FOXO3 Regulates Fetal Hemoglobin Levels in Sickle Cell Anemia

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Sickle Cell Anemia


Fetal Hemoglobin
Variability of Endogenous HbF

Number of patients

Experience level

Ln(%HbF)
Next Generation Sequencing Methods

Genome-wide association studies (GWAS)

- Identified \textit{BCL11A} as a regulator of endogenous HbF
- BCL11A is unlikely to be a good drug target
- \textit{BCL11A} variants account for less than half of the observed variability of HbF

Whole exome sequencing (WES)

- Identifies all variants in protein coding regions
- Identifies rare variants with large effects
- Identifies causal variants
- Has not been applied to modifiers of endogenous HbF
# WES Study Population

171 pediatric sickle cell anemia patients  
HbSS  
Aged 3-18 years

<table>
<thead>
<tr>
<th>Study</th>
<th>Description</th>
<th>Participants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HUSTLE</strong></td>
<td>Hydroxyurea Study of Long-Term Effects</td>
<td>n=120</td>
</tr>
<tr>
<td><strong>SWiTCH</strong></td>
<td>Stroke with Transfusions Changing to Hydroxyurea</td>
<td>n=51</td>
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Linear Regression vs Burden Analysis

Typical Gene

Common Single Variant:Phenotype

Rare Variant Gene:Phenotype
# T2 Burden Analysis Candidates

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Number of nonsynonymous variants</th>
<th>Beta Value (\ln(%HbF))</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
<td>2</td>
<td>-1.5</td>
<td>1.5x10^{-4}</td>
</tr>
<tr>
<td>NKAIN3</td>
<td>Na/K transport</td>
<td>5</td>
<td>-0.6</td>
<td>2.7x10^{-4}</td>
</tr>
<tr>
<td>TNFRSF9</td>
<td>Tumor necrosis factor</td>
<td>5</td>
<td>0.5</td>
<td>3.9x10^{-4}</td>
</tr>
<tr>
<td>FOXO3</td>
<td>Transcriptional activator</td>
<td>7</td>
<td>-0.7</td>
<td>5.6x10^{-4}</td>
</tr>
<tr>
<td>EIF2AK1</td>
<td>Heme-regulated inhibitor kinase</td>
<td>7</td>
<td>-0.3</td>
<td>6.9x10^{-4}</td>
</tr>
</tbody>
</table>
Effect of FOXO3 Variants on 
%HbF

![Box plot showing the effect of FOXO3 variants on baseline HbF percentage]

- Wild-type: n=162
- Variant: n=9

p=0.01
Forkhead box O3

Degradation of FOXO3

Growth factors

AKT

ROS, oxidative stress

AMPK

SIRT1

Erythroid maturation

Degradation of FOXO3

Stress resistance

Longevity

Apoptosis

Cell cycle control

Brunet et al, 1999, Cell 96:857
Location of FOXO3 Variants

DNA binding domain

NLS

NES

TA

- D66N
- A140V
- D283N
- A341T
- P415L
- R548H
- S553F
FUNCTIONAL STUDIES
FOXO3 siRNA knockdown reduces HbF levels in K562 cells

P = 0.008

P = 0.0003
FOXO3 overexpression increases HbF in K562 cells
Primary Erythroid Culture

CD34+ 

Lentiviral infection 
expansion

Erythropoietin 
differentiation

Collect cells and analyze by RT-PCR and Western Blot

Day 0 
Day 5 
Day 7 
Day 14 
Day 21

Expression levels

γ-globin

β-globin
shRNA knockdown of *FOXO3* reduces HbF in primary erythroid cells

Primary erythroid cells

shRNA

P=0.0005

γ-globin/Actin fold change

Control  Scramble-shRNA  FOXO3-shRNA

kDa

90  16

γ-Globin

16

β-Globin

37

Actin

FOXO3

P=0.0001

FOXO3/Actin fold change

Scramble shRNA  FOXO3 shRNA
FOXO3 Inducing Agents May Increase HbF Levels

• AMPK activates FOXO3 through phosphorylation
• Variants in AMPK were also associated with lower HbF levels in our WES study
• Metformin, phenformin, and resveratrol increase AMPK expression levels, and may increase γ-globin through FOXO3
Resveratrol Induces $\gamma$-Globin in PEP

<table>
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<tr>
<th></th>
<th>PEP</th>
<th>DMSO</th>
<th>RES (µM)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>25</td>
<td>50</td>
</tr>
</tbody>
</table>

- **FOXO3**: 90kDa
- **$\gamma$-globin**: 16kDa
- **Actin**: 42kDa
FOXO3 Accumulates in Nucleus with Resveratrol Treatment

- **nuclear**
  - K562
  - DMSO
  - RES (μM) 10, 25, 50

- **cytoplasmic**
  - K562
  - DMSO
  - RES (μM) 10, 25, 50

Detected proteins:
- FOXO3 90 KD
- Lamin A/C 70 KD
- Tubulin 50 KD
FUTURE DIRECTIONS
Remove siblings, samples missing data, degraded samples

1000+ sickle cell patient samples for whole exome sequencing

Remove samples that fail QC

Discovery cohort n=1000

Validation cohort n=500

Identify associations with HbF by linear regression and burden analysis; verify association in validation cohort
Future Plans

- Identify variants associated with HbF
  - N=1000 discovery cohort
  - WES
- Validate associations in second cohort
  - N=500 validation cohort
- Verify associations by functional studies
  - shRNA knockdown in primary erythroid culture
- Identify $\gamma$-globin regulation pathway
- Identify drug target in pathway
- Screen drugs for HbF induction in primary erythroid culture
Future Analyses

Analyze WES data on a new cohort of 1000 SCD patients to investigate further relationships between FOXO3 and γ-globin expression.

a. Identify all non-synonymous FOXO3 gene variants that are associated with reduced HbF levels.

b. Use gene based testing and pathway analysis to determine whether variants in FOXO3 regulatory genes (AMPK, SIRT1) are associated with HbF levels.

c. Use nonbiased SNP and gene based testing to identify all variants that segregate with HbF level in the WES cohort.
Future Analyses

Determine the mechanisms by which *FOXO3* regulates γ-globin expression.

a. Analyze primary human erythroid cells by chromatin immunoprecipitation-sequencing (ChIP-seq) to determine whether FOXO3 binds the γ-globin locus or other loci that regulate HbF (*BCL11A, MYB, KLF1*).

b. Analyze primary human erythroid cells with and without FOXO3 knockdown by RNASEq to identify genes altered by FOXO3 knockdown.

c. Use Gene Set Enrichment Analysis (GSEA) to combine WES, ChIP-Seq and RNASEq analyses.
Conclusions

• Burden analysis of WES data identified seven FOXO3 variants associated with lower endogenous HbF in pediatric sickle cell patients.
• In K562 cells and primary erythroid cells, knockdown of FOXO3 reduced γ-globin levels.
• Overexpression of FOXO3 increased γ-globin levels.
• FOXO3 may be a viable drug target.
• Further work is needed to elucidate the role of FOXO3 in γ-globin regulation.
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