



Predicting Variability in a Monogenetic Inherited Disease

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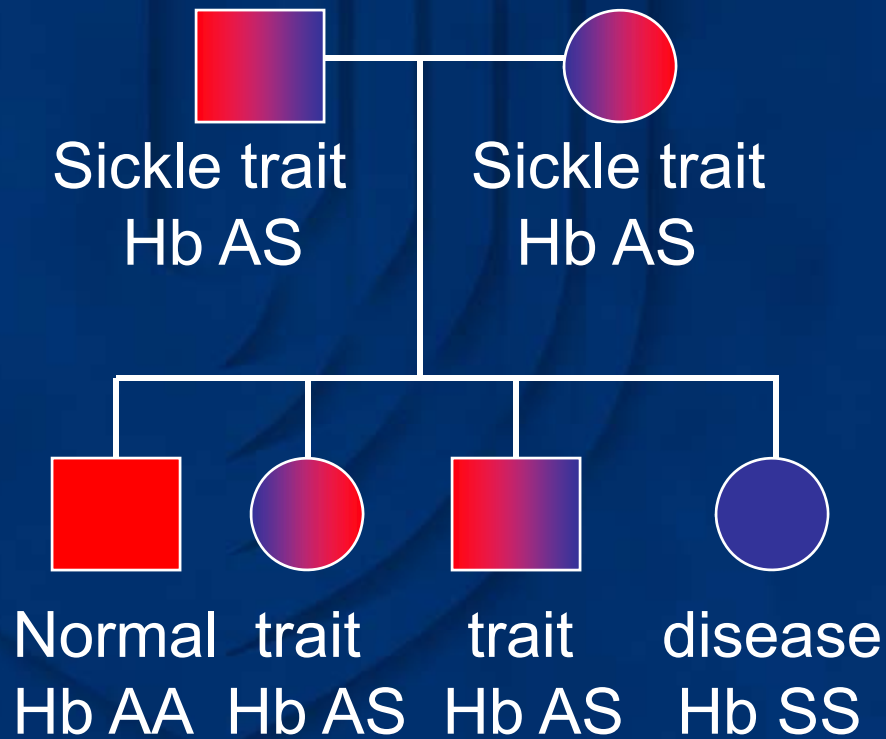
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What Causes Sickle Cell Disease?

- Sickle hemoglobin (Hb S) has one amino acid different from normal hemoglobin (Hb A).
- It has a valine instead of a glutamic acid in position 6 of the β globin chain.
- Everyone with homozygous sickle cell disease (SCD) has this same mutation.
- Hemoglobin with this mutation tends to polymerize into elongated chains when deoxygenated.
- These chains are stiff and deform the red cell from its normal shape.

Sickle Cell Disease: A Recessive Disorder



QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

8% of American blacks and up to 30% of people in some parts of Africa have Hb AS. 1 in 625 newborns in the US have SCD. About 100,000 Americans have SCD. Millions have it worldwide.

The Changing Spectrum of Sickle Cell Disease

- **During the last several decades, the outlook for children born with homozygous hemoglobin S (Hb SS) sickle cell disease (SCD) has improved greatly.**
- **Over 90% of children born with Hb SS now live to adulthood, whereas 30 years ago one-half of individuals in the U.S. with SCD died before age 20.**

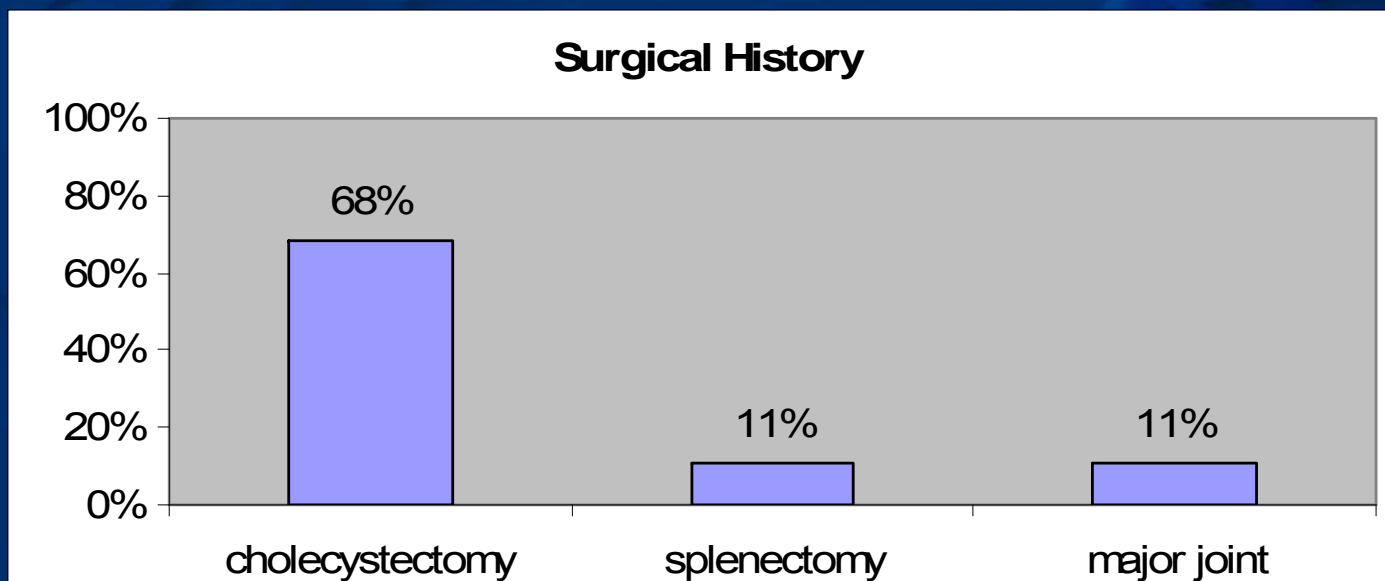
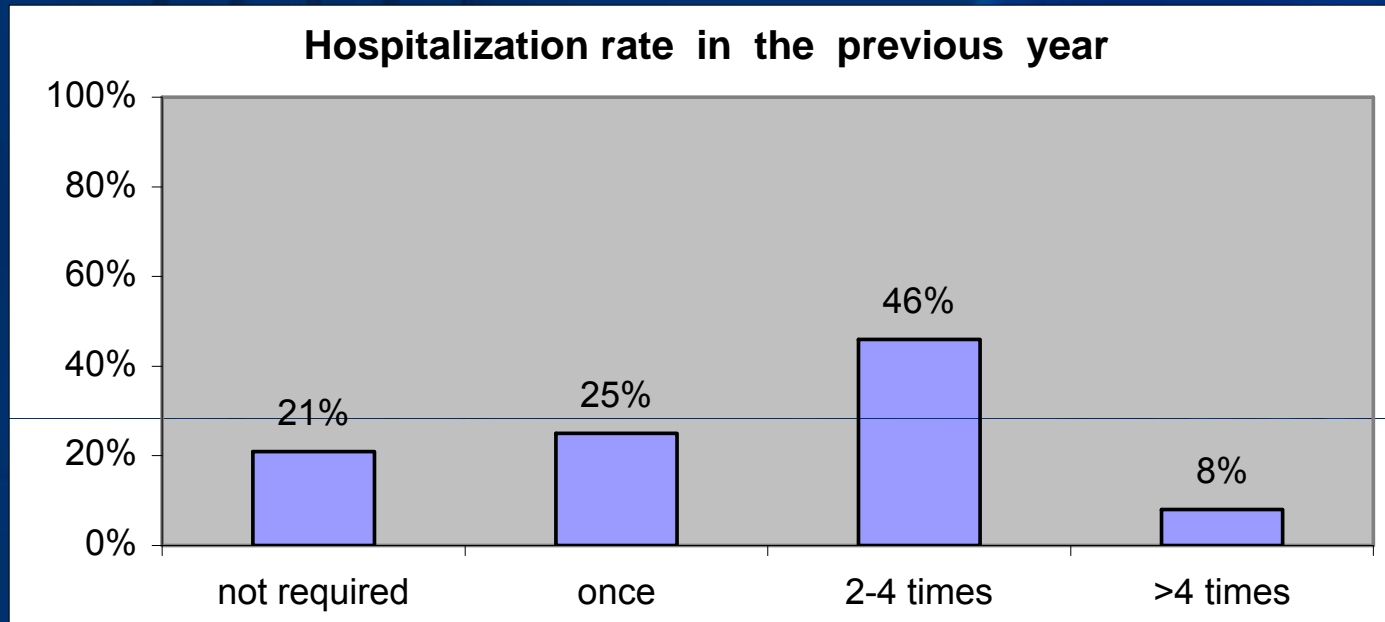
Clinical Picture of Sickle Cell Disease

- Shortened RBC survival and hemolysis
 - Hemolytic Anemia
 - Pulmonary hypertension and other disorders possibly related to nitric oxide bioavailability
 - Cardiac remodeling and diastolic dysfunction related to anemia
 - Gallstones
- Vaso-occlusion
 - Pain: severe acute painful episodes
 - Acute and chronic organ damage
 - Sickle cell nephropathy and renal failure
 - Stroke
 - Acute chest syndrome
 - Priapism
 - Osteonecrosis
 - Skin ulcers

Frequency of Clinical Outcomes in Adults with SCD

| | |
|----------------------------------|------------|
| Acute chest syndrome | 76% |
| Priapism (males only) | 41% |
| Stroke | 16% |
| Transient Ischemic Attack | 5% |
| Seizure | 12% |
| AVN of shoulders or hips | 30% |
| Leg ulcers | 26% |
| Heart failure | 7% |
| Gallstones | 66% |
| Retinopathy | 22% |

Hospitalizations and Surgery



Identifying Risk Factors

- Who will have more or less severe disease?
- Who will develop specific types of end-organ complications, such as kidney failure, stroke, pulmonary hypertension, or cardiac failure?
- Who needs early intervention?
- Will certain interventions be more useful in certain populations?

Benefits of Identifying Risk Factors

- Identify who needs to undergo early screening for end-organ damage.
- Identify who needs prophylactic therapy.
- Identify patients for whom aggressive but riskier therapy (e.g. bone marrow transplantation) might be appropriate.
- By identifying “risk genes,” identify new targets for therapeutic intervention.
- Can we tailor treatment to an individual patients risks?

Outcome Modifying Genes in SCD

- Goal: To identify genetic polymorphisms associated with risk for specific clinical outcomes in SCD.
- Methods
- Enroll > 600 patients from three large sickle cell centers (Duke, UNC, Emory)
- Create a database and sample repository containing detailed phenotypic (clinical) information and both DNA and plasma samples
- Type for over 400 SNPs in > 60 candidate genes
- Perform statistical tests for association with specific clinical outcomes

Candidate Gene Approach

- Genes known to be in pathways thought important to SCD pathophysiology
 - Adhesion
 - Inflammation
 - Coagulation
 - Signal Transduction
 - Cytokine receptors
 - Transporters & ion channels
- Genes known to be important to diseases of the same organ system or type
 - Nitric oxide
 - TGF β pathway (primary pulmonary hypertension)
- We have examined >350 SNPs in > 50 genes.

Definition of Clinical Outcomes

- Priapism
- Pulmonary Hypertension
- Sickle cell nephropathy
- Overall disease severity
- Pain (episodes/year and narcotic usage)
- Left ventricular dysfunction and heart failure
- History of stroke and other CNS events (seizure, TIA)
- Leg ulcers
- Avascular necrosis

Identifying Risk Genes for Priapism

- Priapism is a prolonged, unwanted and usually painful erection of the penis which is not relieved by orgasm or ejaculation.
- Incidence variable in different studies, most often 30-45%, but possibly as high as 89% lifetime risk.
- Priapism can affect psychosocial and psychosexual development in children, as well as sexual function and potency during adult life.

Study Population

- 199 unrelated, adult (>18 years) male patients with Hb SS and Hb S β^0 -thalassemia.
- 83 (42%) reported a history of priapism.
- This is consistent with previous reports of prevalence in other studies.

Methodologic Approach

- We initially analyzed 297 single nucleotide polymorphisms (SNPs) in 49 candidate genes.
- Most of the genes we examined are involved in adhesion, signaling, transport, or coagulation.
- Additionally, we examined genes involved in nitric oxide (NO) biology (*NOS2*, *NOS3*, *ARG2*), since NO may promote vasodilation and reduce inflammation and the adhesive characteristics of endothelium.
- For each SNP, contingency tables and tests of association were constructed for the genotypes as well as for the alleles by the occurrence of priapism.

Results

| ITGAV - rs3768780 | | | |
|-------------------|------------|------------|------------|
| | Priapism | | Row Total |
| Genotype | N | Y | |
| AA | 27 0.73 | 10 0.27 | 37 1.00 |
| AG | 52 0.64 | 29 0.36 | 81 1.00 |
| GG | 35 0.45 | 43 0.55 | 78 1.00 |
| Col Total | 114 | 82 | 196 |

The ITGAV gene encodes an endothelial cell membrane protein (αV integrin) that serves as the primary receptor for adhesion of sickle red cells.

In our sample, 27% of individuals having the AA genotype for the *ITGAV* SNP had priapism, compared with 36% of individuals with the AG genotype and 55% of individuals with the GG genotype. The odds of developing priapism were therefore increased 1.9 times for each additional G allele in the *ITGAV* SNP, with a confidence interval of 1.3 to 2.9.

Results

| AQP1 Grs10244884 | | | |
|------------------|----------|------|-----------|
| | Priapism | | Row Total |
| Genotype | N | Y | |
| TT | 26 | 10 | 36 |
| | 0.72 | 0.28 | 1.00 |
| CT | 59 | 33 | 92 |
| | 0.64 | 0.36 | 1.00 |
| CC | 27 | 39 | 66 |
| | 0.41 | 0.59 | 1.00 |
| Col Total | 114 | 82 | 194 |

AQP1 encodes the water transporter of red cells. It helps keep the red cell hydrated.

28% of individuals having the *TT* genotype for the *AQP1* SNP had priapism, compared with 36% of individuals with the *CT* genotype and 59% of individuals with the *CC* genotype. The odds of developing priapism were therefore approx. 2 times larger for each additional C allele in the *AQP1* SNP, with a confidence interval ranging from 1.3 to 3.2.

Results

| TGFB3 Grs7526590 | | | |
|------------------|------------|-------------------|-------------|
| Genotype | Priapism | | Row Total |
| | N | Y | |
| AA | 88 0.67 | 44 0.33 | 132 1.00 |
| AT | 22 0.41 | 32 0.59 | 54 1.00 |
| TT | 1 0.25 | 3 0.75 | 4 1.00 |
| Col Total | 111 | 79 | 194 |

TGFB3 encodes one of the three types of TGF β receptors and is strongly expressed by endothelial cells. It is critical to endothelial cell migration and transformation (Brown et al, 1999).

The odds of developing priapism were 2.8 times larger for each additional T allele in the TGFB3 SNP. The confidence interval for this odds ratio ranges from 1.6 to 5.1. TGF β signaling may promote the occurrence of priapism through changes in vascular endothelium. Other genes in the TGF β pathway are associated with other outcomes of SCD (Sebastiani et al, 2005; Nolan et al, 2006).

What we have learned about priapism:

- Some genes--such as ITGAV-- that we hypothesized were involved in SCD pathophysiology do indeed appear to be involved in SCD-related priapism.
- Identification of other genes, such as AQP1, give rise to new hypotheses as to what mechanisms are involved in priapism.
- Hopefully, this work will translate into an ability both to identify at-risk patients and to offer them preventive therapies, possibly eventually tailored to their genotype.

Pulmonary Hypertension

- Up to 30% of adult patients with SCD will develop pulmonary hypertension (pHTN).
- Median survival for patients with pHTN was only 25.6 months, vs over 70% survival at the end of 119 months for patients without pHTN. Castro et al 2003
- In our study population, 21% of adult patients with pHTN died within three years, compared with only 4% of the patients without pHTN.

Definition of Clinical Endpoint

- 115 patients underwent echocardiography at Duke or UNC.
- Echoes were classified as c/w pHTN (TRjet ≥ 2.5 m/s), normal, or abnormal but w/out pHTN.
- Analyses were done comparing pHTN to all others, as well as pHTN to normal only.

Individuals With and Without pHTN

| | Patients with pHTN (n=44) | Patients without pHTN | | |
|--------------------------------|------------------------------|-----------------------|--------------------|---------------------------------|
| | | All (n=67) | Normal Echo (n=29) | Left Heart Abnormalities (n=38) |
| Mean Age* (std dev) | 41.09 (12.09) | 32.69 (11.86) | 31.24 (9.23) | 33.79 (13.54) |
| Female:Male Ratio | 1.2 | 1.03 | 1.07 | 1.0 |
| Mean Hgb in g/dL* (std dev) | 7.72 (1.35) | 8.53 (1.49) | 8.56 (1.53) | 8.51 (1.48) |
| Currently on HU Treatment | 53.5% | 54.8% | 62.9% | 48.6% |

***Mean Age was significantly different in patients with pHTN compared to patients without pHTN.**

Nominally Significant SNPs

| Gene | SNP | Fisher's Exact p-value |
|---------------|-------------------|------------------------|
| ACE | rs4317 | 0.02015 |
| ACVRL1 | rs3759178 | 0.01663 |
| ACVRL1 | rs3847859 | 0.00259 |
| ACVRL1 | rs706814 | 0.00978 |
| ADCY6 | rs3730070 | 0.04636 |
| ADRB1 | rs1801253 | 0.00874 |
| BMP6 | rs267192 | 0.00634 |
| BMP6 | rs267196 | 0.00429 |
| BMP6 | rs267201 | 0.01540 |
| BMPR2 | rs17199249 | 0.01051 |
| BMPR2 | <u>rs35711585</u> | 0.01781 |
| CD36 | rs1527479 | 0.03443 |
| CR1 | rs6663530 | 0.02002 |
| F13A1 | <u>rs4960166</u> | 0.00737 |
| FY | rs3027045 | 0.01469 |
| KL | rs1888057 | 0.04323 |
| LCAT | rs5923 | 0.04757 |
| LCAT | hcv2846928 | 0.02203 |
| LCAT | rs7200210 | 0.02464 |
| LOC255411 | rs3729972 | 0.00341 |
| LTA4H | rs10492226 | 0.01894 |
| NOS3 | rs1800780 | 0.04783 |
| SELP | rs2235302 | 0.00050 |
| SERPINC1 | rs2227617 | 0.01730 |
| TGFBR3 | rs10874940 | 0.01205 |
| TGFBR3 | rs284176 | 0.03985 |
| TGFBR3 | rs7526590 | 0.01099 |

Odds Ratios for Multiple Logistic Model for Risk of pHTN*

| Effect | Comparison | Odds Ratio Estimate | Lower CLM | Upper CLM | Likelihood Ratio p-value | FDR p-value |
|--------------------------|------------|---------------------|-----------|-----------|--------------------------|-------------|
| <i>ADRB1</i> rs1801253 | CC vs. GG | 13.7 | 2.2 | 86.0 | 0.00051 | 0.07573 |
| | CG vs. GG | 1.6 | 0.3 | 8.9 | | |
| <i>ACVRL1</i> rs3847859 | AA vs. GG | 10.7 | 1.3 | 87.7 | 0.00498 | 0.24651 |
| | AG vs. GG | 10.6 | 1.7 | 64.9 | | |
| <i>ACVRL1</i> rs706814 | TT vs. AA | 14.4 | 1.4 | 147.4 | 0.00047 | 0.07573 |
| | AT vs. AA | 8.4 | 1.8 | 39.8 | | |
| <i>BMP6</i> rs267192 | TT vs. CC | 2.4 | 0.2 | 24.0 | 0.00094 | 0.09306 |
| | CT vs. CC | 12.9 | 2.5 | 67.1 | | |
| <i>TGFBR3</i> rs10874940 | TT vs. CC | 46.1 | 2.4 | 872.6 | 0.00203 | 0.12058 |
| | CT vs. CC | 4.7 | 1.1 | 20.1 | | |

* Patients with pHTN compared to all other patients, controlling for age, hemoglobin and all other SNPs in model.

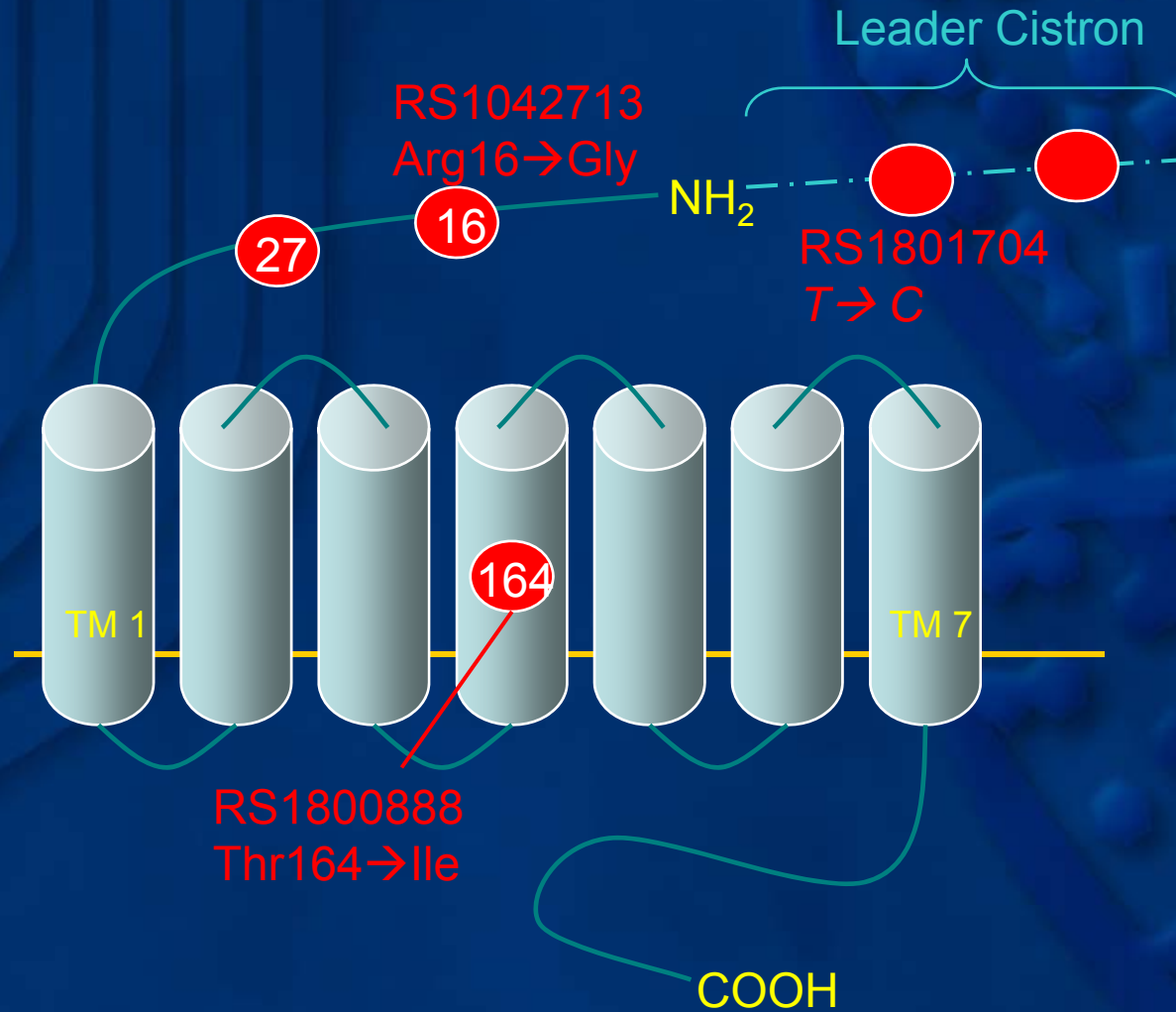
Pathways to pHTN

- In summary, we identified several genetic variants that are associated with the occurrence of pHTN in patients with SCD.
- A model including age, genetic variation in *ADRB1* and genes in the TGF β pathway predicted risk for pHTN in our study population.
- Further evaluation of this model in a prospectively studied cohort of SCD patients is warranted.
- Our findings suggest new targets that might be useful for developing therapies that might reduce the occurrence of pHTN or ameliorate its prognosis.

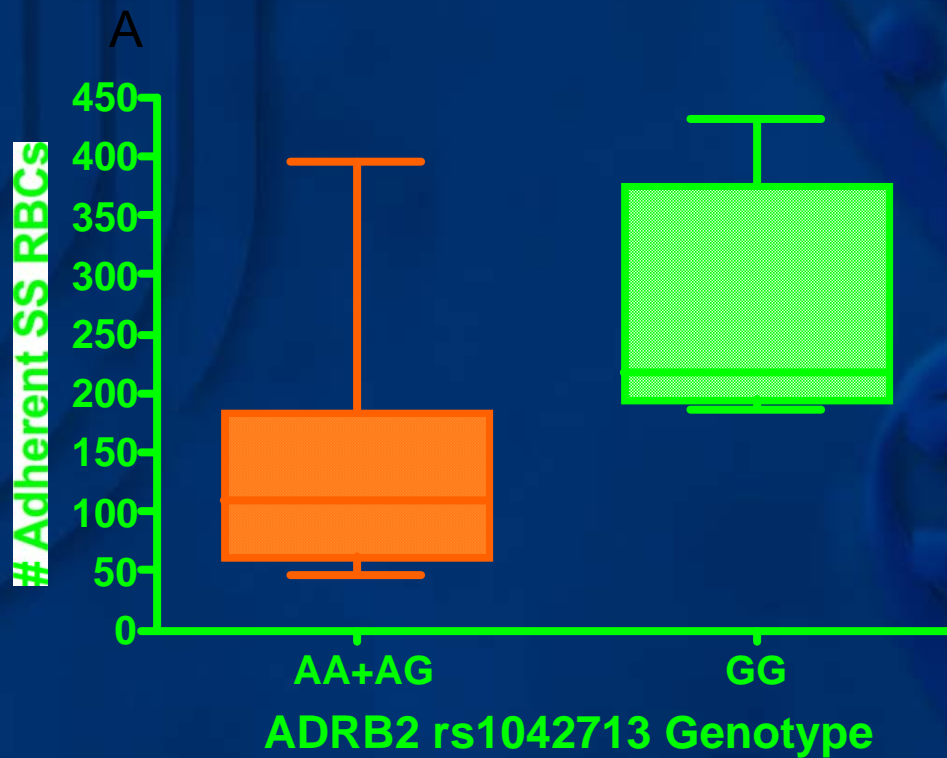
Genetics of Red Cell Adhesion

- Single nucleotide polymorphisms (SNPs) of the β_2 adrenergic receptor gene (ADRB2) have been implicated in clinical variability of several cardiopulmonary disorders.
- Polymorphisms of ADRB2 correlate with the incidence of stroke in sickle cell disease (SCD).
- Stimulation of sickle red cell (SS RBC) adrenergic receptors by epinephrine, which in turn leads to stimulation of adenylate cyclase, activates the B-CAM/LU laminin receptor [Hines et al. 2003].
- We have also shown that activation of B-CAM/LU on SS RBCs occurs in vivo [Zen et al. 2004].
- SS RBCs from different individuals show variable baseline adhesion to laminin, as well as variable responsiveness to stimulation by epinephrine.

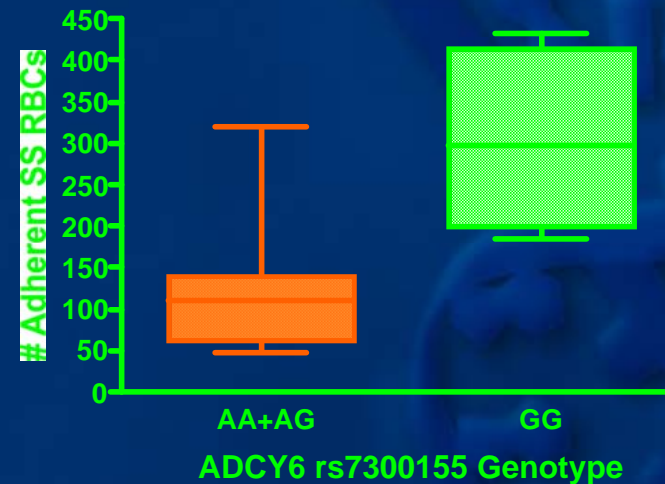
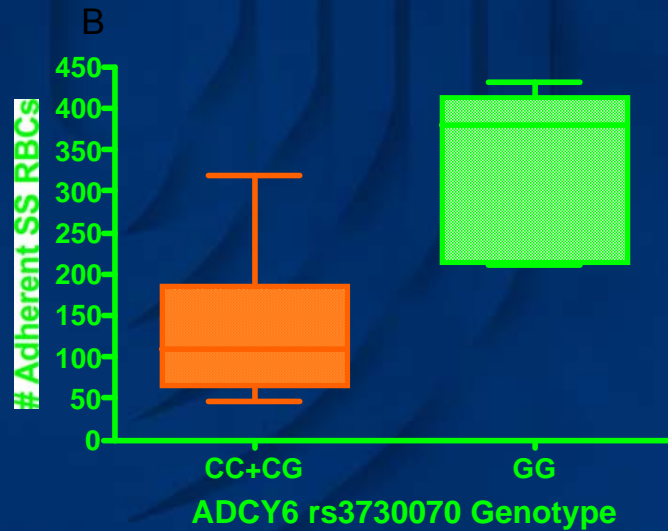
Polymorphisms of ADRB2



Effect of ADRB2 SNP on Adhesion to Laminin



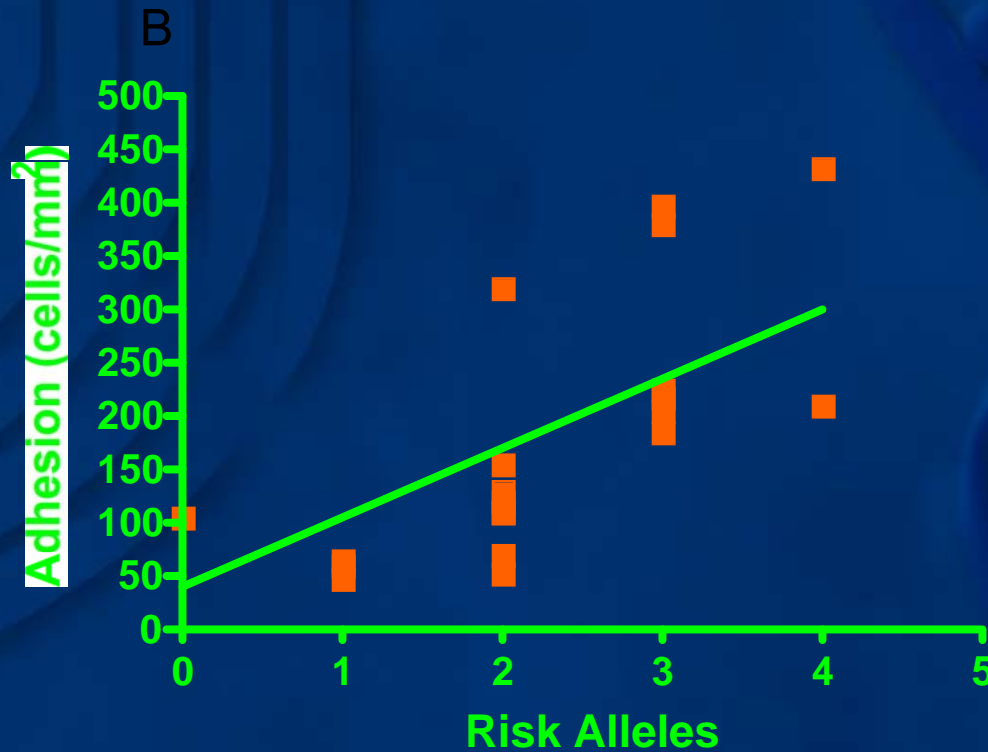
Effect of ADCY6 SNPs on Adhesion to Laminin



SS RBC from patients who were homozygous for either the G allele at SNP rs1042713 of *ADRB2* or the G allele at SNP rs3730070 of *ADCY6* demonstrated significantly increased adhesion to laminin compared to red cells from all other patients studied [$p=0.0002$].



Least squares line for regression of adhesion on the number of G alleles in either *ADRB2* or *ADCY6* ($p < .0001$, $r^2 = .77$) also showed a relationship between the number of “risk” alleles and the degree of adhesion observed.



Activation of Adhesion

- We have recently shown that the B-CAM/LU laminin receptor undergoes activation in response to epinephrine in about 50% of patients.
- Activation also appears to occur *in vivo*, in that some patients' cells appear to be reversibly activated.
- We have also recently shown that epinephrine activation of SS RBCs can lead to vaso-occlusion in a mouse model.

Adhesion, the Stress Response, and Vaso-Occlusion

- The vaso-occlusive crises of SCD have often been associated with stress.
- It is of great interest to identify genetic factors that may lead to variability in individual patients' physiologic responses to stress and their risk of developing vaso-occlusive events.
- We hypothesize that polymorphisms in the signaling processes involved in adhesion may contribute to disease variability .
- Genotype may also help predict the usefulness in individual patients of future anti-adhesive therapy aimed at preventing or reversing activation of adhesion receptors.

Generalizable Conclusions

- Frequency of genetic polymorphisms varies among racial and ethnic groups.
- Genetic polymorphisms may confer different risks in different populations due to either environmental or genetic co-variables.
- Identification of risk alleles may help identify patients who require earlier, more aggressive, or differently targeted treatment.
- Identification of risk alleles can help lead to the discovery of new therapeutic modalities.

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