore, the low expression of SLC39A8 was confirmed in PH mice by using RT-PCR. These finding suggested that SLC39A8 may play an important role in the progression of PH.

The results of GSEA analysis of all DEGs between PH and NC showed that top 5 KEGG pathways were all associated with PH, and 3 of the top 5 Reactome pathways were also involved in the pathogenesis of PH. These results demonstrated the validity of the selected datasets. To further elucidate the potential role of SLC39A8 in PH, we identified SLC39A8-correrlated genes in DEGs and performed GSEA analysis of the correlated DEGs. The results of enriched Wikipathways, Reactome pathways and KEGG pathways suggested that SLC39A8 was intimately linked to cholesterol metabolism.

Studies have shown that metabolic dysfunction is associated with PAH [37-40].The involvement of obesity and lipid metabolism in the development of high-altitude pulmonary hypertension (HAPH) was highlighted in a recent review, which suggests that triglycerides (TGs) and low-density lipoprotein (VLDL) could be predictors of HAPH in early stages, and high BMI is an important contributor to the development of HAPH[44]. Furthermore, the role of imbalanced fatty acid metabolism in pulmonary arterial hypertension (PAH) also has been discussed[39]. It was interesting that body mass index (BMI)[45], obesity[46, 47] high-density lipoprotein (HDL) cholesterol levels[48-51] were correlated with rs13107325 SNP (results Ala-Thr amino acid change at position 391 of the protein) of the Solute Carrier Family 39 Member 8 (SLC39A8) gene in several genome-wide association studies (GWAS).

Considered together, we speculated that SLC39A8 may play a role in PH by regulating cholesterol and/or lipid metabolism, and subsequently identified SLC39A8-related metabolic DEGs by using a Venn diagram. Next, 7 hub SLC39A8-related metabolic DEGs were identified, after analysing the expression of these genes and the correlation of these genes and SLC39A8, 6 hub genes were selected for further study. Finally, of the 6 hub genes, only 4 hub genes such as Acat2, Nsdhl, Fasn, Acly were downregulated in PH mice, while the other two genes such as Fdps and Acss2 were equivalent between two groups.

Of the four genes, NSDHL gene encodes a sterol dehydrogenase or decarboxylase enzyme involved in cholesterol biosynthesis[52], ACLY is a key fatty acids synthesis enzymes, FASN is a key enzyme for the de novo synthesis of fatty acids and ACAT2 is an ER membrane-spanning enzyme converting cholesterol and fatty acid to cholesteryl esters (CEs)[53].

It is known that fatty acid metabolism involves fatty acid synthesis, fatty acid oxidation and cholesterol metabolism[54]. The importance of lipid mechanism in PH[39, 44] has been demonstrated, and imbalanced fatty acid metabolism is reported in heart and lungs of PAH patients [55-57]. A higher rate of de novo fatty acid synthesis was found in PAH-HPASMC[58], and increased expression of FASN were observed in lungs of MCT-treated rats [59] and human PAH pulmonary arterial vascular smooth muscle cells (PAVSMC) [60]. In addition, another fatty acid synthesis enzyme ACLY was also upregulated in PAVSMC[60]. Furthermore, another study reveals that inhibition of FASN is beneficial for endothelial function in PH[61] and improves cardiac function associated with PH[62]. These results demonstrated that the increased FASN is correlated with PH. However, in this study, we found that the expression of FASN and ACLY in PH patients and PH mice were decreased. We speculate that the discrepancy might arise from the difference between cell sample and tissue sample and need to be further studied. Although cholesterol and fatty acids (FA) are essential lipids that play a wide range of physiological roles, excessive polar lipids, such as free cholesterol (FC) and free FA (FFA), are the major risk factors in the body. The stabilized ACAT2 converts cholesterol and FAs to CEs, thereby reducing the lipotoxicity of polar lipids. Previous studies have found HDL-cholesterol reduced [32] in PAH patients, and loss of membrane cholesterol contributes to impaired pulmonary endothelial store-operated Ca^{2+} entry (SOCE) in chronic hypoxia induced PH[33]. Those findings suggested the protective properties of HDL in PAH[34]. In this study, the expression of NSDHL and ACAT2 were decreased, which can decrease the production of cholesterol and increase the toxicity of cholesterol, finally participant in the procession of PH. In conclusion, the results of the present study revealed that SLC39A8 expression is low in pH patients and mice, we firstly identified its potential target genes associated with fatty acid metabolism through bioinformatic prediction, and validated the expression of these genes in PH patients and mice. Finally, we conclude that SLC39A8 may play a pivotal role in the progression of PH by regulating fatty acid and/cholesterol metabolism.

However, some limitations should also be noted in this study. First, the sample size of the included datasets in this study is not big enough. Second, the specific regulatory mechanism between SLC39A8 and the 4 hub genes has not been clearly explored. Therefore, a more detailed investigation of the protective role of SLC39A8 in PH and whether these four hub genes were involved will be required.

Conclusions

Our data presented here was the first, to our knowledge, to show that the expression of SLC39A8 was low in the lung of PH patients by analyzing four publicly available microarray datasets retrieved from GEO database. This result was validated in PH mice by using RT-PCR. Furthermore, based on our current study, our research provided a bioinformatic analysis of SLC39A8 and its correlated metabolic DEGs. The screened hub genes, NSDHL, ACLY, ACAT2, and FASN may be downstream target genes of SLC39A8. However, further study about PH is required for a better understanding of the role of ZIP8 in PH.

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Availability of data

The raw microarray data were deposited in the Gene Expression Omnibus (http:www.ncbi.nlm.nih.gov/projects/geo).

Declaration of interests

The authors have declared that no competing interests exist.

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Figure 1. SLC39A8 expression was downregulated in PH patients and mouse PH models. **(A)** The volcano plots of DEGs in the merged datasets (153 PH and 71 NC, merged from these four datasets), in which 3031 DEGs were downregulated (log2FC > 0, FDR < 0.05) and 2197

DEGs upregulated (log2FC > 0, FDR < 0.05). **(B)** Heatmap of DEGs in the merged datasets **(C)** GSEA analysis shows enriched KEGG pathways. **(D)** GSEA analysis shows enriched Reactome pathways. **(E)** Expression levels of SLC39A8 in PH and NC groups in the merged datasets. **(F)** Expression levels of Slc39a8 in the lungs of normoxia and Su/Hx treated PH mice (n=10). Data were presented as mean±SEM. ***P<0.001.

Figure 2. Correlation analysis of all DEGs and SLC39A8. (A) Heatmap of the top 20 genes positively or negatively correlated with SLC39A8. Red represents positive correlation and blue represents negative correlation. **(B)** Top 5 genes positively correlated with SLC39A8 were displayed. **(C)** Top 5 genes negatively correlated with SLC39A8 were displayed.

Figure 3. The GSEA analysis of SLC39A8-correlated DEGs between PH and NC. (A) GSEA classical plots generated based on NES score in canonical Wikipathways. **(B)** The top 3 WikiPathways are listed respectively. **(C)** GSEA classical plots generated based on NES score in canonical Reactome pathways. **(D)** GSEA classical plots generated based on NES score in canonical KEGG pathways. P.adj<0.05 and false discovery rate (FDR, qvalue)<0.25 were used to indicate significant enrichment score.

Figure 4. Identification of hub genes SLC39A8-correlated metabolic DEGS between PH and NC. (A) Venn diagram of common genes in three groups (1600 transcripts of metabolic genes, 6083 SLC39A8-correlated DEGs, and 5228 DEGs). **(B)** PPI network was constructed by the STRING database and visualized by cytoscape software (v3.9.1), and each blue filled node represents a SLC39A8-related gene; **(C-E)** The top 15 Hub genes were identified via cytoscape software (cytohubba) using MCC (C), Degree (D), and Closeness (E). **(F)** Venn diagram of common genes in these three hub gene sets.

Figure 5. Verification of hub genes expression at the mRNA level. (A) The expression of 7 key SLC39A8-correlated metabolic DEGs in the merged dataset. **(B)** The correlations between 7 key SLC39A8-correlated metabolic DEGs and SLC39A8 were presented independently. **(C)** RT-PCR analysis of the expression of Acat2, Nsdhl, Acly and Fasn in lungs of normoxia and Su/Hx treated PH mice. n=10 for each group. Data are shown as mean ± SEM; *P<0.05, ** P<0.01, ***P<0.001; ns, no significance.

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