The Hypoxic Response Contributes to Altered Gene Expression and Pre-Capillary Pulmonary Hypertension in Patients with Sickle Cell Disease

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Abstract

Background—We postulated that the hypoxic response in sickle cell disease (SCD) contributes to altered gene expression and pulmonary hypertension, a complication associated with early mortality.

Methods and Results—To identify genes regulated by the hypoxic response and not other effects of chronic anemia, we compared expression variation in peripheral blood mononuclear cells from 13 SCD subjects with hemoglobin SS genotype and 15 Chuvash polycythemia subjects (VHL^{R200W} homozygotes with constitutive up-regulation of hypoxia inducible factors in the absence of anemia or hypoxia). At 5% false discovery rate, 1040 genes exhibited >1.15 fold change in both conditions; 297 were up-regulated and 743 down-regulated including *MAPK8* encoding a mitogen-activated protein kinase important for apoptosis, T-cell differentiation and inflammatory responses. Association mapping with a focus on local regulatory polymorphisms in 61 SCD patients identified expression quantitative trait loci (eQTL) for 103 of these hypoxia response genes. In a University of Illinois SCD cohort the A allele of a *MAPK8* eQTL, rs10857560, was associated with pre-capillary pulmonary hypertension defined as mean pulmonary artery pressure ≥25 and pulmonary capillary wedge pressure ≤15 mm Hg at right heart catheterization (allele frequency=0.66; OR=13.8, P=0.00036, n=238). This association was confirmed in an independent Walk-PHaSST cohort (allele frequency=0.65; OR=11.3, P=0.0025, n=519). The homozygous AA genotype of rs10857560 was associated with decreased *MAPK8* expression and present in all 14 identified pre-capillary pulmonary hypertension cases among the combined 757 patients.

Conclusions—Our study demonstrates a prominent hypoxic transcription component in SCD and a *MAPK8* eQTL associated with pre-capillary pulmonary hypertension.

Keywords

sickle cell disease; *MAPK8*; hypoxic response; expression quantitative trait loci; association mapping; pre-capillary pulmonary hypertension

Sickle cell disease (SCD) is due to homozygosity for a Glu6Val mutation in *HBB* (sickle cell anemia; hemoglobin SS) or to compound heterozygous forms like hemoglobin SC disease and hemoglobin S-β thalassemia. Investigation of the pathophysiology of SCD complications has focused on the adverse effects of vaso-occlusion, chronic inflammation, and hemolysis.[1] Little attention has been given to the up-regulation of the hypoxic response. Erythropoietin expression sensitively reflects tissue oxygenation status,[2] and hypoxia inducible factor (HIF)-α, the master regulator of the body's response to hypoxia, was discovered by studying the regulation of the erythropoietin gene.[3] SCD is characterized by high circulating erythropoietin concentrations under basal circumstances,[4] indicating that this chronic anemia is accompanied by chronic up-regulation of the hypoxic response. Hypoxia influences diverse cellular and metabolic processes,[5] and both chronic and acute hypoxia cause morbidity and mortality associated with pulmonary and brain edema, aberrant metabolism, and pulmonary hypertension.[6, 7]

A substantial body of evidence also indicates that normoxic activation of HIF-1α is involved in the etiology of various forms of group 1 pulmonary hypertension through changes in

mitochondrial redox signaling, fission and numbers, and is critical to the development of a proliferative, apoptosis-resistant phenotype in pulmonary vascular cells.[8-10] Furthermore, placental growth factor activates HIF-1α in normoxia and has been associated with elevated systolic pulmonary artery pressures in SCD.[11] Hypoxia has broad effects on gene expression, but this phenomenon has been almost entirely investigated *in vitro*[12] or in animal models.[13] We postulated that the up-regulated hypoxic response in SCD might contribute substantially to altered gene expression and to pulmonary hypertension, an important clinical complication that is associated with early mortality.[14]

To address this hypothesis, we prospectively compared clinical data and genomic profiles of SCD and Chuvash polycythemia (CP) subjects. Similar to SCD, CP is a monogenic hematologic disorder characterized by an up-regulated hypoxic response.[15] Unlike SCD, CP patients are not anemic and the hypoxic response occurs at normoxia. Specifically, homozygosity for the *VHL*[R200W] mutation leads to post-translational stabilization of the alpha subunits of HIF at normoxia via decreased binding of these subunits to the mutant VHL protein, which normally marks them for destruction through the proteasome.[15, 16] As the result, increased levels of HIF-1 and HIF-2 lead to altered transcription of a number of genes.[15] Given the lack of the confounding effect of anemia to the presence of the hypoxic response in CP, we leveraged this characteristic to investigate the contribution of the hypoxic response in SCD.

Materials and Methods

Study strategy

The study was prospectively designed to compare clinical manifestations and peripheral blood mononuclear cell (PBMC) genomic profiles of SCD and CP subjects with the hypothesis that shared hypoxia-induced pathways may underlie the risk for pulmonary hypertension. The scheme of the study is summarized in Figure 1. We first identified altered gene expression associated with SCD by comparing 13 African-American hemoglobin SS subjects and 16 African-American hemoglobin AA control individuals. On the same array platform we profiled hypoxia-induced gene expression under normoxia by comparing 15 Chuvash *VHL*^*R200W* homozygotes and 16 Chuvash *VHL* wildtype individuals. Intersecting the two sets of genes identified hypoxia-induced gene expression in hemoglobin SS subjects. We further mapped expression quantitative trait loci (eQTL) for these genes in SCD patients and carried out genetic association between the identified eQTL and pulmonary hypertension phenotypes in two additional SCD cohorts.

Study subjects

The study was approved by the IRBs of the participating institutions and all subjects provided written informed consent.

Howard University cohort—Thirty-three hemoglobin SS, seven hemoglobin SC, two hemoglobin Sβ⁺-thalassemia and 17 hemoglobin AA adult African-Americans subjects were studied.

Chuvash polycythemia (CP) cohort—Fifteen VHL^{R200W} homozygotes and 16 wildtype controls from Chuvashia, Russia with serum ferritin concentration   21 μg/L were studied.

University of Chicago cohort—Twenty-four hemoglobin SS individuals[17] were included for determining hypoxic expression quantitative trait loci (eQTL).

University of Illinois at Chicago (UIC) cohort—One hundred eighty-two hemoglobin SS, 43 hemoglobin SC, 15 hemoglobin Sβ⁺-thalassemia, six hemoglobin Sβ⁰-thalassemia, one hemoglobin SO Arab subjects were tested for genetic association between eQTL and pulmonary hypertension. Genotyping was carried out using Affymetrix Axiom genome-wide Pan-African array. Genotype information in the ~1 Mb regions around each of 103 target genes was used to impute genotypes at the identified eQTL. Only these eQTL genotypes were used in clinical associations. Twenty-seven subjects with elevated tricuspid regurgitation velocity (TRV) and clinical suspicion of pulmonary hypertension underwent right heart catheterization: eight had pre-capillary pulmonary hypertension defined as mean pulmonary artery pressure (PAP)   25 mm Hg and pulmonary capillary wedge pressure (PCWP)   15 mm Hg, nine had post-capillary pulmonary hypertension defined as mean PAP   25 mm Hg and PCWP >15 mm Hg, and 10 did not have pulmonary hypertension based on mean PAP <25 mm Hg. Absence of pulmonary hypertension was defined as mean PAP <25 mm Hg if right heart catheterization was performed or TRV <2.5 m/sec if catheterization was not performed.

Walk-PHaSST cohort—Clinical phenotypes and genotype data for SNPs located within the *MAPK8* gene and its ±500 Kb flanking regions were obtained for 393 hemoglobin SS, 99 hemoglobin SC, 19 hemoglobin Sβ⁰-thalassemia and 11 hemoglobin Sβ⁺-thalassemia subjects from the Walk–Treatment of Pulmonary Hypertension and Sickle Cell Disease with Sildenafil Therapy (Walk-PHaSST) study.[4] Patients recruited at UIC were excluded from this analysis to avoid overlap with the UIC cohort. A subgroup of 17 of the 56 subjects with TRV   3.0 m/sec underwent right heart catheterization, and pre-capillary and post-capillary pulmonary hypertension were defined as above. Six of these subjects had pre-capillary pulmonary hypertension, three had post-capillary pulmonary hypertension and eight had mean PAP <25 mm Hg. Genotyping was carried out using Illumina Human 610-Quad SNP array.

Clinical testing

Serum ferritin concentration was determined by enzyme immunoassay (Ramco Laboratories Inc., Stafford, TX) and serum erythropoietin (EPO) by enzyme linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). Echocardiography measurements were performed according to American Society of Echocardiography guidelines.[18]

RNA isolation and expression profiling

PBMCs were isolated from 10 ml of EDTA-anticoagulated blood. Total RNA was extracted using TRIzol® (Invitrogen, CA) and quality assessed using nanodrop (Thermoscientific, Waltham, MA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total

RNA was submitted to University of Chicago Functional Genomics Center for whole transcript sense target labeling assay, hybridization to the *Affymetrix* Human Exon 1.0 ST Array, and washing and scanning (Affymetrix, Inc., Santa Clara, CA).

Microarray data preprocessing

Twenty-five-mer probe sequences were aligned to human genome assembly GRCh37 allowing 2 mismatches.[19] Probes with perfect unique match to the genome were selected. We removed probes that interrogated multiple gene transcripts and that contained SNPs with 1% minor allele frequency in dbSNP dataset (v135). Probe level intensities were $\log_2$ transformed, background corrected[20] and quantile normalized.[21] Probe intensity was subtracted by the corresponding probe mean across samples. Gene-level expression intensities were summarized as mean probe intensity within each transcript cluster. In total, 16,642 autosomal transcript clusters (gene-level) were included.

Statistical analysis of clinical data and gene expression variation

Wilcoxon's rank sum test and Fisher's exact test were applied for comparison of continuous and categorical clinical covariates, respectively, between patients and controls for both the CP and Howard University SCD cohorts. To compare gene expression levels between two groups (VHL^{R200W} homozygote subjects versus VHL wildtype controls, hydroxyurea-treated versus non-treated hemoglobin SS individuals, or hemoglobin SS versus hemoglobin AA), for each gene we tested the null hypothesis H_0: expression$_{group1}$= expression$_{group2}$. A d-statistic[22] was calculated for each gene. The d-statistic is a modified t-statistic that stabilizes statistical variance for low expression genes thereby improving across-gene comparison. We performed 100 permutations to estimate false discovery rate (FDR).[23] Statistical analyses were carried out using R.[24]

eQTL mapping

Twenty-eight hemoglobin SS, seven hemoglobin SC and two hemoglobin Sβ^+-thalassemia subjects from Howard University and 24 hemoglobin SS patients from the University of Chicago were pooled. Batch effect of two hybridization batches was corrected by an empirical Bayes approach implemented in ComBat[25]. Affymetrix Genome-Wide Human SNP Array 6.0 was used for SNP genotyping. Among the 868,157 autosomal SNPs, 94% had call rate > 90% across the 61 samples. The per sample genotype rate was > 91% with a median of 98%. Considering our small sample size, we analyzed SNPs with 100% call rate. SNPs with known autosomal locations, minor allele frequency >10%, and not significantly deviated from Hardy–Weinberg equilibrium ($P > 10^{-4}$) were selected, resulting in 321,919 SNPs. To test for heterogeneity in ancestral admixture among the 61 subjects, we selected 148,365 SNPs for principal component analysis[26], which were pruned from the 321,919 SNPs by pair-wise $r^2 > 0.3$ using PLINK [27]. A total of 27,717 SNPs were local to 1,040 hypoxia response genes, defined as SNPs located in the regions from upstream 100 Kb of gene start to downstream 100 Kb of gene stop. We also carried out principal component analysis for the expression data of the analyzed 15,775 genes, and the top 11 axes were regressed out to improve detection sensitivity. Gene expression levels were regressed on allelic dosage of SNP. To estimate FDR, sample labels were permuted 100 times for gene

expression traits. Hypoxic eQTL with FDR <0.05 were then pruned by linkage disequilibrium (LD, r^2 >0.3).

Association mapping of hypoxic eQTL with clinical phenotypes

SNPs within the target genes of eQTL and their +/- 500Kb flanking regions were imputed for both UIC and Walk-PHaSST cohorts, using HapMap Phase II panels as reference.[28] Armitage trend test and logistic regression were applied to test the association between eQTL and pulmonary hypertension phenotypes in the UIC and Walk-PHaSST SCD cohorts with an additive genetic model. In logistic regression, age and SCD genotype (hemoglobin SS/ $S\beta^0$-thalassemia/SO Arab or hemoglobin SC /$S\beta^+$ thalassemia) were included as covariates. For the UIC cohort, the top five principal components estimated from genome-wide SNP data were also included in the logistic linear model to account for population stratification. The Walk-PHaSST cohort was relatively homogenous for population stratification as previously reported.[29]

Hypoxic transcription in pre-capillary pulmonary hypertension

We selected seven SCD patients diagnosed with pre-capillary pulmonary hypertension and seven hemoglobin AA African-American control individuals. Total RNA, 500-2,000 ng, was reverse transcribed by Superscript III reverse transcriptase in 20 μL reactions using Oligo(dT)$_{12-18}$ primers (Invitrogen, Carlsbad, CA). Gene specific primers were obtained from a primer database[30] and further selected against regions containing multiple gene transcripts or SNPs. Primers were tested by polymerase chain reaction (PCR) in 10 μL reactions using Platinum Taq DNA polymerase (Invitrogen, Carlsbad , CA) followed by running on 3% agrose gel. For quantitative PCR, a standard curve method was applied. cDNA for each sample was diluted at 1:20, 4 μL of which was used with iTaq Universal SYBR Green Supermix (BIO-RAD, Hercules, CA) in 10 μL reactions. A fast thermal cycling protocol was applied using the Applied Biosystems ViiA™ 7 real-time PCR system (Applied Biosystems, Foster City, CA) at 95° for 30 sec, followed by 40 cycles at 95° for 3 sec and 60° for 30 sec. For each sample/gene pair, cycling thresholds were estimated as the average of duplicate reactions. The specificity of the quantitative PCR was further confirmed by melting curve analysis.

Results

Altered gene expression in sickle cell anemia

Howard University sickle cell anemia subjects had lower hemoglobin concentrations and hemoglobin O_2 saturations and higher concentrations of erythropoietin, ferritin and markers of hemolysis than control subjects (Supplemental Table 1). This cohort prospectively included a proportion of patients with high TRV. Seven hemoglobin SC and two hemoglobin $S\beta^+$-thalassemia individuals were also among this cohort (Supplemental Table 2).

Comparison of PBMC gene expression between 20 hemoglobin SS patients with and 13 without hydroxyurea treatment revealed a large number of genes mildly suppressed by hydroxyurea treatment (Supplemental Figure 1). We therefore excluded those on

hydroxyurea treatment from further analysis. The final cohort consisted of 13 hemoglobin SS patients and 16 hemoglobin AA controls (one hemoglobin AA individual was excluded due to iron deficiency) matched for age (P>0.9) and gender (P=0.7). More than 10,000 genes exhibited altered expression in hemoglobin SS patients at FDR 0.05, the majority showing modest up-regulation (Figure 2A). Using a threshold of FDR <0.05 and >1.15 fold change, 784 genes were up-regulated genes (Supplemental Table 3) and 1,245 down-regulated (Supplemental Table 4) in hemoglobin SS patients. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways and GO (Gene Ontology) biological processes that were significantly altered in hemoglobin SS subjects are shown in Supplemental Table 5. KEGG hematopoietic cell lineage, NOD-like receptor signaling, and complement and coagulation cascades pathways were significantly enriched in up-regulated genes whereas T cell receptor signaling, aminoacyl-tRNA biosynthesis, taste transduction, and cell cycle pathways were significantly enriched in down-regulated genes ($P_{adjusted}$ <0.05, fold enrichment >2.5).

Hypoxic transcriptional response in sickle cell anemia

Identification of genes regulated by the hypoxic response in VHL^{R200W} homozygotes—To identify genes specifically regulated by the hypoxic response and not other effects of chronic anemia, we prospectively studied a Chuvash polycythemia (CP) cohort consisting of 15 VHL^{R200W} homozygotes with elevated hemoglobin and serum EPO concentrations[15] and 16 VHL wildtype controls (Supplemental Table 6). Similar to the Howard University hemoglobin SS cohort, this cohort prospectively included a high proportion of patients with elevated TRV. Gene expression of PBMCs was profiled on Affymetrix exon array in an identical manner to the hemoglobin SS cohort. Similar to the hemoglobin SS cohort, expression levels of about 10,000 genes were altered in VHL^{R200W} homozygotes at 5% FDR, the majority with modest up-regulation (Figure 2A). Using a threshold of 1.15 fold change, 474 genes were induced and 1,194 genes were suppressed in VHL^{R200W} homozygotes.

Common transcriptional responses in sickle cell anemia and Chuvash polycythemia—Similar to hemoglobin SS patients, the KEGG T cell receptor signaling pathway was significantly enriched with genes down-regulated in the CP patients. Furthermore, 'inflammatory response' and 'negative regulation of apoptosis' were among seven GO biological processes enriched with up-regulated genes in both hemoglobin SS and CP subjects (P_{adjust} <0.05, fold enrichment >2.5, Supplemental Table 5). Regression coefficients of differential gene expression were highly correlated between sickle cell anemia and CP (Spearman's ρ=0.73) (Figure 1B), suggesting that 53% of expression variation in hemoglobin SS patients is related to hypoxic transcriptional responses. Applying thresholds of FDR <0.05 and fold change >1.15, 1,040 genes displayed altered regulation in both hemoglobin SS patients and VHL^{R200W} homozygotes, with 297 up-regulated (hypoxia-induced, Supplemental Table 7) and 743 down-regulated (hypoxia-suppressed, Supplemental Table 8). These common genes, which we hereafter refer to as hypoxia response genes, represented 51% of the altered genes in the hemoglobin SS patients and 63% in the VHL^{R200W} homozygotes. To verify the specificity of the hypoxic response genes identified under these conditions, we compared the genomic profile of hemoglobin SS with that of Down syndrome, a congenital condition with no involvement of a canonical hypoxia

pathway, in a publicly available PBMC expression dataset using the same array platform.[31] We did not observe any correlation between expression variation in hemoglobin SS and Down syndrome subjects (Figure 2C).

To further explore the gene regulation cascades, we mapped the hypoxia response genes to the Reactome functional interaction database[32] and identified several hub genes that may play important roles in gene-gene interactions (Figure 3). *MAPK8* interacted with the greatest number of genes in the network. Down-regulation of *MAPK8* and its directly interacting partners *ATF2*, and *MAP3K7* are consistent with a suppression of stress-induced apoptosis, while down-regulation of *STAT4* and subunits of the T-cell receptor complex are consistent with a suppression of T-cell activation.

HIF-1 target genes induced in sickle cell anemia—To search for corroboration of an over-representation of hypoxic transcriptional responses in sickle cell anemia we examined the expression of *HIF1A* (hypoxia inducible factor 1, alpha subunit) and known genes regulated by HIF. *HIF1A* was induced in hemoglobin SS patients by 1.3 fold at FDR 0.05, although, as expected, not in VHL^{R200W} homozygotes who were not anemic. In addition, genes up-regulated >1.15-fold in hemoglobin SS patients were enriched for known targets of HIF[33] by 6.4-fold (binomial test $P=8\times10^{-13}$). Genes up-regulated in Hb SS subjects were also enriched for hypoxia response genes expressed in vascular endothelial cells[12] by 2.5-fold (binomial test $P= 0.00024$).

Genetic regulation of hypoxic response genes in SCD patients

To assess the genetic contribution to hypoxic transcriptional variation among SCD patients, we mapped eQTL for the 1,040 hypoxia response genes. For the Howard University SCD cohort we obtained both gene expression and whole genome genotyping data for 37 individuals, including 28 hemoglobin SS, seven hemoglobin SC and two hemoglobin Sβ+-thalassemia patients. These data were pooled with expression and genotype data of 24 hemoglobin SS patients from the University of Chicago cohort. Principal component analysis[26] of 148,365 SNPs suggested little heterogeneity in ancestral admixture among the 61 subjects (Supplemental Figure 2). We associated gene expression levels of the 1,040 hypoxia response genes with allelic dosage of 27,717 local SNPs, defined as SNPs positioned within ± 100 Kb of target genes. We focused on local SNPs because local regulatory polymorphisms have strong effects on gene expression in general, and because the restricted analysis greatly reduced the burden of multiple comparisons. At 5% FDR, 25 eQTL were detected for 15 hypoxia-induced genes and 276 eQTL were detected for 88 hypoxia-suppressed genes. After pruning for LD ($r^2 > 0.3$), we identified 126 hypoxic eQTL (Supplemental Table 9).

Association between risk for pre-capillary pulmonary hypertension and a *MAPK8* eQTL

Pulmonary hypertension documented by right heart catheterization is associated with early mortality in patients with sickle cell anemia.[14, 34, 35] Because hypoxic transcriptional response may constitute an important pathogenic condition for SCD progression, hypoxic eQTL potentially underlie heterogeneity in risk of pulmonary hypertension. To test this hypothesis, we examined the association between hypoxic eQTL and pulmonary

hypertension phenotypes in an independent UIC cohort in which right heart catheterization measurements were available. For direct comparison, we imputed eQTL genotypes for the UIC cohort. Genotypes for 109 eQTL were imputed with high quality (imputation $r^2 > 0.9$) for 92 hypoxic genes. We tested three pulmonary hypertension-related phenotypes defined in Methods: pulmonary hypertension, pre-capillary pulmonary hypertension, and post-capillary pulmonary hypertension. Setting a threshold of P <0.05 after Bonferroni correction, an association between the A allele of *MAPK8* eQTL rs10857560 and pre-capillary pulmonary hypertension was detected among all analyzed patients (OR=13.8, nominal P=0.00036, n=238) using a logistic regression model in which age and hemoglobin genotype, two known pulmonary hypertension predictors, were included as covariates. Significant associations were not found for the categories of pulmonary hypertension and post-capillary pulmonary hypertension.

To validate the results we further tested the association of rs10857560 with pre-capillary pulmonary hypertension in an additional independent cohort. We imputed genotype for rs10857560 (imputation $r^2=0.99$) in the Walk-PHaSST cohort. With adjustment for age and hemoglobin genotype, the A allele of rs10857560 was significantly associated with pre-capillary pulmonary hypertension with consistent allelic direction as observed in the UIC cohort (OR=11.3, P= 0.0025, n=519, see Table 1 for details). The A allele of rs10857560 is the ancestral allele and had frequencies of 0.66 in the UIC cohort and 0.65 in the Walk-PHaSST cohort. This allele was associated with low *MAPK8* expression (Figure 4A). The relationship between the A allele and pre-capillary pulmonary hypertension in the combined UIC and Walk-PHaSST cohorts is depicted in Figure 4B. The homozygous AA genotype of rs10857560 was present in all 14 identified pre-capillary pulmonary hypertension cases versus 310 (42%) of the 743 SCD patients among whom pre-capillary pulmonary hypertension was not identified (P=6×10^{-6} by the Fisher exact test). Plotting the association P values of expression (Figure 4C) and pre-capillary pulmonary hypertension (Figure 4D) phenotypes in a ~1 Mb region revealed a relatively broad association peak at *MAPK8* and its proximal regulatory regions. Therefore, due to LD, it is unclear whether the causal regulatory polymorphism is located within the *MAPK8* gene or its promoter. Genetic associations between SNPs in the *MAPK8* region and pulmonary hypertension in general have not been reported to our knowledge.

Altered hypoxic gene expression in SCD patients with pre-capillary pulmonary hypertension

To confirm altered hypoxic transcriptional responses in the identified pre-capillary pulmonary hypertension cases, we selected seven SCD patients diagnosed with pre-capillary pulmonary hypertension and seven healthy African-American controls in the UIC cohort for the assessment of expression levels of 16 genes using reverse transcription (Supplemental Table 10) followed by quantitative PCR (RT-qPCR, Supplemental Table 11). The fold changes of gene expression determined by RT-qPCR highly correlated with those estimated by microarray profiling (Pearson's $r^2=0.94$), with the direction of gene expression alteration consistent between the two platforms for all 16 genes (Figure 5). In particular, we confirmed the up-regulation of several known HIF targets including *FECH*, *BNIP3L*, *PLAUR*, and *HK1*

as well as the unexpected down-regulation of HIF targets *PDK1* and *ETS1* in the PBMCs of pre-capillary pulmonary hypertension patients.

Discussion

The results of the present study indicate that the hypoxic response is a global feature of sickle cell anemia, as suggested by the strong correlation of altered gene expression profiles in hemoglobin SS subjects and *VHL^R200W^* homozygote polycythemic subjects. Over 50% of PBMC gene expression variation in hemoglobin SS patients may be related to the hypoxic response. Most importantly, the hypoxia down-regulated gene, *MAPK8,* appeared to play an important role in hypoxic gene regulation (Figure 3) and an eQTL of this gene (rs10857560) was associated with right heart catheterization-documented pre-capillary pulmonary hypertension in SCD (Figure 4).

MAPK8, also known as JNK1, is a member of the mitogen-activated protein kinase family that is involved in multiple cellular processes including proliferation, differentiation and transcriptional regulation.[36] It has a prominent role in promoting apoptosis, both through increasing the expression of pro-apototic genes in the nucleus and through facilitating pro-apoptotic pathways originating in the mitochondria.[37] In the present study, expression of *MAPK8* was down-regulated in SCD and CP where the hypoxic response is up-regulated, and the A allele of rs10857560 was associated with a further decrease in expression in SCD. Homozygosity for the A allele was present in all 14 pre-capillary pulmonary hypertension patients we examined, suggesting that the dosage effect of the A allele on *MAPK8* gene expression might contribute to the pathogenic mechanism of pre-capillary pulmonary hypertension. Abnormal proliferation of pulmonary vascular smooth muscle cells and resistance to apoptosis is a prominent feature of pulmonary arterial hypertension.[38] Therefore decreased MAPK8 pro-apoptotic activity in homozygotes for the A allele of rs10857560 is a plausible explanation for the association observed in this study.

Some other investigations are consistent with an association of decreased MAPK8 activity with pulmonary hypertension. Down-regulation of *MAPK8* may contribute to the pathogenesis of IL-6-induced pulmonary hypertension in mice.[39] Absence of *MAPK8* in experimental mice is associated with preferential differentiation of Th2 versus Th1 cells and enhanced production of Th2 cytokines,[40] and the Th2 response in mice has been associated with pulmonary arterial remodeling when other risk factors are present.[41] TGF-β signaling up-regulates MAPK8 kinase activity,[42] and genetic variants of *BMPR2* and other genes in the TGF-β signaling pathway have been reported to associate with risk of pre-capillary pulmonary hypertension. However, MAPK8 has dual roles in activating or suppressing the mitochondrial apoptosis pathway and in signaling cell survival depending on the cellular context.[43, 44] Furthermore, some studies implicate increased MAPK8 activity with pulmonary complications.[45-47]

Pre-capillary pulmonary hypertension in SCD is a complex disease to which both environmental and genetic variations contribute, as underscored by our finding that only a minority of the SCD patients with homozygosity for the *MAPK8* rs10857560 A allele were found to have pulmonary hypertension. Besides *MAPK8*, genetic variants affecting the

expression or function of other genes in key pathways may also contribute to the heterogeneity in disease risk. In the present study, a number of genes that interact with *MAPK8* (Figure 3) and inhibit apoptosis, including *atf2, map3k7, map3k4,* and *traf5,* were hypoxia down-regulated but we did not observe eQTLs in these genes. We validated the up-regulation of *SOD2* and *BNIP3L* in SCD patients with pre-capillary pulmonary hypertension, genes that may play anti-apoptosis roles by suppression of superoxide anion radical production and by repair of mitochondria damage under hypoxia conditions. Genes involved in inflammatory responses were induced in both VHL^{R200W} homozygotes and hemoglobin SS subjects, and inflammatory pathways are implicated in some forms of pulmonary hypertension.[10]

eQTL is a key method to dissect complex traits.[48] Genetic effects tend to explain a greater proportion of variation for gene expression phenotypes than for organism level phenotypes such as disease predisposition.[49] The restriction to potential local eQTL in clinical associations in the present study effectively reduced the multiple comparisons by >1000 fold (321,919 SNPs versus 126 SNPs) while prioritizing the analysis toward genetic polymorphisms having strong phenotypic effects and clear functional interpretation.

There are a number of limitations to our study. The patients with SCD were mostly African Americans whereas the Chuvash subjects were Caucasians. Only a minority of patients in the UIC and Walk-PHaSST cohorts with echocardiographic evidence of elevated systolic pulmonary artery pressure underwent right heart catheterization to determine mean pulmonary arterial pressure. Therefore the actual prevalence of pre-capillary pulmonary hypertension is no doubt higher than the observed prevalence of 14 of 757 or 1.8%. Sample size is critical in association mapping of polygenic clinical traits such as pulmonary hypertension; our study may not have detected some contributing genetic variations due to limited sample size. Furthermore, the focus on SNPs within ±100 kb potentially excluded any eQTL that acts at a distance. Nevertheless, the association of decreased expression of *MAPK8* with pre-capillary pulmonary hypertension but not post-capillary pulmonary hypertension in the present study points to the possible importance of *MAPK8* for the health of the pulmonary arterial vasculature in the setting of the high flow state and hemolytic vasculopathy that characterize SCD.

Future studies should examine the molecular role of *MAPK8* in protection from pre-capillary pulmonary hypertension in SCD, and whether interventions targeting this pathway can be developed for prevention or treatment. Genotyping for rs10857560 might also be a useful screening test for pre-capillary pulmonary hypertension in SCD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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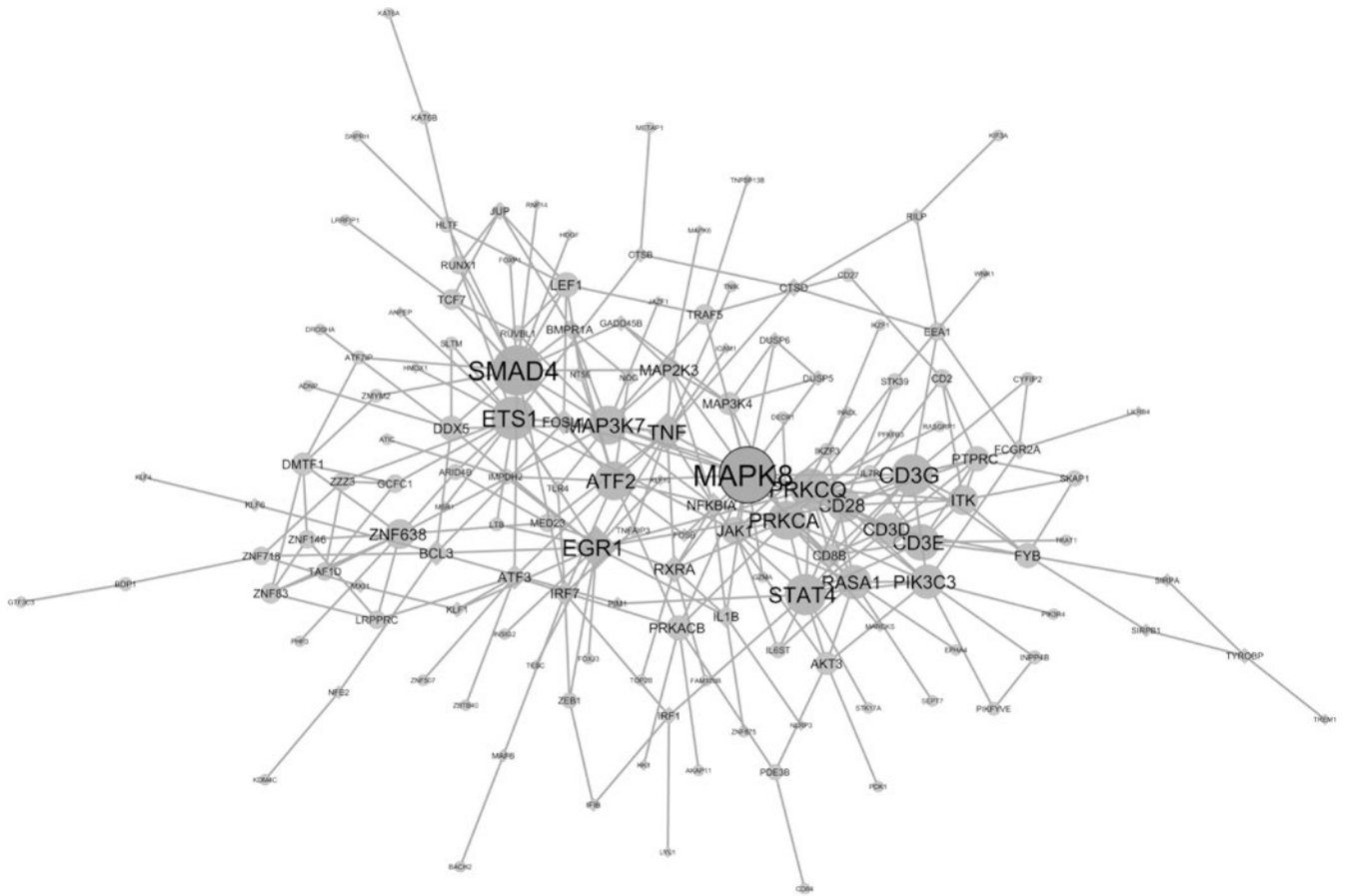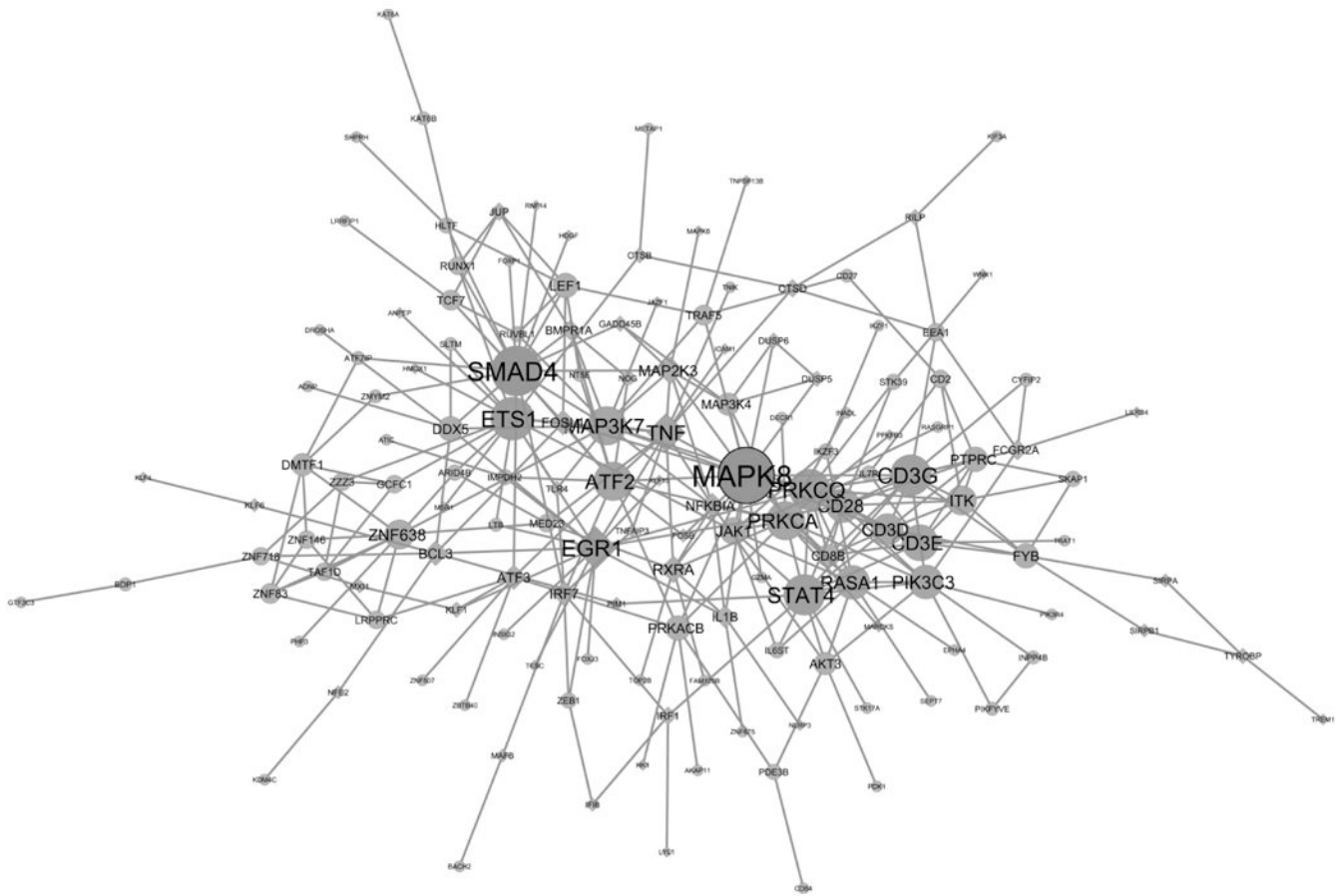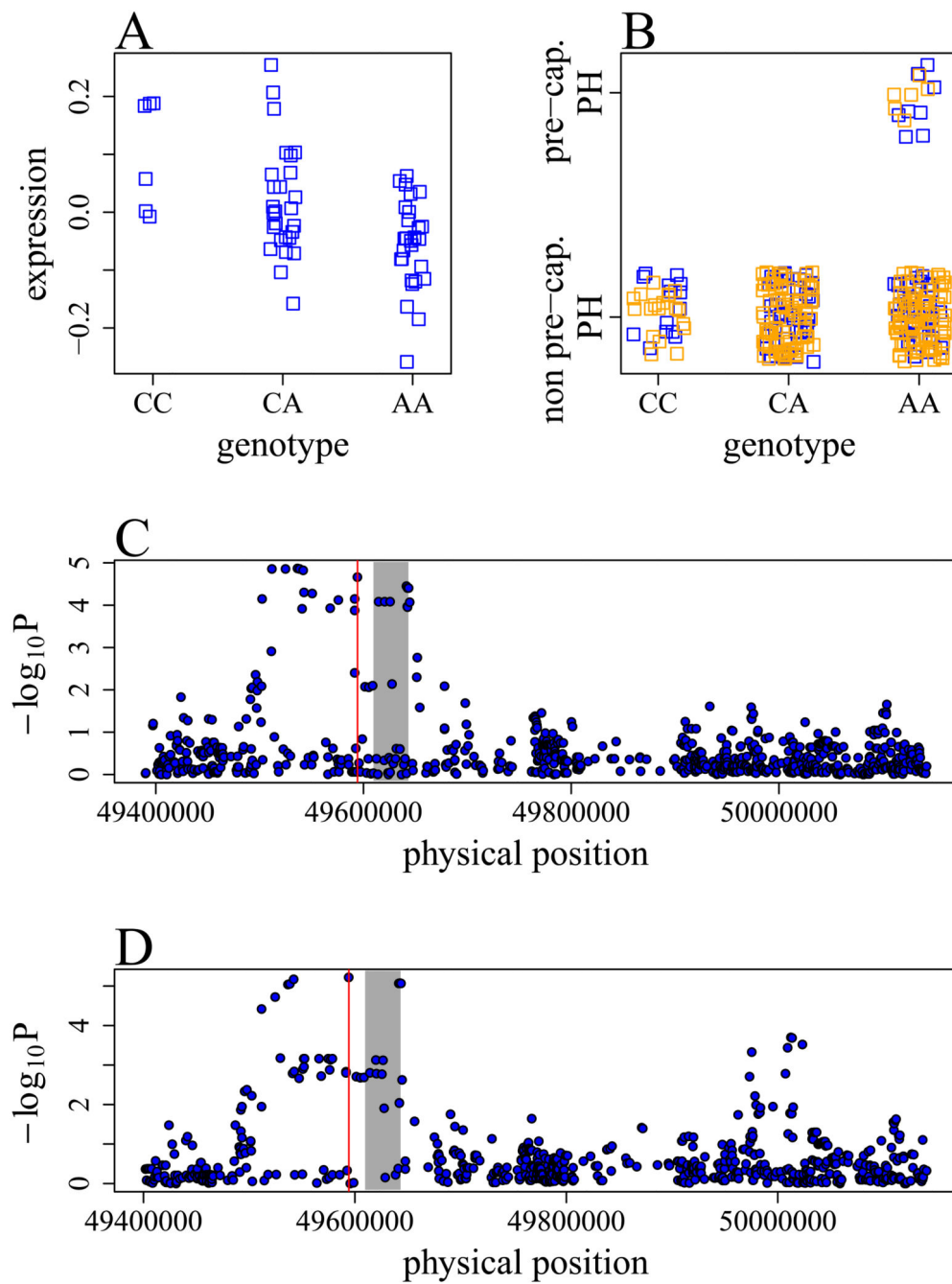
Figure 1.
The schema of the study.
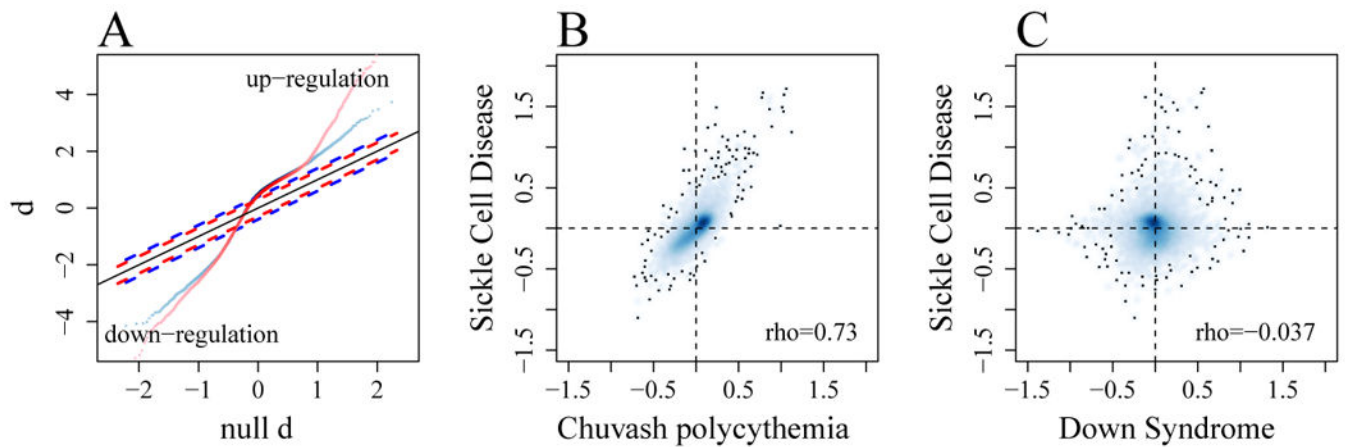
Figure 2.
Altered gene expression profiles in hemoglobin SS patients and *VHL^R200W* homozygotes
were highly correlated. (A) Q-Q plot of d-scores from real data against d-scores from 100
permutations for comparison between *VHL^R200W* and *VHL* wildtype (blue) and between
hemoglobin SS and hemoglobin AA (red). The dashed lines represented thresholds at 5%
FDR. A large number of genes showed modest up-regulation in the disease subjects, as
indicated by the overall up-shift of d-scores relative to the null distributions. (B) Correlation
of differential expression (regression coefficients of genes) in hemoglobin SS and
homozygous *VHL^R200W*. (C) Correlation of differential expression in hemoglobin SS and
Down syndrome (C). In B and C, Spearman's ρ is presented.

Figure 3.
Gene regulation network of hypoxic transcription in sickle cell anemia. Genes up-regulated are diamond-shaped and genes down-regulated ellipse-shaped. The size of the nodes is proportional to their corresponding number of interactions among the network. *MAPK8* is marked by a black border.

Figure 4.
Association of *MAPK8* expression and risk of pre-capillary pulmonary hypertension with SNP rs10857560. (A) Relative expression of *MAPK8* plotted against rs10857560 genotype in the combined Howard University and UC cohorts. (B) Pre-capillary pulmonary hypertension (pre-cap. PH) and non pre-capillary pulmonary (non pre-cap. PH) cases plotted against rs10857560 genotype in the combined UIC (blue points) and Walk-PHaSST (orange points) cohorts. (C) The -log₁₀ *P*-value of expression association plotted against chromosome position for SNPs located within *MAPK8* and its ±500 Kb regions. (D) The

combined -$\log_{10}$ P-value of pre-capillary pulmonary hypertension association of the UIC and Walk-PHaSST cohorts plotted against chromosome position for SNPs within *MAPK8* and its ±500 Kb regions. In (C) and (D) the position of rs10857560 is labeled by a red vertical line and the region of *MAPK8* is highlighted grey.
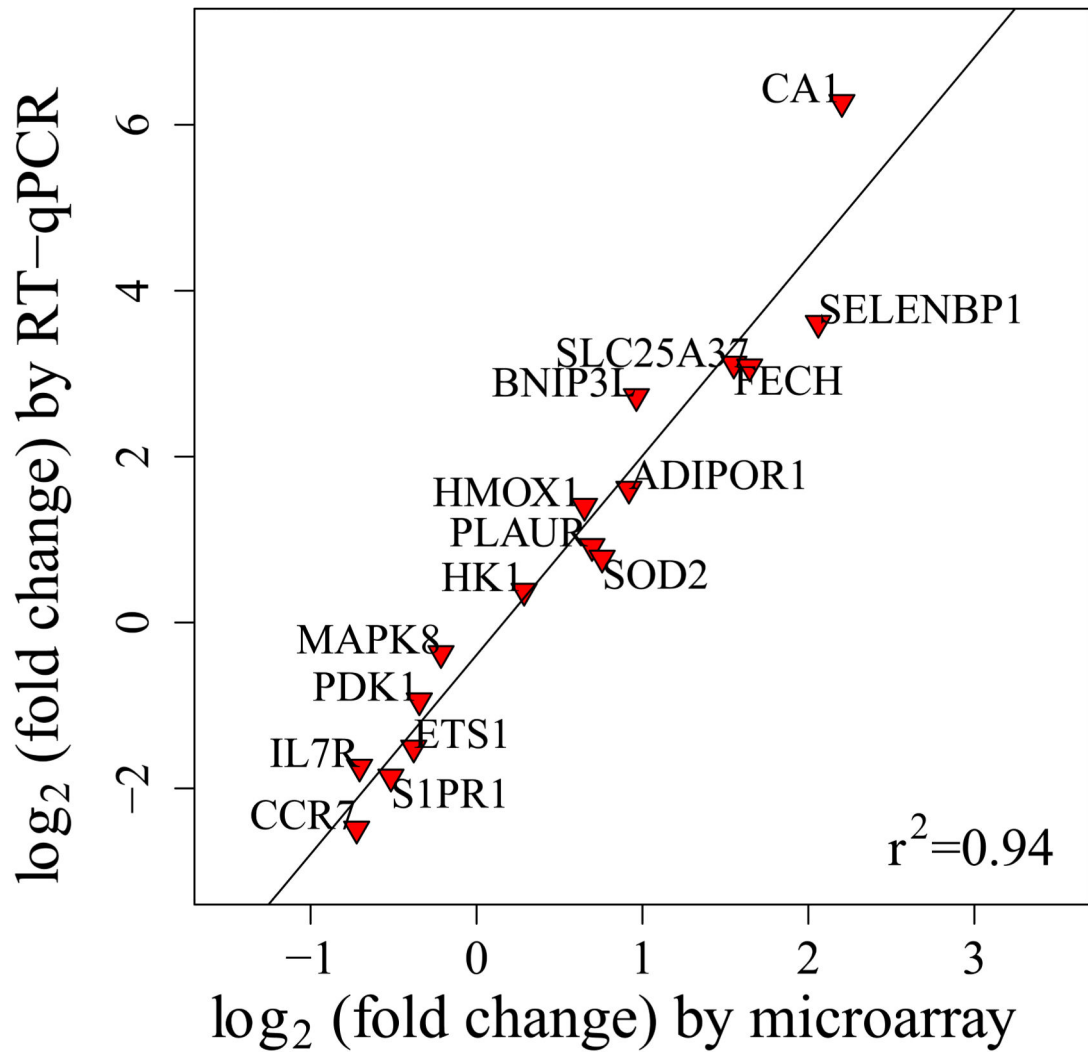
Figure 5.
Hypoxic transcriptional response in SCD patients diagnosed with pre-capillary pulmonary hypertension. The slope of the $\log_2$ fold change estimated by RT-qPCR against the $\log_2$ fold change estimated by microarray is 2.4, suggesting that fold change estimated by microarray could be conservative compared to RT-qPCR. Alternatively, hypoxic transcription could be further elevated in pre-capillary pulmonary hypertension patients.

Table 1

The association between pre-capillary pulmonary hypertension and the A allele of rs10857560 in the UIC and Walk-PHaSST SCD cohorts

Cohort	Samples analyzed	rs10857560 genotype:Non pre-capillary pulmonary hypertension			rs10857560 genotype:pre-capillary pulmonary hypertension			Armitage trend test		logistic regression	
		CC	CA	AA	CC	CA	AA	r^a	P	OR^b	P^c
UIC	All patients	28	108	94	0	0	8	0.19	0.0039	13.8	0.00036
	TRV <2.5 m/sec or patients with right heart cath^d	14	40	34	0	0	8	0.30	0.0033	17.9	0.000099
Walk-PHaSST	All patients	64	233	216	0	0	6	0.11	0.011	11.3	0.0025
	TRV <2.5 m/sec or patients with right heart cath^d	19	82	87	0	0	6	0.17	0.019	11.0	0.0052

^{a}Pearson's r;

^{b}estimated by generalized linear model using the bias-reduction method;

^{c}estimated by χ^2 test with one degree of freedom;

^{d}non pre-capillary pulmonary hypertension defined by TRV <2.5 m/sec or by right heart catheterization.