PROJECT TITLE: Phenotypic and genotypic evaluation of a large Manitoban kindred with hereditary xerocytosis

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SUMMARY:
The hereditary stomatocytoses are a group of heterogeneous conditions associated with chronic red cell haemolysis for which the causative genetic mutations are not known. We investigated 137 members of a large Manitoban kindred with phenotypic findings consistent with hereditary xerocytosis. The objectives of this study were to systematically characterize the disease phenotype and to define the chromosomal region carrying the disease locus. The mode of inheritance was autosomal dominant. Affected family members were found to have well-compensated haemolysis, associated with an elevated MCHC, decreased osmotic fragility, decreased haptoglobin, and increased indirect hyperbilirubinemia. Cholelithiasis and progressive iron loading were common, despite normal haemoglobin levels. Quantitative erythrocyte morphologic evaluation revealed increased schistocytes, target cells, reticulocytes, and eccentrocytes in affected individuals; stomatocytes were however not increased. Using DNA linkage analysis, we confirmed the localization of the disease phenotype to chromosome 16q, and we refined the candidate region to 16q24.2 – 16qter, a 2.4 million base pair interval containing 51 known or predicted genes. Exome sequencing, and subsequent bioinformatic analysis identified a single gene mutation within a red cell membrane mechanosensitve ion channel that was present in all affected family members, but in no unaffected individual. Functional studies are necessary to clarify the influence of the identified mutation with regard to erythrocyte structure and function.

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INTRODUCTION

The hereditary stomatocytoses are a rare, diverse, group of clinical conditions associated with chronic haemolysis and an increase in erythrocyte membrane permeability to monovalent cations.[1,2] In the absence of defined causative mutations, sub-classification of hereditary stomatocytoses has been based on phenotypic parameters including clinical characteristics, red cell osmotic fragility and temperature-dependent cation leak.[3,4] The rarity of these conditions and the variability in clinical presentation can lead to delay in diagnosis or misdiagnosis, with hereditary stomatocytosis sometimes being confused for the much more common hereditary spherocytosis.

The most common form of hereditary stomatocytosis is hereditary xerocytosis (OMIM 194380; also called dehydrated hereditary stomatocytosis; DHS), first reported by Miller *et al.* in 1971.[5] Hereditary xerocytosis (HX) is a clinically mild, autosomal dominant condition characterized by well compensated haemolysis, macrocytosis, reticulocytosis, intermittent jaundice and a propensity to iron overload.[6,7] In some kindreds, hereditary xerocytosis has been associated with perinatal fluid effusions, pseudohyperkalemia, or both.[8-10] While there have been recent advances in the genetics of other subtypes of hereditary stomatocytoses,[11,12] the relevant gene and causative mutations responsible for hereditary xerocytosis remain unknown. Previous family studies have linked the disease phenotype to markers on chromosome 16q,[10,13,14] but also to chromosome 2p.[15]

In this report, we describe a large Manitoban kindred with haemolysis associated with decreased red cell osmotic fragility and indices consistent with hereditary xerocytosis. The objectives of this study were to systematically characterize the clinical features of the haemolytic process and to define the chromosomal region linked to the disease phenotype.

HYPOTHESIS

An inherited defect, likely affecting an ion transport protein, is responsible for haemolysis in members of a large Manitoban kindred.

OBJECTIVES

Specific objectives

1. Describe the pattern of inheritance for this haemolytic process within the kindred.
2. Characterize and compare the clinical features and biochemical abnormalities among affected and unaffected family members.
3. Conduct DNA linkage analysis to confirm that the causative gene is located on chromosome 16, and if so, to undertake candidate gene analysis to identify the underlying mutation responsible for the haemolytic process.

MATERIALS AND METHODS

Study Protocol

Prior to commencement of this research, we drafted a study protocol which detailed all methods and analytic techniques to be employed during the conduct of this study. This included the identification of the study population, development of the pedigree, specific haematologic testing and laboratory procedures, and methods related to DNA linkage analysis. Methods were also devised to quantitate erythrocyte peripheral blood morphology. A complete time-line for all study components is outlined in Table I.

Application for funding

With input from the investigative team, an application for funding was drafted by myself, revised, and submitted to the Manitoba Medical Services Foundation (MMSF). Funding was awarded in December 2010 ($20,000) and was used to offset the costs of the laboratory testing and genetic linkage analysis.

Study participants

We drafted and submitted an ethics application which was approved by the Health Research Ethics Board of the University of Manitoba (Winnipeg, Canada). In collaboration with the index case, we organized a family reunion in St. Lazare, Manitoba. Family members (n=137) from a single kindred were enrolled in this study; informed consent was obtained from all participants.

Development of the pedigree

With assistance from the proposita, we constructed a preliminary pedigree using the Cyrillic program (version 2.0, Oxfordshire, UK). Pedigree development continued as an iterative process, and was confirmed, revised and expanded with information received during interviews with family members.

Characterization of the clinical phenotype

A brief medical history was elicited from each participant or parent, including signs, symptoms and potential consequences of haemolytic anaemia, organ dysfunction or complications related to iron overload. A limited physical examination was performed to assess the presence of splenomegaly and to establish whether each participant had undergone prior abdominal surgery. To further characterize the haemolytic process and to determine whether this disorder was consistent with previously described cases of hereditary xerocytosis, laboratory investigations were conducted. All tests were conducted at the Health Sciences Center, Winnipeg, Canada.

Haematological analysis

Five ml of blood were collected in ethylenediaminetetraacetic acid (EDTA) from each participating family member, and stored at 4°C prior to testing. A complete blood count, including a 5-part white cell differential and reticulocyte count, was performed on all participants using a Sysmex XE-2100 (Sysmex Corporation, Kobe, Japan) automated haematology analyzer. Peripheral blood films were prepared at the time of collection, air-dried and stained using a

Sakura Manual Slide Stainer. Osmotic fragility testing was performed on 10 affected family members according to standard operating protocols.[16,17]

Red cell morphology was quantitatively evaluated by two independent observers. Following an initial training exercise where the observers agreed upon reference red cell morphology,[18,19] a calibration exercise was performed. The trained observers, blinded to the other's responses and to identifying case data, adjudicated the red cell morphology in 5 high power fields from 10 enrolled participants. Agreement (for the presence of stomatocytes, target cells, schistocytes and eccentrocytes) between observers was determined using the kappa statistic.[20] 'Almost perfect'[20] inter-observer agreement allowed the remaining slides to be adjudicated by a single observer.

Biochemical analysis

Five ml of blood were obtained from each participant, and stored at room temperature prior to testing. Bilirubin (total and direct), haptoglobin, alanine aminotransferase (ALT) activity, and ferritin measurements were measured using a Cobas 600 analyzer (Roche, Basel, Switzerland). Plasma haemoglobin concentration was measured by scanning spectrophotometry with a Cary 50 Bio UV-visible spectrophotometer (Varian, Palo Alto, California).

To rule out glycolytic enzyme deficiencies[21] as a potential cause of anaemia, hexokinase, glucose-6-phosphate isomerase, phosphofructokinase, phosphoglycerate kinase, pyruvate kinase, glucose-6-phosphate dehydrogenase, adenylate kinase and adenosine deaminase activities were measured in two affected individuals.

Microsatellite analysis and linkage determinations

Based on the clinically determined phenotype, 51 participants (23 affected individuals) were genotyped for the polymorphic microsatellite markers *D16S2624*, *D16S3073*, *D16S539*, *D16S3074*, *D16S2621* and *D16S3026* that map within the 16q22.2-16q24.3 interval. Genomic DNA was isolated from the buffy coat of a peripheral blood sample drawn into EDTA. Approximately 100ng of DNA was amplified by polymerase chain reaction (PCR) in the presence of marker-specific ^{32}P end-labeled forward primer and unlabeled reverse primer in a reaction mixture detailed elsewhere.[22] Reactions were terminated by the addition of stop buffer. Each sample was boiled for 5 min and placed on ice. Products were separated on 6% polyacrylamide gels containing urea at 69W for 70-150 min depending on the expected size of the amplified products.[22] Gels were transferred to filter paper, dried under vacuum at 72°C for 80 min and exposed to Biomax-XR (Kodak) film for 3-12 hr at room temperature.

Linkage data was generated from both paternal and maternal meioses. Lods (logarithm (base 10) of odds) were determined at assumed recombination fractions (theta) 0.00, 0.05, 0.10, 0.20, 0.30 and 0.40.[23] Peak lods and peak recombination fractions were determined as described by Conneally et al.[24] Continuous allelic alignments were generated manually.

Statistical Analysis

The following descriptive statistics were generated: frequency analysis (percentages) for categorical variables and means (and standard deviations [SDs]) or medians (and interquartile ranges [IQRs]) for continuous variables. Between-group comparisons were assessed using the

Student's t-test or the Wilcoxon rank-sum test for continuous variables, or the chi-square test for categorical variables. All statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC). The confidence limits and p values reported reflect an alpha level of 0.05.

RESULTS

Clinical phenotype

The complete family pedigree captured 237 individuals spanning five generations; Figure I depicts a partial pedigree with only the affected family units. Blood samples were obtained from 111 related individuals and 26 genetically unrelated spouses. The average age of the study participants was 29 years (range 8 months to 76 years); 48% were male.

The phenotypic classification of affected and unaffected individuals was based on the bimodal and non-overlapping distribution of reticulocyte counts between the two groups (Figure II). The mean percent reticulocyte count of unaffected subjects (related family members and unrelated spouses) was 1.1% (± 0.4%, range 0.5-2.3%). Affected individuals had a mean percent reticulocyte count of 9.7% (± 2.6%, range 5.3-14.6%). Based on these determinations, the haemolytic process appeared to be inherited in an autosomal dominant fashion, with complete penetrance (Figure I).

Histories of anaemia (46% vs. 8%), jaundice (45% vs. 4%), red or brown urine (45% vs. 1%), red cell transfusion (17% vs. 1%), and either gallstones or cholecystectomy (41% vs. 4%) were significantly ($p < 0.01$) more prevalent in affected than unaffected individuals (Table II). There was no association between the haemolytic phenotype and a history of neuromuscular, cardiovascular, pulmonary, renal, hepatic, or endocrine disorders. Despite a mean percent reticulocyte count of 9.7%, affected individuals were not anaemic and their haemoglobin concentrations were not statistically different from unaffected individuals (135 ± 12 g/L vs. 138 ± 14 g/L, $p = 0.26$). Consistent with haemolysis, affected family members had significantly elevated indirect bilirubin levels (32 ± 8 μmol/L vs. 5 ± 3 μmol/L, $p < 0.01$) and decreased haptoglobin levels (0.4 ± 0.3 g/L vs. 1.2 ± 0.7 g/L, $p < 0.01$) as compared to unaffected family members or unrelated individuals. Serum ferritin was elevated in all age tertiles in affected individuals compared to non-affected or unrelated individuals, and 7/29 affected individuals had a serum ferritin >900 μg/L (Table II, Figure III). Glycolytic enzyme activities (n=2), haemoglobin electrophoresis (n=5) and screening for unstable haemoglobin species (n=5) were normal in the affected individuals tested. Osmotic fragility testing (n=10) showed that red blood cells from affected individuals were resistant to lysis in progressively hypotonic saline solutions.

Red cell morphological assessments revealed that target cells, schistocytes and eccentrocytes were increased among affected individuals as compared to unaffected or unrelated individuals (Figure IV). Eccentrocytes, defined as red blood cells with haemoglobin puddled at the periphery,[19] represented the most prominent abnormal red cell phenotype. The number of stomatocytes per high-powered field was not statistically different (2.50 ± 2.40 vs. 2.73 ± 4.24, $p=0.76$) between affected and unaffected individuals (Table II, Figure V).

DNA linkage analysis

Because the haemolytic process occurring in this family was consistent with hereditary xerocytosis, we undertook genetic linkage studies using six microsatellite markers located within the 16q22.2 – 16q24.3 interval. Lod scores for linkage were determined in each family with each microsatellite marker and are summarized in Table III. Significant lod scores (>+3.00) were established between the *HX* causing gene and *D16S3074*, *D16S2621* and *D16S3026*. Three recombinants were observed between *HX*:*D16S3074*, two between *HX:D16S2621* and none between *HX*:*D16S3026*. These recombination events allowed us to define the centromeric boundary of the region containing the disease-causing gene as between *D16S2621* and *D16S3026*. This observation is supported by the family haplotype illustrated in Figure VI, where the affected son inherited his affected father's normal haplotype until *D16S2621*, and then the *HX* causing allele (horizontal line indicates the cross-over) with allele 5 of *D16S3026*.

YEAR II

At the American Society of Hematology conference (December 2010) we presented data which characterized the inheritance and clinical phenotype of the haemolytic process. Our results, and more specifically, the large size of our pedigree, attracted interest and invitations to collaborate from several investigators from the United States and Europe. Though unexpected and not a planned for component of the original BSc. (Med) proposal, we established a formal collaboration with Dr. Patrick Gallagher from Yale University (New Haven, CT). Our comprehensive clinical characterization and large kindred provided a unique opportunity for further genetic investigation, and the collaboration provided us with an opportunity to expedite efforts to identify the causative genetic mutation. I accepted the invitation, and completed much of the second summer at Yale University, in the genetics laboratory of Dr. Gallagher. To facilitate this inter-institution collaboration, I applied for and received a Trainee Award from the American Society of Hematology.

The objective of the second summer of research was to conduct genetic studies that would assist in the eventual identification of the genetic mutation responsible for hereditary xerocytosis.

Under the direction of Dr. Gallagher, whole exome sequencing was performed on 3 affected individuals and 2 unaffected individuals. A validation study was designed using Dual Labeled BHQ® (Black Hole Quencher) Probes (Biosearch Technologies) to prove consistent inheritance of the mutation in affected individuals of the Manitoban kindred. The same assay was also used to screen unrelated individuals affected with hereditary xerocytosis to establish the presence of the mutation identified by whole exome sequencing.

Exon primers were designed to further investigate the candidate gene in unrelated individuals presumably affected with hereditary xerocytosis. Primers for each exon were used to amplify the DNA of affected individuals. Exon PCR was performed on 14 unrelated individuals with phenotypic descriptions consistent with hereditary xerocytosis. DNA sequencing was then performed using a Hitachi 3130x/ Genetic Analyzer to identify other mutations in the causative gene to corroborate preliminary findings.

Exome sequencing in 3 affected individuals and 2 unaffected individuals from our family identified 3 candidate genes in the region previously delineated by linkage analysis. When analyzed in conjunction with unrelated individuals affected with hereditary xerocytosis, a single gene was identified as the most likely candidate.

TaqMan® validation assays, using BHQ® Probes, confirmed the identified mutation in all 29 affected individuals within the Manitoban kindred, which was absent in all 70 unaffected individuals tested in our family. The same assay was also used to screen 14 unrelated individuals presumably affected with hereditary xerocytosis, and the identical missense mutation was identified in 2 unrelated individuals.

Further investigation of the gene of interest using exon PCR and sequencing identified a different mutation in the same candidate gene within a second family (Rochester, NY) with hereditary xerocytosis. The identified mutation in the Rochester family was consistent amongst all 5 affected individuals, and was absent in the one unaffected individual tested.

DISCUSSION

In a large Manitoban kindred we have identified an autosomal dominant haemolytic disorder associated with an elevated MCHC and decreased osmotic fragility, a phenotype most consistent with what has been previously described as hereditary xerocytosis. We also found evidence of iron loading in affected individuals, despite normal haemoglobin levels. DNA linkage analysis has allowed us to narrow the location of the disease locus to chromosome 16q24.2 – 16qter, a region containing 51 known or predicted genes.

Given the phenotypic and clinical heterogeneity of disorders that have been characterized as hereditary xerocytosis, we scored the red blood cell morphologic abnormalities to systematically quantify the presence of abnormal erythrocytes in the peripheral blood. Using this methodology we found no difference in the mean number of stomatocytes between affected and unaffected individuals. Affected individuals had more target cells and schistocytes than unaffected family members. The most frequent abnormality identified was dehydrated appearing cells previously referred to as desiccytes[25,26] or eccentrocytes.[19]

Although the mean haemoglobin levels in affected subjects were normal (and not different from the unaffected subjects), the disease phenotype was associated with clinically important sequelae. Almost half of affected individuals reported a history of transient anaemia, often in conjunction with an acute illness (presumably as a consequence of suppression of reticulocytosis, although a transient increase in red cell haemolysis could not be excluded). In addition, nearly half of affected individuals had symptomatic gallstone disease.

Perhaps most importantly, affected individuals demonstrated a propensity to iron load, even in the absence of anaemia or transfusion. Compared with unaffected or unrelated individuals, serum ferritin in affected family members was elevated in all age tertiles. Iron loading in hereditary xerocytosis was first highlighted by Stewart et al., and has been reported by other investigators.[3,6,12] The mechanism for this process is poorly understood, but the normal

haemoglobin concentration in our kindred suggests that it is mediated through a hypoxia-independent process. Such hypoxia-independent mediators of iron regulation include growth differentiation factor 15 (GDF15) and twisted gastrulation 1 (Twsg1), two transforming growth factor-beta (TGFβ) family modulators that are generated during ineffective erythropoiesis that have been shown to down-regulate hepcidin.[27-29] Further studies are required to better understand the regulation of the observed iron overloading in this family.

Three previous studies have mapped a phenotypically similar *HX* locus to chromosome 16q.[10,13,14] The first and second of these reports defined the region from *D16S511* – 16qter, a 20 centimorgan (cM) region containing 104 known and predicted genes.[13,14] Studying 10 families with the pleiotropic syndrome hereditary xerocytosis, Grootenboer et al., assigned the locus to 16q23-16q24, a 9 cM interval containing approximately 20 genes.[10] Despite these assignments, the causative gene and underlying mutation(s) have not yet been determined. The difficulty in identifying the causative gene may be due to an increased amount of gene duplication that is known to occur in the 16q telomeric region.[30] In our study, the large informative family has allowed us to both confirm linkage to chromosome 16q, and more importantly to reduce the size of the *D16S511* – 16qter[13,14] candidate gene interval to *D16S2621* - 16qter, a region containing 51 known and predicted genes.[31] From our family analysis (Table III), 18% recombination between *HX:D16S3073* and 11% recombination between *HX:D16S539* strongly suggests that the causative gene is not located between 16q23 – 16q24 as previously reported.[10] Definition of the 2.4 cM region we have identified was facilitated by haplotype analysis and the identification of a critical recombination event occurring between *HX* and *D16S2621,* which defines the centromeric boundary of the region, and supports the placement of *HX* distal to *D16S2621*.

The consistent inheritance of the identified mutation in our kindred, in corroboration with the presence of the identical mutation in two unrelated affected individuals, and the identification of an alternate mutation within the same gene in a second (Rochester, NY) kindred, strongly suggests that the causative mutation responsible for the disease phenotype resides within a single gene on the long arm of chromosome 16. An alternative explanation could be linkage disequilibrium, that is the association of alleles at two different loci, and thus confirmatory functional studies are being developed to prove that the identified mutation is in fact causative. Due to ongoing studies and institutional agreements between centers, the name of particular gene under investigation has been withheld from this report.

Strengths and limitations

Our study has several strengths and limitations that warrant consideration. The large size and informative nature of the family have allowed us to thoroughly characterize the disease phenotype. Through the quantitative evaluation of red cell morphology we were able to demonstrate the lack of stomatocytes in a disease phenotype that would otherwise be characterized as dehydrated hereditary stomatocytosis. This study has also allowed us to substantially reduce the size of the candidate gene interval. A potential limitation of this study is the unavailability of ion flux studies that have previously been used to characterize similar disease phenotypes. The phenotype of this kindred, however, is consistent with previous phenotypic descriptions characterized as dehydrated hereditary stomatocytosis.[5,25,3]

CONCLUSION

We have identified a large kindred with autosomal dominant hereditary xerocytosis. We have confirmed the linkage of the disease phenotype to chromosome 16q, and have further refined the locus to 16q24.2-16qter. Mutations within a single gene may be responsible for the hereditary xerocytosis phenotype; however, confirmatory studies and functional analyses are required.

FUTURE DIRECTIONS

Functional studies are required to prove the mutation identified with whole exome sequencing is causative. cDNA including the identified mutation will be introduced into a vector, and incorporated into an erythroid cell line to deduce variation in protein expression. Functional studies that will characterize the pathobiological effect of the expressed protein are in the planning phase.

PROJECT MILESTONES

This project and research experience has provided me with a range of learning opportunities that will be invaluable to my future career. During my time in the BSc. (Med) program, I was fortunate to have achieved several personal and professional milestones (Table IV). In a relatively short period of time, with strong mentorship and institutional support, I learned how to create a study protocol, how to successfully prepare a grant submission, and how to write an abstract for a research conference. This project afforded me the opportunity to present my work as a poster at an international conference, and facilitated the development of strong collaborative relationships at both the University of Manitoba and Yale University. In the final stages of my BSc. (Med) project, I learned and experienced the process involved in preparing a manuscript for submission to a biomedical journal. This project as a whole consistently demonstrated the importance of a detailed, well-thought out and achievable plan to facilitate success in research, but also highlighted the need for flexibility based on results or unanticipated opportunity.

CONTRIBUTIONS

I would like to thank my primary supervisor, Dr. Ryan Zarychanski, for his continued support and mentorship throughout this project. Ryan consistently challenged me throughout the development and execution of this project, and in doing so provided me with a strong appreciation and respect for biomedical research, for which I am very grateful.

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Dr. SJ Israels	Dr. BN Chodirker	Dr. PG Gallagher

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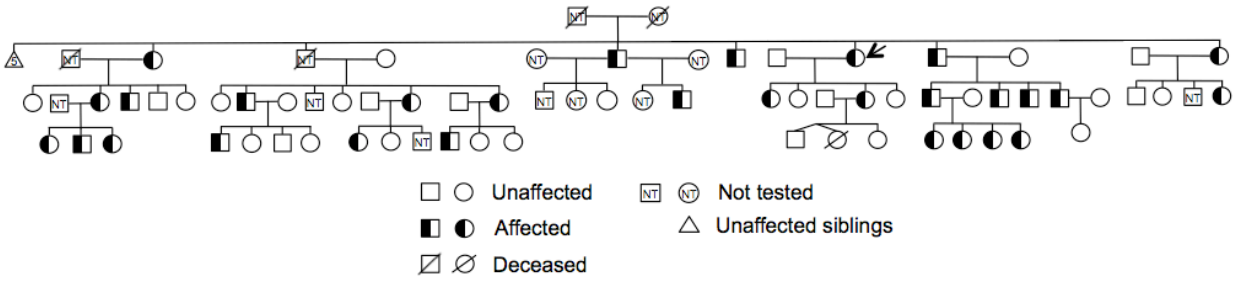

Figure I. Partial family pedigree highlighting autosomal dominant inheritance pattern of haemolytic disease based on the distribution of reticulocyte counts. Arrow indicates proposita.

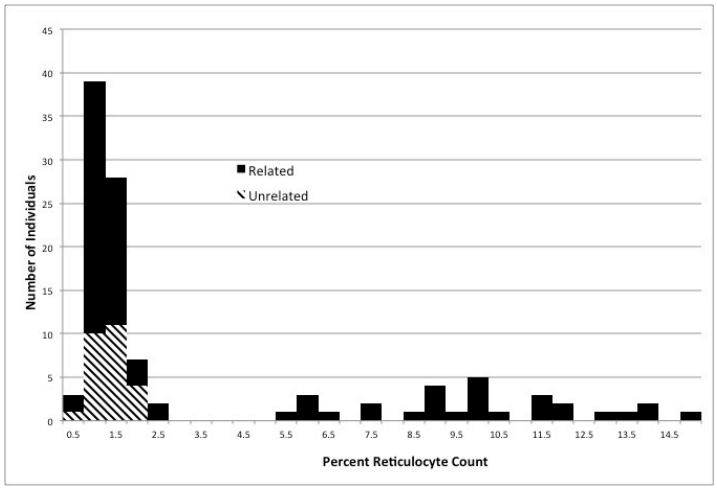

Figure II. The distribution of reticulocyte counts was bimodal with no overlap allowing individuals to be classified as phenotypically affected or non-affected.

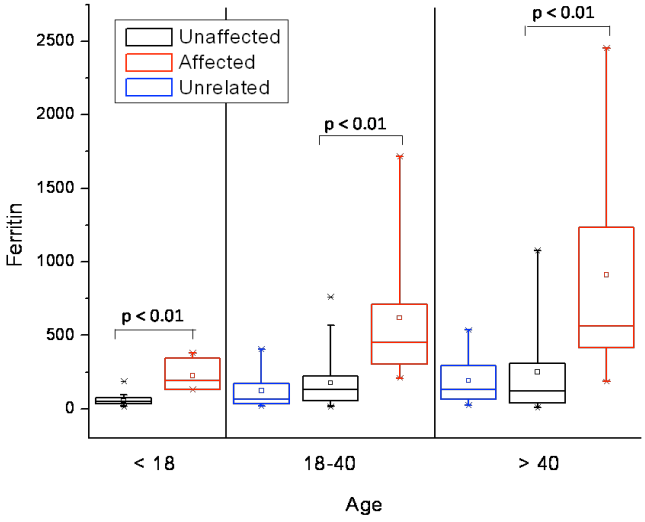

Figure III. Serum ferritin was elevated in all age tertiles in affected individuals compared to non-affected or unrelated individuals.

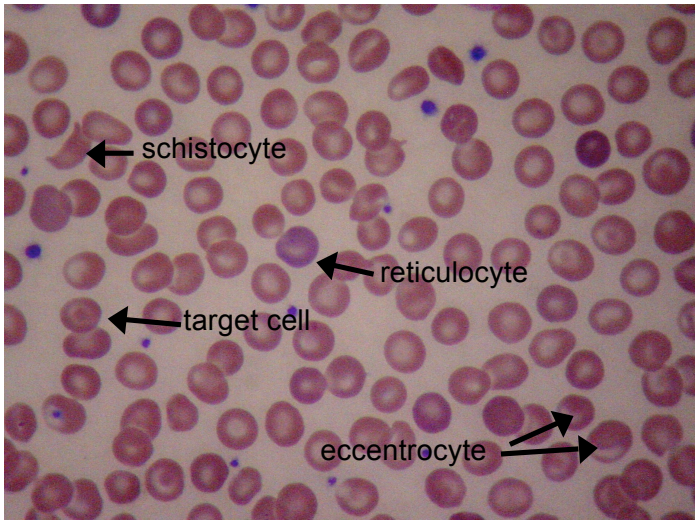

Figure IV. Peripheral blood film demonstrating a schistocyte, target cell, reticulocyte, and eccentrocyte (red cell with haemoglobin puddled in the periphery).

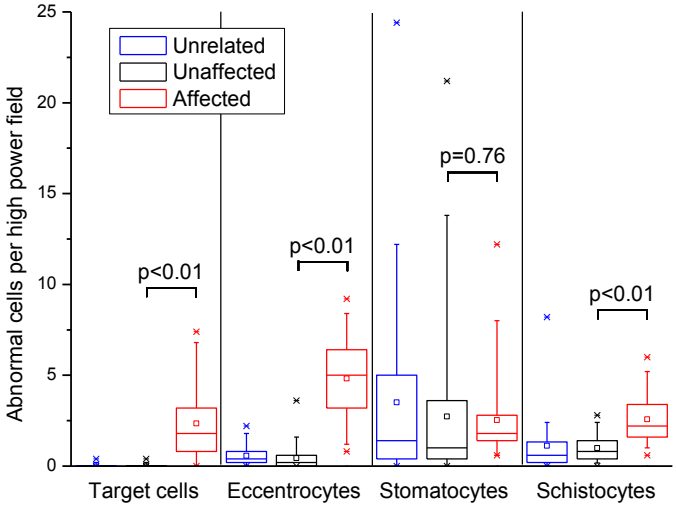

Figure V. Quantitative red cell morphology. Target cells and eccentrocytes were significantly elevated in affected individuals. There was no statistically significant difference between stomatocytes in affected and unaffected individuals. Reported cell counts reflect the average of 5 high power fields per slide.

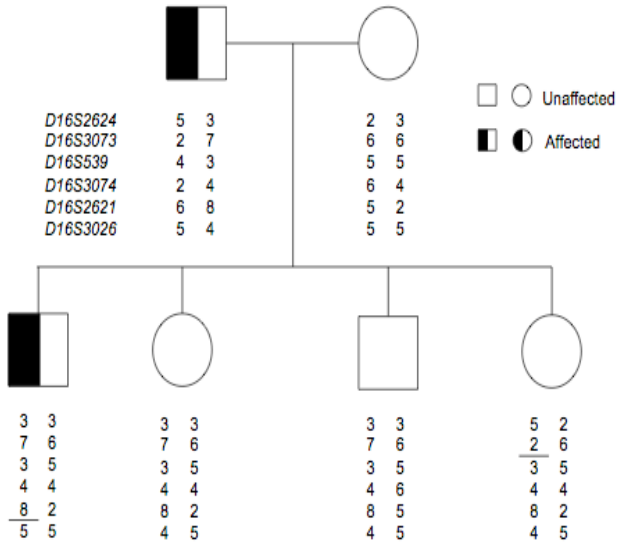

Figure VI. Family haplotype diagram. The unaffected daughter (far right) received her father's *HX* carrying chromosome (indicated by allele 5 of *D16S2624* and allele 2 of *D16S3073)* until *D16S3073*. Following a recombination event, (indicated by horizontal line) she inherited her father's normal chromosome with the non-disease associated alleles. Her affected brother inherited his father's normal chromosome until *D16S2621*, and then his *HX* carrying chromosome (horizontal line indicates the cross-over) with allele 5 of *D16S3026*. This critical recombinant allowed us to assign the disease causing gene to a chromosomal segment distal to *D16S2621*.

Table I: Progress and time-line

Year 1:	**Year 2:**
Preparation of ethics submission	Completion of linkage analysis
Development of study protocol and application for funding	Collaboration with Yale University
Organization of family reunion	Exome sequencing
Preparation of a complete family pedigree	Candidate gene analysis
Haematological and biochemical data collection	Preparation and submission of manuscript for publication
Linkage analysis	Preparation and submission of BSc. (Med) final report

Table II: Clinical characteristics and laboratory analyses

	Affected n = 29	**Unaffected n = 77**	**P value**	**Unrelated n = 26**
Males (n, %)	12 (41)	41 (53)		12 (46)
Age (mean, SD)	34.8 (20.2)	22.0 (16.0)		45.7 (14.0)
History (n, %)				
Anemia	13 (46)	6 (8)	<0.01	1 (4)
Transfusion	5 (17)	1 (1)	<0.01	1 (4)
Jaundice	13 (45)	3 (4)	<0.01	0 (0)
Red or brown urine	13 (45)	1 (1)	<0.01	0 (0)
Gallstones or cholecystectomy	12 (41)	3 (4)	<0.01	2 (8)
Hematology (mean, SD)				
Hemoglobin (g/L)	135 (12.4)	138 (14.3)	0.26	140 (11.0)
MCV (fl)	96.7 (5.5)	87.3 (5.2)	<0.01	88.6 (3.1)
MCHC (g/L)	366 (5.9)	338 (9.1)	<0.01	337 (7.1)
Percent reticulocytes	9.7 (2.6)	1.1 (0.4)	<0.01	1.2 (0.4)
Absolute reticulocytes	367 (101)	51 (18)	<0.01	53 (15)
White blood cells ($x10^{12}$/L)	6.7 (1.6)	7.3 (2.1)	0.15	7.3 (1.9)
Platelets ($x10^{12}$/L)	261 (80)	278 (85)	0.38	251 (56)
Biochemistry (mean, SD)				
Indirect bilirubin (µmol/L)	32 (18)	5 (3)	<0.01	7 (5)
Haptoglobin (g/L)	0.4 (0.3)	1.2 (0.7)	<0.01	1.3 (0.6)
ALT (IU/L)	23 (14)	23 (14)	0.85	27 (18)
Ferritin (µg/L) (median, IQR)				
(Age) < 18	194 (134, 345)	51 (34, 74)	<0.01	-
18-39	478 (316, 813)	134 (53, 225)	<0.01	65 (36, 172)
≥40	570 (534, 1235)	158 (85, 334)	<0.01	131 (63, 293)

Table III: Total peak lods and recombination fractions (for combined paternal and maternal meioses) between the *HX* causing gene and six chromosome 16q microsatellite markers.

	$\hat{z}$	$\hat{\theta}$
HX:*D16S2624*	0.556	0.32
HX:*D16S3073*	2.170	0.18
HX:*D16S539*	2.647	0.11
HX:*D16S3074*	3.850	0.10
HX:*D16S2621*	3.718	0.05
HX:*D16S3026*	4.816	0.00

Table IV: Milestones and achievements related to BSc. (Med) project

2010 – **ASH Abstract**: BL Houston, DS Houston, SJ Israels, G Coghlan, BN Chodirker, T Zelinski, R Zarychanski. Phenotypic evaluation of a family cohort with hemolytic anemia. (American Society of Hematology, Orlando, Florida, 2010) [Poster]
2010 – American Society of Hematology **ASH Travel Award**
2011 – **MMSF Funding Award Recipient** ($20 000)
2011 – American Society of Hematology – **Trainee Research Award**
2011 – American Society of Hematology – **ASH Travel Award**
2011 – **1st Prize** in the Clinical Oral Presentation for the 12th Annual CancerCare Manitoba Foundation Research Day
2011 – **ASH Abstract:** BL Houston, T Zelinski, DS Houston, SJ Israels, G Coghlan, BN Chodirker, PG Gallagher, R Zarychanski. Genetic linkage of the dehydrated hereditary stomatocytosis locus to chromosome 16 in two kindreds, and refinement of candidate region to 16q24.2 – 16qter. (Submitted to the American Society of Hematology, San Diego, Florida, 2011)
2011 – **Manuscript accepted for publication**: BL Houston, T Zelinski, SJ Israels, G Coghlan, BN Chodirker, PG Gallagher, DS Houston, R Zarychanski, Refinement of the Hereditary Xerocytosis Locus on Chromosome 16q in a Large Canadian Kindred, 2011, *Blood Cells Molecules and Diseases. In press*