Congenital Dyserythropoietic Anemia type III (CDA III) - diagnostics, genetics and morbidity

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”What makes the desert beautiful,” said the little prince, ”is that somewhere it hides a well…”

Antoine de Saint-Exupéry

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Abstract

The Congenital Dyserythropoietic Anemias (CDA) are rare hereditary hemolytic disorders with large bi- to multi-nucleated erythroblasts in the bone marrow. Hemolysis is negative in a direct antiglobulin test (DAT). Based on morphology and clinical picture, three major forms of CDAs, type I, II, and III have been defined. CDA III, dominantly inherited, constitutes the rarest type with a majority of cases belonging to a family in Västerbotten, Sweden. The genetic background of CDA I and CDA II has been linked to mutations in *CDAN1* and *SEC23B* respectively. The mutation of CDA III has been linked to 15q22 in earlier studies.

In this project we have defined the causative genetic lesion in two families with CDA III. The novel mutation *KIF23* c.2747C>G (p.P916R) was shown to segregate with CDA III in the Swedish and American CDA III families and was absent in 356 healthy controls. *KIF23* encodes mitotic kinesin-like protein 1 (MKLP1), which plays a central role in the last step of cytokinesis. RNAi-based knock-down and rescue experiments demonstrated that the p.P916R mutation causes cytokinesis failure in HeLa cells, resulting in increasing number of bi-nuclear cells, consistent with appearance of large multinucleated erythroblasts in CDA III patients. We conclude that CDA III is caused by a mutation in *KIF23*, encoding MKLP1, a conserved mitotic kinesin crucial for cytokinesis.

Flow cytometry with eosin-5´-maleimide (EMA), anti-CD55 and anti-CD59 is commonly used when investigating non-autoimmune hemolytic anemias. Reduced fluorescence of EMA, typically detected in hereditary spherocytosis, is also seen in CDA II, while reduction of CD55 and CD59 characterizes paroxysmal nocturnal hemoglobinuria (PNH). We studied the flow cytometric profile of EMA, CD55, and CD59 on erythrocytes in CDA III. We found no abnormality of the erythrocyte membrane in CDA III and concluded that standard flow cytometry cannot be used to discriminate between CDA III and normal controls.

In CDA I and CDA II a majority of patients, including those who are not transfusion dependent, suffer from iron overload, which, according to earlier studies, is not the case in CDA III. We found that individuals of the Västerbotten CDA III family carry mutations in the hemochromatosis (*HFE*) gene. Three CDA III patients with heterozygous or compound *HFE* mutations need treatment with phlebotomy due to iron overload. One of them carries heterozygous H63D mutation, which is not reported to lead to iron overload by itself in otherwise healthy individuals. We propose that molecular genetic testing of the *HFE* gene is indicated in all patients with CDA, including CDA III.

Abbreviations

AGLT	Acid glycerol lysis time test
ASF1	Anti-silencing function protein 1
CDA	Congenital dyserythropoietic anemia
CLL	Chronic lymphatic leukemia
DAT	Direct antiglobulin test
EMA	Eosin-5´-maleimide
ER	Endoplasmic reticulum
ERFE	Erythroferrone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDF15	Growth differentiation factor 15
GPA	Glycophorin A
Hb	Hemoglobin
HEMPAS	Hereditary erythroblastic multinuclearity associated with a positive acidified-serum test
HE	Hereditary elliptocytosis
HH	Hereditary hemochromatosis
HPP	Hereditary pyropoikilocytosis
HS	Hereditary spherocytosis
IL-6	Interleukin-6
LD	Lactate dehydrogenase
MCF	Mean channel fluorescence
MCV	Mean cellular volume
MGUS	Monoclonal gammopathy of unknown significance
MKLP1	Mitotic kinesin-like protein 1
MRI	Magnetic resonance imaging
MVK	Mevalonate kinase gene
NGS	Next generation sequencing
OF	Osmotic fragility test

PCR	Polymerase chain reaction
PNH	Paroxysmal nocturnal hemoglobinuria
RHAG	Rh-associated glycoprotein
RT- PCR	Reverse transcription PCR
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tf	Transferrin
TfR	Transferrin receptor
TTP	Thrombotic thrombocytopenic purpura
TSAT	Transferrin iron saturation
TWSG1	Twisted gastrulation BMP signaling modulator 1
Wt	Wild type

Populärvetenskaplig sammanfattning på svenska

Kongenitala dyserytropoetiska anemier (CDA) är en grupp ovanliga ärftliga anemier med ineffektiv bildning av röda blodkroppar (erytropoes) och varierande grad av anemi. Baserat på benmärgsmorfologi, klinisk bild och laboratoriefynd indelas CDA i flera undergrupper, varav I-III varit kända sedan länge. De senaste decennierna har nya former identifierats, vilka klassificerats som typ IV-VII, framförallt baserat på enstaka fallrapporter. CDA I-III är de mest studerade. Gemensamma karakteristika utgörs av icke autoimmun hemolys samt dyserytropoes med stora, dubbel- till fler-kärniga, erytroblaster i benmärgen.

CDA III utgör den ovanligaste formen av de dyserytropoetiska anemierna. Den globala prevalensen av CDA III är svårbedömd men majoriteten av rapporterade fall tillhör en släkt med ursprung i Västerbotten. I denna familj har individer med CDA III kartlagts 6 generationer bakåt, till mitten av 1800-talet. Totalt har 47 individer fått diagnosen CDA III. Denna anemiform har ett dominant nedärvningsmönster och en benmärgsbild som präglas av aktiv erytropoes med stora mångkärniga erytroblaster. Den kliniska bilden varierar från mild till måttlig hemolytisk anemi. I tidigare arbeten av Sandström och medarbetare påvisades ett antal diagnostiska kriterier för CDA III, baserat på benmärgsmorfologi och perifera blodprover med analys av hemolysparametrar samt tymidinkinas. Den sjukdomsorsakande genen lokaliserades till 15q22. Individer med CDA III visades ha ökad förekomst av ögonbottenförändringar (angioid streaks) samt ökad risk att drabbas av blodcancern multipelt myelom och dess förstadium monoklonal gammopati utan signifikans (MGUS).

Vid utredning av patient med icke autoimmun hemolytisk anemi bör flödescytometri utföras med frågeställningarna hereditär sfärocytos (HS) samt paroxysmal nocturn hemoglobinuri (PNH). HS, med en prevalens i norra Europa på 1 % och i norra Sverige upp mot 5 %, är en ärftlig hemolytisk anemi med varierande grad av anemi och hemolysorsakade komplikationer i form av gulsot (ikterus) och gallstensproblematik. Svårighetsgraden varierar även på individnivå, med försämrad symtombild vid såväl bakteriella som virusorsakade infektioner. PNH bör alltid uteslutas vid icke autoimmun hemolys på grund av de allvarliga komplikationer, framförallt tromboser och organpåverkan, som denna sjukdom kan medföra. Flödescytometri med eosin-5´-maleimid (EMA) visar minskat upptag hos röda blodkroppar vid HS, medan minskat uttryck av CD59 på ytan av såväl röda som vita blodceller utgör ett karakteristikum för PNH. Vid EMA-flöde ses liknande mönster vid CDA II som vid HS.

Vid CDA I och II utgör järninlagring ett stort kliniskt problem. Att järnnivåerna är ökade vid hemolytiska tillstånd är vanligt, beroende på den ökade destruktionen av röda blodkroppar samt påverkan på hepcidinregleringen. Hepcidin, ett hormon med central roll i järnomsättningen, ser till att absorptionen av järn från tarmen och frisättningen av järn från kroppens järnlager justeras efter aktuellt behov. Vid hemolys och ineffektiv erytropoes fungerar inte denna regleringsmekanism optimalt. Trots detta har järndepåerna vid CDA III i tidigare studier ej visat sig förhöjda, sannolikt beroende på att patienterna, på grund av intravasal hemolys, kontinuerligt förlorar järn i urinen.

Hos i övrigt friska personer utgör, i norra Europa, hereditär hemokromatos den vanligaste orsaken till förhöjda järnnivåer. Sjukdomen nedärvs autosomalt recessivt och orsakas av mutationer i *HFE*-genen, vilket leder till ökat upptag av järn från tarmen. Muterat anlag på båda kromosomerna (homozygot mutation) krävs för att utveckla ökade järnnivåer, men även hos homozygoter är det bara ca 20 % som utvecklar sjukdomen. I Sverige är prevalensen av hereditär hemokromatos 0,5 %, men i Norrland är sjukdomen vanligare. Omkring 7 % av befolkningen är anlagsbärare. Obehandlad kan sjukdomen leda till inlagring av järn i inre organ med organsvikt som följd. De vanligaste symtomen är ledvärk, lever och hjärtpåverkan samt diabetes. Om behandling i form av regelbundna blodtappningar påbörjas i tid kan denna utveckling helt förhindras. Diagnostiken består av genetisk analys avseende *HFE*-mutationer tillsammans med provtagning avseende järn, transferrinmättnad (TSAT) och ferritin i serum. I samband med vårt projekt uppmärksammades att mutationer i *HFE*-genen förekommer hos individer i Västerbottenssläkten.

Syfte

- att identifiera den mutation som ger upphov till CDA III.

- att undersöka ytan på de röda blodkropparna vid CDA III, med de flödescytometriska analyser som, i klinisk rutin, utförs vid utredning av icke-autoimmun hemolytisk anemi.

- att kartlägga förekomsten av hereditär hemokromatos i Västerbottenssläkten samt undersöka den kliniska bilden vid hereditär hemokromatos hos individer med och utan CDA III.

Patienter och metoder Kartläggning av Västerbottenssläkten gjordes av Bergström och Jacobsson i början av 1960-talet och uppdaterades av Sandström och medarbetare i mitten av 90-talet. Släktträdet sträcker sig över sex generationer och tar sin början i mitten av 1800-talet. Sedan 1990-talet har ytterligare barn tillkommit i släkten och en uppdatering av släktträdet har genomförts i detta projekt. DNA har samlats in och nytillkomna individer har utretts med avseende på CDA III. I tidigare arbete av Sandström och medarbetare insamlades DNA på alla då levande individer i släkten. Totalt finns idag DNA preparerat från 60 individer i denna släkt.

För att få fram kandidatgener har i arbete I DNA analyserats med molekylärgenetisk metod i form av next generation sequencing (NSG). För ytterligare konfirmerande studier av mutation i *KIF23* har PCR-baserade metoder använts.

I arbete II har flödescytometri med EMA samt anti CD55 och anti CD59 använts i enlighet med klinisk rutin. Flödescytometri utfördes på perifert blod från 16 CDA III positiva individer och 14 CDA III negativa syskon.

I arbete III har *HFE*-mutationsstatus analyserats med PCR-baserad metod. *HFE*-mutationsstatus bedömdes på totalt 58 individer i släkten, 37 CDA III positiva och 21 CDA III negativa syskon. För vidare analys med koppling till järnstatus och hemolysprover inkluderades 32 CDA III positiva och 18 CDA III negativa i studien. TSAT, ferritinnivåer och hemolysparametrar analyserades enligt klinisk rutin på laboratoriet för klinisk kemi.

Resultat I arbete I har vi identifierat den genetiska mutation i *KIF23* som ger upphov till CDA III. Mutationen leder till dysfunktionellt KIF23, även kallat Mitosis Kinesin-Like Protein 1 (MKLP1). Detta protein har en central roll i bildningen och funktionen av den så kallade ”midbody”, vilken reglerar avslutningen av celldelningen. Mutationen leder till att cellen inte delar sig trots bildning av två eller flera cellkärnor. Störning i midbody-funktionen har experimentellt visats ge upphov till flerkärniga celler.

I arbete II har vi visat att flödescytometri med EMA ger ett obetydligt ökat upptag hos erytrocyter vid CDA III, sannolikt beroende på ökad cellvolym hos de röda blodkropparna. Flödescytometri med anti CD55 och anti CD59 ger helt normalt utfall. Flödescytometri kan alltså inte användas för diagnostik av CDA III. CDA III riskerar inte heller att misstas för HS eller PNH vid flödescytometrisk analys av röda blodkroppar.

I arbete III har förekomsten av *HFE*-mutationer i Västerbottenssläkten kartlagts och klinisk presentation avseende järnbalansen har undersökts hos

CDA III positiva individer och deras syskon. Bland CDA III negativa syskon har en individ med homozygot mutation och ökade järnnivåer påbörjat blodtappningar. Av de CDA III positiva patienterna hade ingen homozygot mutation men tre befanns bära på heterozygot *HFE*-mutation tillsammans med laboratoriemässiga fynd förenliga med ökade järndepåer. Alla tre har påbörjat blodtappningar med sjunkande ferritin och TSAT som följd. Tappningarna har tolererats väl trots den kontinuerliga hemolys som föreligger vid CDA III. Ingen av de CDA III negativa syskonen befanns ha störd järnomsättning trots förekomst av heterozygot *HFE*-mutation, vilket är förväntat, då heterozygot *HFE*-mutation ej ger upphov till ökade järndepåer hos i övrigt friska personer.

Diskussion I arbete I har vi fastslagit den molekylärgenetiska bakgrunden till CDA III, vilket bidragit till ökad förståelse för celldelningens slutstadium. Kunskap om *KIF23* och dess protein KIF23 (MKLP1) kan utgöra en viktig pusselbit avseende utveckling av flerkärniga celler vid exempelvis olika cancerformer. Den höga proliferationen hos många cancerceller ställer också krav på ökad tillgång till proteiner, såsom KIF23, vilka möjliggör celldelning. Att *KIF23* är uppreglerat vid flera tumörtyper är känt. Överuttryck finns beskrivet vid såväl hjärntumören gliom som lung-, bröst-, lever- och magsäcks-cancer. Nedreglering av *KIF23* har också visat att celldelningen i dessa cancerformer avstannat.

Individer med CDA III har ökad risk at utveckla blodcancern myelom. Vi genomför nu insamling av data från Cancerregistret och Dödsorsaksregistret samt planerar morfologiska studier av obduktionspreparat från individer med CDA III. Detta för att undersöka om även annan cancersjukdom kan kopplas till *KIF23* mutation, samt om flerkärniga celler även förekommer i andra cellinjer än den röda blodkroppsbildningen. Vi avser också att studera effekterna av den specifika genetiska mutationen i *KIF23* efter att ha infört denna i försöksdjur.

I arbete II har vi undersökt ytan på de röda blodkropparna vid CDA III med de flödescytometriska metoder som utgör basen vid utredning av icke-autoimmun hemolys. Den flödescytometriska profilen vid CDA II kan förväxlas med HS, vilket kan medföra risk för feldiagnostik tidigt i utredningsförloppet av icke-autoimmun hemolytisk anemi. I detta projekt visar vi att så inte är fallet beträffande CDA III. Vid negativt svar på flödescytometri med EMA samt CD59 antikropp bör benmärgsundersökning utföras, vilken i förekommande fall kommer att inge misstanke om CDA. Molekylärgenetisk diagnostik är därefter att föredra för ytterligare subklassificering, vilket numera är möjligt för alla tre huvudtyperna av CDA.

I arbete III visar vi att hereditär hemokromatos förekommer i Västerbottenssläkten. Vid homozygot mutation av *HFE*-genen kan detta, hos

friska CDA III negativa syskon, leda till ökad risk för järninlagring. Utveckling av hereditär hemokromatos förebyggs med blodtappning. Hos CDA III positiva individer kan även heterozygot mutation leda till patologiska järnnivåer. Kombinationen av CDA II och hereditär hemokromatos finns beskriven hos en patient från Italien. Blodtappningar var i detta fall svåra att genomföra på grund av patientens anemi, men våra patienter med CDA III har tolererat behandlingen väl. Även hos individer med blodcancern myelodysplastiskt syndrom eller hemoglobinopatin thalassemi har heterozygot *HFE*-mutation visats tillräcklig för att leda till patologisk järninlagring. Vårt arbete bekräftar att heterozygot *HFE*-mutation är tillräckligt för att orsaka höga järnnivåer, när mutationen förekommer tillsammans med annan sjukdom med ineffektiv erytropoes, såsom CDA III. Eftersom mutationer i *HFE*-genen är vanligt förekommande i befolkningen, bör screening avseende dessa utföras hos patienter med annan hematologisk sjukdom med påverkad järnomsättning, såsom vid hemolytiska sjukdomar och tillstånd med ineffektiv erytropoes.

Original papers

This thesis is based on the following studies, and referred to by their roman numerals in the text.

I. **Maria Liljeholm,** Andrew F. Irvine, Ann-Louise Vikberg, Anna Norberg, Stacy Month, Herbert Sandström, Anders Wahlin, Masanori Mishima, Irina Golovleva. Congenital Dyserythropoietic Anemia type III (CDA III) is caused by a mutation in kinesin family member, *KIF23*.

Blood 2013;121(23):4791-9

II. **Maria Liljeholm**, Elisabeth Grönlund, Irina Golovleva, Herbert Sandström, Anders Wahlin. Erythrocyte Flow Cytometric Analysis in Congenital Dyserythropoietic Anemia Type III - Evaluation of Eosin-5´-Maleimide, CD55, and CD59.

J Blood Disord Transfus 2013;4:172. doi:10.4172/2155-9864.1000172

III. **Maria Liljeholm**, Ann-Louise Vikberg, Irina Golovleva, Herbert Sandström, Anders Wahlin. Congenital Dyserythropoietic Anemia type III and primary hemochromatosis; coexistence of mutations in *KIF23* and *HFE*.

J Hematol Blood Disord 2016;1(2):203

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Introduction

Congenital Dyserythropoietic Anemias (CDA) is a group of rare hereditary disorders with large bi- to multi-nucleated erythroblasts in the bone marrow (Figure 1). Granulopoiesis and thrombopoiesis are normal. Hemolysis, which is negative in a direct antiglobulin test (DAT), is continuously ongoing and takes place intra- as well as extra-vascular. Laboratory signs are, as in other hemolytic anemias, due to increased hemoglobin (Hb) turnover with elevated lactate dehydrogenase (LD), hyperbilirubinemia and low or absent haptoglobin. Due to ineffective dyserythropoiesis, reticulocytes are not elevated, which is often the case in other hemolytic diseases (1). Three major forms of CDA, type I, II and III, were defined by Heimpel and Wendt in the late 1960ies, based on morphology and clinical picture (2). Over the years, some minor entities have been subclassified as sporadic CDA III and CDA variants (IV-VII), mainly referring to single case reports (3-6). Types I - III are the most closely studied and characterized forms (Table 1). The subclassification set by Heimpel and Wendt is still used in clinical practice, though advances in molecular genetics have brought new light on the spectrum of CDAs (7-9). As a molecular diagnostic approach is now available, some new entities have been defined. Moreover, the evolving molecular field together with decades of knowledge on morphology and clinical picture, make CDAs ideal models for studying cell division and cellular functions, not only in erythropoiesis (9).

CDA I

This autosomal recessive disorder has been reported in approximately 120 cases from 109 families in Europe, with dominance from the Western countries (10). Other reported cases mainly originate from a Bedouin tribe, living in the Negev desert in the south of Israel (11). The bone marrow reveals erythroid hyperplasia with up to 7 % of erythroblasts showing binuclearity and occasionally three or more nuclei in the same cell. Internuclear chromatin bridges, connecting almost terminally divided erythroblasts compose a characteristic sign (12). In electron microscopy studies, erythroblast nuclei appear as “Swiss cheese” due to the spongy pattern of heterochromatin and widening of nuclear pores resulting in invaginations of the cytoplasm into the nuclear area (13). The degree of anemia varies from mild to moderate. The need of transfusions during childhood is not exceptional, but in adults transfusion requirement is rare, often connected to pregnancy or severe infections. Clinical manifestations refer to symptoms due to anemia, mild jaundice and gallstones. In many cases a palpable spleen is observed (14). In adult patients, secondary hemochromatosis is usually seen, requiring chelation therapy, in order to prevent organ damage (15). Non-

hematologic abnormalities, mainly skeleton malformations of hands and feet, short stature and pigeon chest may be present (16, 17). Treatment with alfa-interferon has been shown to raise Hb and reduce dyserythropoiesis as well as ferritin levels (18, 19).

In 1998, linkage mapping in four Bedouin families localized the responsible gene to 15q15.1-15.3, and a few years later mutations in the *CDAN1*-gene encoding codanin-1 was found (20, 21). The protein is localized to heterochromatin and is cell cycle regulated, accumulating in S-phase (22). Furthermore, codanin-1 has been shown to interact with anti-silencing factor 1 (Asf1), a histone chaperone centrally involved in nucleosome assembly and disassembly (23). In 2013, a mutation in *C15ORF41* was detected in a few *CDAN1* negative cases (24). The specific function of the protein is unknown but it seems to interact with Asf1b, indicating a connection to heterochromatin regulation also in *CDAN1* negative CDA I (24).

CDA II

With approximately 450 cases from 350 families worldwide, CDA II constitutes the most reported CDA subtype (10). Most cases originate from Europe, especially from Italy (10, 25). In early literature the disorder was differentiated from other CDAs by an acidified serum test (Ham's test) and referred to as HEMPAS (Hereditary erythroblastic multinuclearity associated with a positive acidified-serum test) (26). The bone marrow is hypercellular with marked erythropoiesis, characterized by approximately 10 % binucleated cells among the erythroid progenitors. Pseudo-Gaucher cells are seen in a majority of cases (12, 27). Double membranes of the erythrocytes are visible at electron microscopy, and consist of proteins originating from the endoplasmic reticulum (ER) (28). Studies of the erythrocyte membrane show abnormal glycosylation of several transmembranous proteins, in particular anion exchanger 1, also called band 3 (29-31). The inheritance pattern is autosomal recessive. The degree of anemia varies from mild to severe with up to 10 % of cases in need of red cell transfusions on a regular basis. Jaundice, biliary stones and splenomegaly are often present, and a majority of patients develop iron overload, even in the absence of transfusions (32). Therapeutic approaches with interferon have not been successful in CDA II. Splenectomy seems to have a slight effect on Hb levels but does not affect the progression of iron overload, even in non-transfused patients (32). A few patients with severe CDA II, some with coexistence of thalassemia, have undergone allogeneic stem cell transplantation (33, 34). Regarding the epidemiology of CDA II, it is of course important to consider coexistence of thalassemia, especially in severe cases.

In 2009 two research groups detected the causative gene *SEC23B*, earlier localized to chromosome 20 (35, 36). The encoded protein SEC23B, is a component of cytoplasmic coat protein II (COP II), which is centrally involved in transportation of membrane and protein components from the ER towards the Golgi apparatus (37). Approximately 80 mutations have been found throughout the gene, giving rise to slightly different phenotypes (38). Two founder mutations have been described in the Italian population (39).

CDA III

This is the rarest form of the three major subtypes of CDA. Globally, a familial form has been detected in three families. The majority originates from a family in Västerbotten, Sweden, and the others include a few cases reported from one family in the United States and one family in Argentina (40, 41). In addition, some sporadic cases have been diagnosed and reported as CDA III. These are single case reports with diverse phenotypes, including hepatosplenomegaly, iron overload and mental retardation, which are not present in familial CDA III (4, 42-45). The mode of inheritance is dominant in the familial cases but appears to be recessive in the sporadic forms (4). The earliest reported familial CDA III cases, a mother and her three children from the American family, were described in 1951 by Wolff and von Hofe and referred to as "familial erythroid multinuclearity" (40). In 1962, the first cases of the Swedish family were described by Bergström et al (46). The pedigree composed 15 affected individuals, reaching back to a couple living in the middle of the 19th century. Today the pedigree of the Swedish Västerbotten CDA III family covers six generations with 47 diagnosed CDA III cases. Throughout the years the family has been closely studied, defining morphology features, as well as laboratory and clinical signs (47). Bone marrow microscopy reveals erythroid hyperplasia with bi- and multinucleated erythroblasts, some with up to 12 nuclei in the same cell, so called gigantoblasts (46, 48). Electron microscopy studies show non-specific signs such as intranuclear clefts and ironladen mitochondria (48). Observations of the erythrocyte membrane, performed in two patients, revealed a slight reduction of glycosylation of band 3 while CD44, CD47, CD59 and Rh-related proteins appeared to be normal (48).

Laboratory signs are consistent with hemolysis with absent haptoglobin and elevated LD and bilirubinemia. Anemia is usually mild and the need of transfusions is very rare. In contrast to CDA I and II, a major part of hemolysis takes place intravascular, leading to hemosiderinuria with loss of iron in the urine. This has been believed to explain why iron overload has not been detected in patients with CDA III (47). Serum thymidine kinase is highly

elevated in CDA III and has been used as a diagnostic tool in the CDA III family when bone marrow examination has not been feasible (49). Splenomegaly is uncommon in these patients but about 20 % of cases occasionally experience jaundice and biliary symptoms. Weakness, fatigue and headache have been reported in approximately one third of the patients, symptoms that are aggravated in pregnancy or infection (50).

Although not statistically significant, Bergström et al noted a high frequency of cancer in the medical records of the CDA III cases observed in their study (46). Sandström et al confirmed a connection between CDA III and the precancerous plasma cell disorder monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (47). Angioid streaks, rarely occurring retinal changes in patients with other hemolytic disorders such as thalassemia and sickle cell anemia, were found in some CDA III patients (51). The causative gene was localized to 15q21-q25 (52).

In the present study we focus on the Västerbotten CDA III family, defining the genetic cause of the disease, analysing the erythrocyte membrane with flow cytometry, and report that iron overload is present in this family, attributable to coexistence of hereditary mutations of the *HFE* gene. We are presently investigating the incidence of cancer in this family.

CDA variants

All reported CDAs do not fulfil the criteria of the CDA subtypes described above. According to bone marrow findings and clinical phenotype they have been diagnosed as CDA IV-VII, including only a few patients in each group. In addition, some cases do not fit into these groups either and are regarded as CDA variants (3-6). Hence, CDA subtypes represent a very heterogeneous group, probably originating from different genetic defects connected to erythroid regulation. Some genes, as *KLF1* and *GATA-1,* encoding erythroid transcription factors and mutations in genes where CDA is a part of a clinical syndrome have been described. CDA type IV is caused by mutation in *KLF1*, while *GATA-1* mutation has been detected in X-linked thrombocytopenia with CDA (53-55).

CDA may constitute one of several clinical presentations in syndromes such as Majeed syndrome (mutation in *LPIN2*), CDA with exocrine pancreatic insufficiency and calvarial hyperostosis (mutation in *COX4I2*), and the inborn error of metabolism, mevalonate kinase deficiency (mutation in *MVK)* (56-58).

Table 1. Characteristics of the three major forms of CDA

	CDA I	CDA II	CDA III familial	CDA III sporadic
No of reported cases	~ 170	~ 450	3 families	<20
Inheritance	Recessive	Recessive	Dominant	Various
Associated disorders	Skeletal abnormality Iron overload	Iron overload	MGUS , Myeloma Angioid streaks	Hepato splenomegaly Mental retardation
Morphology	Bi-multi-nuclearity Internuclear bridges	Bi-multi-nuclearity	Giganto-blasts	Giganto-blasts
Electron microscopy	Spongy hetero chromatin	Double membrane	Intranuclear clefts	Various
Glycosylation of red cell membrane	Some abnormality	Marked abnormality	Some abnormality	Not studied
Flow cytometry	Not studied	Band 3 ↓	Normal CD59*, Band 3*	Not studied
Causative gene	*CDAN1*	*SEC23B*	*KIF23**	Unknown
Coexisting hereditary disorders	None reported	Thalassemia Hereditary hemochromatosis	Hereditary hemochromatosis*	Various

* Demonstrated in this project

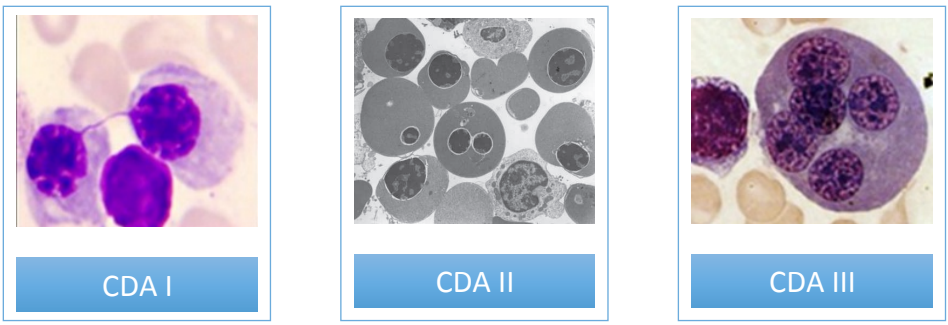

Figure 1. Bone marrow smears in the major forms of CDA. Erythroblasts with internuclear bridge in CDA I, a binuclear erythroblast in CDA II, and a gigantoblast in CDA III.
CDA I and CDA II photos reprinted with permission from Dr Tamary and Prof Iolascon, respectively.

Cell cycle, mitosis and cytokinesis

The cell cycle represents the events that enable a cell to duplicate. The process is strictly regulated, assuring genetic material to transfer properly through generations of cells. Correct segregation of chromosomes and the ability to fulfil cell division is highly dependent on microtubules and kinesins, which are the most central proteins of these processes (59, 60). The microtubules are filamentous structures running through the cell, building up the cytoskeleton and enabling transportation through the cell. The movement of the tubules and the ability to move cargo along the cytoskeleton is carried out by kinesins, so called motor proteins, with the ability to walk along the microtubules. More than 30 kinesins have been defined in the different stages of the cell cycle (59).

The cell cycle is either divided into five phases, GAP0 (G0), GAP1 (G1), Synthesis (S), GAP2 (G2), and Mitosis (M) or the three major stages of interphase, mitosis, and cytokinesis (61, 62). In G0, the cell is per definition not in cell cycle, since it is not dividing or preparing for cell division but fulfilling the tasks it was destined for. Going into G1 the cell grows and gets ready for DNA synthesis. When it is fully prepared for this task it enters the next phase, the S phase, where DNA is replicated. Thereafter it enters the phase of mitosis, which ends in cell division (61).

The definitions of interphase, mitosis and cytokinesis enable a more detailed description of the cell cycle since interphase and mitosis are subdivided into several steps and cytokinesis composes an entity of its own (61, 62). G1, S and G2 correspond to interphase while M includes mitosis and cytokinesis. Here I will focus on the steps of mitosis and cytokinesis.

Mitosis is divided into four major steps, prophase, metaphase, anaphase and telophase. In prophase, the chromatin condensates into the 92 chromosomes present after DNA duplication. The cell poles form as centrosomes are pulled apart by microtubules to opposite sides of the cell, forming the "mitotic spindle". In metaphase the chromosomes line up at the equator of the cell, each chromosome attached to one of the cell poles by microtubules. Sister chromatids line up tightly together, enabling segregation to opposite poles during anaphase. At the end of anaphase the mitotic spindle elongates as parallel microtubules form the "central spindle", further forming the cleavage furrow. At the end of telophase the furrow is contracted into the formation of the midbody, a dense bundle of microtubules that tightly connects to the plasma membrane (63).

The cell now enters the last step of the cell cycle, cytokinesis, which involves the processes resulting in the final division of the cells. As the daughter cells are defined, the midbody elongates forming an intercellular bridge. This structure is essential for final cleavage of the cytoplasm, as it enables membrane trafficking and membrane fusion (64).

Mutations of genes encoding for microtubules or kinesins can affect the cell cycle at all stages, depending on the gene involved. Malfunction of proteins, essential in cell division can cause damaged cells due to failure of correct segregation of chromosomes**.** In addition, impaired regulation mechanisms of the cell cycle can allow cells to divide out of control, being one of the fundamental conditions in cancer development**.**

In CDA III, erythropoiesis seems to be affected by failure of cytokinesis, since multiple nuclei gather in the same cell. Hence, mitosis seems to proceed properly until the very final step of cell separation. In this project we identify the causative gene in CDA III and show that the encoded protein is centrally involved in the final steps of cytokinesis.

Red cell membrane

The red cell is unique among human cells. In its mature form all cell organelles are lost, including the nucleus. Consequently, the properties of the cell are fully dependent on the membrane and the cytoskeleton. Apart from the skeletal network, the cytosol mainly consists of the oxygen transport protein hemoglobin (Hb). During the 120 days lifespan of an erythrocyte, the ability to undergo reversible deformation is essential for the capacity to pass through small capillaries, carrying oxygen to all parts of the body. The cell membrane is very strong and elastic, closely interacting with the cytoskeleton. Even as the red cell is highly extended passing through capillary vessels, this structure enables it to retain the surface area. This property is very important to protect the cell from undergoing hemolysis. The membrane consists of a bilayer of cholesterol and phospholipids, penetrated by transmembranous proteins anchoring the two-dimensional spectrin-based network of the cytoskeleton. More than 50 types of proteins penetrate the membrane in various quantities. About half of them carry different blood group antigens such as the Rhesus protein (Rh) and the oligosaccharides A and B (65).

The transmembrane proteins enable transportation through the membrane, adherence to other cells and activation of signalling pathways (65). The most abundant membrane proteins are band 3, also called anion exchanger 1 (AE1), and glycophorin A (GPA). Interacting with other proteins, they form vertical chains, linking the membrane to the cytoskeleton. Band 3 is a large protein whose integral part serves as a transporter of bicarbonate (HCO_3^-) and chloride (Cl^-) through the membrane. The cytosolic part links to spectrin through the anchoring proteins protein 4.2 and ankyrin. The function of band 3 can be influenced by horizontal interaction with GPA and the Rh-complex, which includes Rh-associated glycoprotein (RhAg), glycophorin B (GPB), intracellular adhesion molecule-4 (ICAM-4) and integrin associated glycoprotein (IAP) (66, 67). Dysfunction of the vertical linkage system leads to failure of red cell integrity and results in hemolysis.

Attached to the external surface of the membrane are glycolipids and glycoproteins, forming a glycocalyx surrounding the cell. Many of these molecules belong to the blood group system or are part of the complement system. The latter plays an important role in the defence against pathogens, and in the removal of damaged cells. Activation of the cascade system causes a cascade of protein activation resulting in hemolysis due to the formation of a membrane attack complex (MAC), which forms pores through the membrane. Regulation of complement activation is essential to protect normal red cells from undergoing hemolysis. Two of the most relevant proteins, in regard to hemolytic disorders, are the decay accelerating factor (DAF) and MAC-

inhibitory protein (MAC-IP) (68). Dysfunction of these proteins causes hemolysis due to failure of complement inhibition.

Hemolytic disorders

The first thing that must be defined in a patient with hemolysis is whether it is caused by autoantibodies or not. This is done by the direct antiglobulin test (DAT), also referred to as Coomb's test. The test reveals autoantibodies to the patient´s own red cells and is an important tool to distinguish between autoimmune and non-autoimmune mediated hemolysis. The most common form is caused by IgG-autoantibodies and often responds promptly to corticosteroids, while complement activating autoimmunity, through IgM-autoantibodies, is more difficult to treat. Autoimmune hemolysis is often connected to other diseases such as lymphoproliferative disorders, other malignancies, or autoimmune diseases (69).

DAT-negative hemolysis

Non-autoimmune hemolytic disorders or DAT-negative hemolysis is a diverse group of dysfunctions of red cells, leading to increased red cell turnover. Lysis is caused by alterations of the membrane, complement regulation, Hb-chains, or enzymes involved in red cell metabolism.

The clinical appearance varies widely from asymptomatic cases to requirement of regular transfusions and chelation therapy. A majority of these disorders are constitutional but the symptoms of hemolysis often vary throughout life, with more active episodes during, for example, infection or pregnancy. However, some disorders, such as paroxysmal nocturnal hemoglobinuria (PNH), are acquired and can be connected with other disorders or malignancies of the bone marrow.

Enzymopathies such as deficiency of glucose-6-phosphate dehydrogenase and pyruvate kinase, and hemoglobinopathies such as thalassemia and sickle cell anemia represent common causes of DAT-negative hemolysis in a global perspective. These diseases compose entities of its own, and pathogenesis and diagnostic approach in these disorders will not be further discussed here**.** Rather I will focus on the rarer forms of DAT-negative hemolytic disorders, the membranopathies and complement-induced hemolysis, where CDA is an important differential diagnosis to bear in mind.

Membranopathies

Hereditary spherocytosis (HS) is the most common hereditary membrane disorder in Caucasians with an incidence of about 1:2000. The disease is caused by deficiency or dysfunction of one of the proteins connected to the band 3 vertical linkage system: that is band 3, protein 4.2, ankyrin or spectrin. Pattern of inheritance is mostly dominant even if recessive cases have been described (70).

Hereditary elliptocytosis (HE) has a similar prevalence as HS but is more common in malaria endemic regions. Hereditary pyropoikilocytosis (HPP) and South Asian ovalocytosis (SAO) compose variants of the disease. HE and SAO are dominantly inherited while HPP has a recessive trait. The elliptic shape of the red cells in HE and HPP is due to dysfunction of the interaction between the cytoskeleton proteins actin, spectrin and protein 4.1. In SAO the interaction of band 3 and ankyrin is disturbed (70).

In the dominantly inherited disorder hereditary stomatocytosis, widening of the membrane pores causes defects in ion and water transportation (71). As in HS, the red cells fail to undergo proper deformation under stress, leading to increased phagocytosis of erythrocytes, mainly in the spleen.

Complement induced hemolysis

PNH is an uncommon acquired hemolytic disorder, sometimes connected to disorders such as aplastic anemia (AA) and bone marrow malignancies such as myelodysplastic syndrome (MDS). Intravascular hemolysis is due to deficiencies of DAF and MAC-IP, which causes inability to inhibit the progress of the complement cascade (72). Except for symptoms of anemia, thrombotic events and organ failure, such as pulmonary hypertension and kidney failure, are common complications.

Congenital dyserythropoietic anemia

Among the CDAs, the red cells in CDA II carry a double membrane and the glycosylation of band 3 is altered (12). In CDA I and CDA III minor aberrations have been found in membrane proteins in studies of single patients, including band 3 in CDA III (48, 73).

Diagnostic approach in DAT-negative hemolysis

Estimation of erythrocyte fragility

Malfunction of proteins involved in the vertical linkage system or cytoskeleton can result in red cells with spherical appearance, so called spherocytes. These cells fail to retain their surface area under deformation, which causes fragility and hemolysis. This vulnerability can be tested by different resistance tests. In the osmotic fragility (OF) test, the proportion of hemolysis is determined when red cells are suspended in a hypotonic solution. In the acid glycerol lysis time test (AGLT) and the Pink test, lysis is measured after suspension in buffered glycerol solutions (74).

Irrespective of the cause, these tests determine inability to resist stress but cannot differ between different membrane and cytoskeleton defects (71, 74). Both HS and CDA II will appear with raised fragility in the OF-test and shortened lysis time in the AGLT-test (74). In CDA III osmotic fragility has appeared normal, when studied in ten patients (46).

Flow cytometry

Surface antigens, identified by immunophenotyping, are named according to the Clusters of Differentiation (CD) protocol. They are present in various combinations in different cell types and stages of maturation and serve mainly as receptors or ligands. More than 350 CDs have been identified in human cells so far (75). By binding monoclonal antibodies to CDs, single cells can be identified by immunophenotyping or flow cytometry. In flow cytometry, a monoclonal antibody attached to a fluorochrome adheres to a specific CD or membrane protein. As each single cell passes through a laser beam, the fluorescence is detected and spotted in a scatter plot, enabling characterization of single cells.

In erythrocytes, the surface antigens MAC-IP and DAF are named CD59 and CD55, respectively. The transmembrane protein IAP is referred to as CD47. Consequently, deficiencies or abnormalities of these antigens can be detected by flow cytometry using antibodies against these CDs (76). Thus, the diagnosis of PNH mainly relies on flow cytometry with anti-CD55 and anti-CD59 (77).

The dye eosin-5´-maleimide (EMA) interacts with the first extracellular loop of band 3 and some Rh-related integral proteins, including CD47, and has become an important tool in the diagnostics of membranopathies of red cells (78, 79). HS, HPP and CDA II show reduced fluorescence with this method (80, 81).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

In this method denaturized proteins are separated by size as they pass through a gel. Band 3 will appear as the third band when this method is applied on the red cell membrane. Missing bands or bands with altered migration activity reveal deficiencies or structural changes of membrane proteins. The method can be useful in clinical practice when other methods have failed to give a distinct diagnosis (71). In HS, SDS-PAGE will, in 90 % of cases, reveal deficiencies or abnormal migration of band 3, ankyrin, spectrin or protein 4.2. In CDA II, band 3 will have a faster migration and appear slightly thinner compared to normal (12). Studies of two patients with CDA III showed no major abnormalities apart from a minor alteration of migration of band 3, indicating minor reduction of glycosylation of this protein (48).

Diagnostic considerations

As many genes, causing DAT-negative hemolytic anemias, have been defined, molecular approaches can be helpful to set the diagnosis. However, molecular screening of a panel of known involved genes is not yet available in clinical routine (70). Thus, the initial investigation with resistance tests and flow cytometry still constitutes the investigational basis of DAT-negative hemolysis. As CDA II can be misdiagnosed as HS using both the resistance tests and flow cytometry with EMA, the investigation should be complemented with a bone marrow aspirate. In case of CDA, further subtyping should be done by molecular genetics. In this work we investigate the erythrocyte membrane in CDA III with flow cytometric analysis, used in clinical routine in the diagnostic work-up of DAT-negative hemolysis.

Iron

Iron homeostasis

Being an important part of the Hb-molecule, with a major capacity to link oxygen, iron (Fe) plays a crucial role in the transportation of oxygen around the body. On the other hand, excess and accumulation of iron is highly toxic, causing organ failure due to toxic oxidative processes. To keep a favourable balance of this two-edged metal, iron homeostasis needs to be strictly regulated.

The body contains about four to five grams of iron, whereof about two thirds bound in the red cells. Iron can neither be produced nor degraded by the body. Thus, intake through absorption in the duodenum needs to be in equilibrium with losses, mainly through the stools. Daily intake of iron is about 10-20 mg per day, of which about 10 % is absorbed. After absorption by iron transporters on the surface of enterocyte, iron is further transported to the blood by the iron exchange channel, ferroportin. When released into the blood, iron binds to transferrin (Tf) and is further transported around in the body as a Tf-Fe-complex. A majority (approx. 80 %) is directed to the bone marrow to take part in erythropoiesis. The remaining 20 % is transported to other organs to participate in metabolic processes. The Fe-Tf-complex is internalized into cells by the transferrin receptors (TfRs). Excess of cellular iron is stored mainly as ferritin and to a minor extent as hemosiderin. The pool of reserve iron is mainly kept in hepatocytes and macrophages (82).

Erythrocytes are degraded mainly in the spleen and iron is then redirected to the bone marrow to be recycled in the build-up of red cells. A healthy individual loses about 1-2 mg of iron every day through epithelial shedding, mainly from the intestine, but also from the skin and urinary tract. In addition, fertile women lose iron every month due to menstruation (82).

Despite periods of larger losses as in delivery or in minor trauma, iron plasma concentration and iron reserves are kept relatively constant due to the feedback mechanism of hepcidin. Hepcidin is a small peptide, produced by the liver, which binds to and inhibits the iron transporter ferroportin. In a healthy individual, a situation of iron deficiency with diminished reserves and low plasma iron concentration causes reduced production of hepcidin. This results in a reduction of ferroportin inhibition and consequently enhanced ability to absorb iron from the food. In parallel, iron release from the storages in hepatocytes and macrophages is enhanced. On the other hand, in a situation with filled iron stores, hepcidin is increased, resulting in inhibition of ferroportin. This leads to reduction of iron uptake by enterocytes and decreased release of iron from macrophages and hepatocytes (83).

Hepcidin is also known to be suppressed by ineffective erythropoiesis, through an erythroid regulation pathway. A pathway that is not clearly understood, but begins to clarify. Overexpression of Growth Differentiation Factor 15 (GDF15) in CDA I and CDA II has, as in thalassemia, been shown to limit hepcidin expression (84-86). However, in more recent studies GDF15 has not been found to contribute to hepcidin suppression in anemias with effective erythropoiesis such as iron deficiency, anemia of chronic disease and myeloproliferative malignancies (87). Its role in ineffective erythropoiesis remains to be elucidated. In 2014, Krautz et al identified the new hormone erythroferrone (ERFE), produced by erythroblasts in response to erythropoietin. Further they showed that ERFE is needed for the mediation of hepcidin suppression in situations with expanded erythropoiesis in mice after phlebotomy or EPO-injections. In mice with thalassemia, thus with ineffective erythropoiesis, ERFE was shown to be highly expressed, leading to suppression of hepcidin (88). Levels of ERFE in CDAs have not been reported.

Iron overload

Iron overload is a big clinical problem in patients with chronic anemias, in need of regular transfusions. In hemolytic disorders, such as thalassemia and CDA I and CDA II, iron overload is most often present even in non-transfused patients. As described above, this is due to disturbed regulation of hepcidin, caused by ineffective erythropoiesis (84-86, 88). In addition, many patients with thalassemia, CDA I and CDA II suffer from secondary hemochromatosis due to transfusions. In clinical practice, iron overload can be estimated by ferritin, transferrin iron saturation (TSAT) and magnetic resonance imaging (MRI) of the liver or heart, or eventually liver biopsy. Treatment is based on chelation therapy or, in cases without marked anemia, regular phlebotomies.

Hereditary hemochromatosis (HH) is a recessive disorder, caused by mutations in the hemochromatosis gene (*HFE*). The HFE protein, situated on the surface of hepatocytes, plays a central role in the feedback system of hepcidin synthesis. HFE influences the expression of hepcidin through interaction with TfR, increasing hepcidin synthesis as TfRs are activated. Dysfunctional HFE leads to failure of this delicate feedback system and hepcidin synthesis is not increased even in a situation of iron overload. Two *HFE* mutations (C282Y and H63D) are frequently found in Europe (89). Homozygous mutation of C282Y or compound heterozygosity of C282Y and H63D may result in clinical signs of hemochromatosis. Penetrance is low, estimated in the literature to 10-30 % (89). Heterozygous C282Y is found in 7 % of the Swedish population (90). Heterozygosity for H63D is even more frequently found all over the world, with a prevalence of about 10-20 %, but does not by itself lead to significant iron overload in otherwise healthy individuals (89).

In earlier studies, patients with CDA III were not found to have higher ferritin levels compared to their healthy siblings (47). This has been explained by the continuous loss of iron in the urine, due to intravascular hemolysis present in CDA III.

However, in this project we found that some individuals in the Västerbotten CDA III family did have increased levels of ferritin. As mutations of the *HFE* gene were found in these individuals we decided to screen the entire family for C282Y and H63D. In this project we study the prevalence of *HFE* mutations in the Västerbotten CDA III family and investigate the clinical appearance concerning iron balance in patients with coexistence of CDA III and mutation of the *HFE* gene.

AIMS

The overall aim of this project was to investigate the genetic etiology of CDA III.

Furthermore, we wanted to study CDA III in a clinical perspective, concerning diagnostic approach and morbidity connected to iron metabolism.

Specific aims were

- to identify the causative gene in CDA III.

- to examine the erythrocyte membrane in CDA III with flow cytometry.

- to study the presence and clinical effect of *HFE* mutations in individuals of the Västerbotten CDA III family.

Study population and methods

Study population

Individuals from the Västerbotten CDA III family form the basis of these studies. Furthermore, six individuals from the American CDA III family are included in paper I. The Västerbotten CDA III family consists of six generations reaching back to the middle of the 19th century. In this project, the pedigree has been updated and complemented with a few children in the 6th generation and some adults. Today the family consists of 85 individuals with confirmed risk of having CDA III. CDA III has been established in 47 of them, while 28 are non-affected siblings. Except for the two probands, eight individuals have not been examined, five are deceased, one lives abroad, and two did not want to participate (Figure 2).

In paper I, analyses were performed on DNA from 60 individuals from the Västerbotten CDA III family (39 CDA III positive and 21 CDA III negative siblings) and six individuals from the American CDA III family (four affected and two unaffected). Furthermore, DNA from 356 control individuals from a geographically matched Swedish population was analysed. CDA III status was confirmed by Hb, haptoglobin, LD, thymidine kinase or bone marrow morphology.

In paper II, 16 individuals with CDA III and 14 non-affected siblings were included in the flow cytometry analyses with EMA. Three normal controls were used per assay. Flow cytometry with anti-CD55 and anti-CD59 was performed on erythrocytes from 12 CDA III positive and 7 CDA III negative relatives with one normal control per assay.

In paper III, *HFE* genotyping was performed in 37 CDA III positive and 21 CDA III negative siblings. Further evaluation, concerning hematological parameters and iron status, was performed in 32 CDA III patients and 18 CDA III negative siblings.

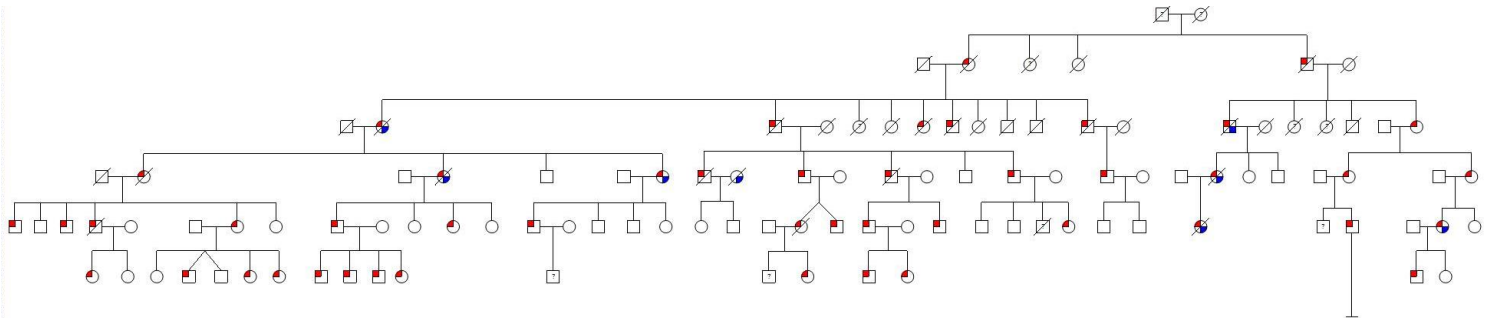

Figure 2. Pedigree of the Västerbotten CDA III family.

? Not examined

CDA III

MGUS/myeloma

Methods

Paper I

Targeted sequencing and data analysis

DNA samples from 1 affected and 1 unaffected sibling from the Västerbotten CDA III family were sent to Ambry Genetics (Aliso Viejo, CA) (http://www.ambrygen.com) for targeted sequencing of the candidate region at 15q23. As a result of haplotype reevaluation with microsatellite markers, the earlier defined region between 15q21-25, was reduced to an interval of 2.41 Mb between markers D15S100 and D15S1050 (69571161-71981252), based on a current version of genome sequence (hg 19, BUILD 37.2). For further analyses we decided to cover the region of 3.54 Mb between markers D15S100 and D15S980 (69571161 - 73113373) (Figure 3).

Figure 3. After haplotype reevaluation, the disease-causative gene was expected to reside in a region of 2.4 Mb. For further analyses with targeted re-sequencing, a region of 3.5 Mb was chosen.
Reprinted with permission from Blood. Paper I. CDA III is caused by mutation in *KIF23*.

Library preparation and indexing were performed using Roche NimbleGen 385k sequence capture array target enrichment, and 54-bp paired-end processing was done using the Illumina GAIIx (San Diego, CA). Initial data processing and base calling, including extraction of cluster intensities, was done using RTA1.8 (SCS version 2.8). Sequence quality filtering script was executed in the Illumina CASAVA software (version 1.7.0). The reads were mapped against University of California Santa Cruz hg19.

Bioinformatics

All bioinformatics tools were available via the Alamut software version 2.0 (Interactive Biosoftware, Rouen, France).

Sequence variants found in the two sisters analysed with targeted sequencing were investigated by bioinformatics to evaluate impact on protein function. Missense mutations were analysed by Sorting Intolerant from Tolerant (http://sift.jcvi.org) and Polymorphism Phenotyping (http://genetics.bwh.harvard.edu/pph). Variants detected in intronic sequences were analysed with the splice site prediction programs GeneSplicer (http://www.cbcb.umd.edu/software/GeneSplicer) and Splice Site Finder (www.genet.sickkids.on.ca/ali/splicesitefinder).

Sequencing of KIF23, MYO9A and TLE3

For bidirectional sequencing of *KIF23* (MIM605064, ENSG00000137807), intronic sequences adjacent to exon 21 were amplified from genomic DNA. Sequences for *KIF23* primers were 21F:5´gctcattttggaggaacagaa and 21R:5´gggagttcctgatgaagtgg, designed with Primer3 software**.** Amplification and sequencing were performed by polymerase chain reaction (PCR). The products of sequencing reactions were analysed on ABI 3500xL Dx Genetic Analyser (Applied Biosystems, Foster City, CA).

Sequencing of *MYO9A* and two *TLE3* variants was similarly done by PCR. Primers are reported in Paper I.

Segregation analysis

To investigate whether mutations of *KIF23, MYO9A* or *TLE3* segregated in the CDA III family, restriction fragment length polymorphism analyses was done. For digestion of *KIF23* exon 21, PCR-products were digested by endonuclease, *Hpy*CH4IV, separated by electrophoresis on an agarose gel and visualized after staining with ethidium bromide.

KIF23 expression

Two known isoforms of KIF23, generated by alternative splicing of exon 18, are described in the literature (91). One full-length transcript containing exon 18, ENST00000260363, encoding a 960 AA protein and one form lacking exon 18 (Δ18), ENST00000559279, encoding a 856 AA protein. RNA from normal tissues was obtained from First Choice Human Total RNA Survey Panel (Ambion Life Technologies, Carlsbad, CA). We used RNA from brain, colon, heart, kidney, liver, lung, small intestine, spleen, thymus, placenta, ovary,

skeletal muscles, prostate, testis, and thyroid. In addition, RNA was extracted from lymphocytes of whole blood of a CDA III patient and one control case. Reverse transcription (RT)-PCR was performed with *KIF23*-specific primers to detect the two known transcripts, along with control primers for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). RT-PCR products were separated and visualised as in the segregation analyses. Primers are reported in Paper I.

KIF23 functional analysis

Knock-down and rescue analyses were done by the research group of Masanori Mishima, Associate Professor, Division of Biomedical Cell Biology at University of Warwick in the United Kingdom. Analyses were done in HeLa cells. Interfering RNA (siRNA), targeted to the 3´-untranslated region of *KIF23* was used to block translation. DNA construct of the *KIF23* **Δ**18 (ENST00000559279) with or without P916R mutation was introduced into the cells, both tagged with a green fluorescent protein (GFP). Time lapse observation by differential interference contrast and GFP fluorescence microscopy was performed to visualize the process of cell division and cytokinesis.

Statistical analysis

No statistical analyses were done to confirm the relationship between CDA III and *KIF23* c.2747G>C since 100 % of CDA III patients expressed the mutation and none of the CDA III negative siblings nor the control population carried the mutation. Data on cytokinesis failure were analysed by the research group of M Mishima, with generalized linear model for binomial data using R (http://www.r-project.org).

Paper II

Peripheral blood was obtained for analyses of Hb, erythrocyte mean cellular volume (MCV), and flow cytometric assays.

Flow cytometry

Flow cytometric assays, measuring fluorescence intensity of intact red cells after incubation with EMA were performed on a BD Biosciences FACS Calibur flow cytometer and Mean channel fluorescence (MCF) levels were determined by Cellquest software. Mean channel fluorescence (MCF) was determined for 30000 events. Each assay contained one study sample from a patient of the Västerbotten CDA III family together with three normal controls, randomly

selected from the Department of Laboratory Chemistry, University Hospital of Umeå.

In flow cytometry regarding CD55 and CD59, each study sample was analysed together with one normal control. 15000 events were recorded. A threshold for populations of erythrocytes expressing CD55 and CD59 in the normal control was identified and set at the same level in the study sample.

Statistical analyses

Ratios of MCF in study samples and controls in each assay were calculated. Study sample MCF and control MCF values were normally distributed according to Kolmogorov-Smirnov test. Results were presented as MCF ratios and range. The Sign test was used to compare MCF of study samples and their normal controls. $P < 0.05$ was regarded as significant. Pearson's correlation test was used for analysis of correlation between EMA fluorescence and MCV.

Paper III

Detection of HFE mutations

HFE genotyping was performed as in clinical routine, at the Department of Clinical Genetics, University Hospital of Umeå. Applied Biosystems® Assay-by-design TaqMan probes were used to establish presence of C282Y and H63D.

Clinical appearance

Peripheral blood was obtained for analyses of iron status (ferritin and TSAT) and hematological parameters (Hb and LD). Ferritin and TSAT were analysed according to standard procedures at the Department of Laboratory Chemistry, University Hospital of Umeå. Samples were collected between 1997 and 2015. All individuals with *HFE* mutation and elevated ferritin or TSAT were offered clinical and laboratory investigation to confirm or reject iron overload. Phlebotomy was initiated in individuals with iron overload.

Statistical analyses

Ferritin, TSAT, Hb and LD are presented as median values and range. Two-sided Mann-Whitney U-test was used for comparison between groups. $P < 0.05$ was regarded as significant.

Results

Paper I

CDA III and KIF23

Targeted sequencing of two individuals in the Västerbotten CDA III family, revealed ten unique sequence variants which were present in the CDA III positive woman and absent in her unaffected sister. Six homozygous variants were excluded from further analyses, since CDA III is dominantly inherited. As shown in table 2, four sequence variants in three genes were further investigated. *KIF23* was the most promising candidate gene since the others were either found, by the bioinformatics tools, to be relatively common in the general population or did not result in any shift of amino acid (Table 2). Sanger sequencing confirmed sequence variants in *KIF23, MYO9A* and *TLE3*. As shown in figure 4, the amino acid substitution due to *KIF23* c.2747G>C results in p.P916R (Figure 4).

Table 2. Heterozygous sequence variants in CDA III. Mutation, amino acid change, and evaluation by bioinformatics tools concerning mutational consequence on protein function and prevalence in general population.

Gene	Nucleotide change	Amino acid change	SIFT[1]	PolyPhen[2]	MAF[3]
KIF23	c.2747C>G	p.916P>R	Not tolerated	Deleterious	Not available
MYO9A	c.4892T>A	p.1631N>I	Not tolerated	Possibly damaging	A=0.011/24
TLE3	c.615G>C	p.205S>S	Not known	Not known	Not available
TLE3	IVS297+3T>CT		Not known	Not known	C=0.228/497

1. Sorting Intolerant From Tolerant. 2. Polymorphism Phenotyping
3. Minor Allele Frequency (established in 1000 Genome project)

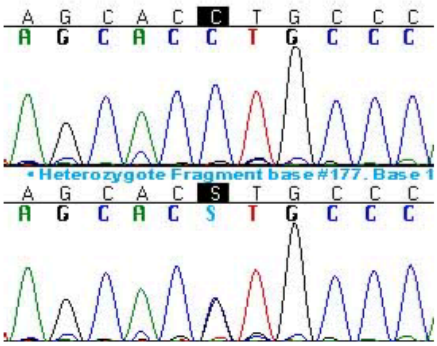

Figure 4. DNA sequence of wt *KIF23* (upper) and heterozygous *KIF23* c.2747C>G (lower) with mutation position marked in black.
Reprinted with permission from Blood. Paper I. CDA III is caused by mutation in *KIF23*.

Segregation analysis was performed for all sequence variants shown in table 2. *MYO9A* and the two *TLE3* variants did not segregate with CDA III in the Västerbotten CDA III family. In addition the *MYO9A* variant was detected in 6.5 % of the matched controls, a frequency much higher than previously reported (Table 2).

KIF23 c.2747C>G was found to segregate with CDA III in the Västerbotten CDA III family as well as in the American CDA III family (Figure 5). The mutation was present in heterozygous form in 100 % of CDA III positive patients and in none of the unaffected siblings. Furthermore the mutation was absent in 356 healthy controls from a matched Swedish population. The p.P916R mutation has not been described in the literature previously and was thus novel to both the Swedish and the American family.

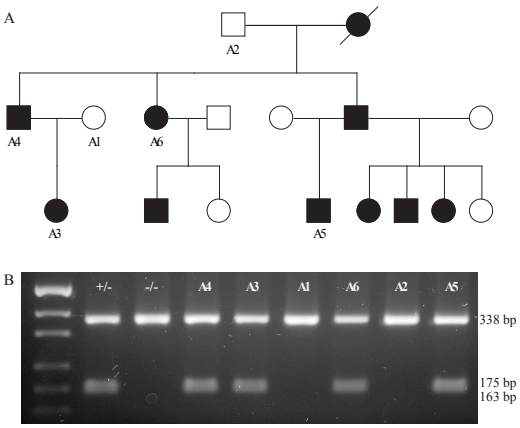

Figure 5. *KIF23* c.2747C>G segregates with CDA III.
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A: Pedigree of the American family, black boxes indicating CDA III and unfilled boxes indicating unaffected siblings.
B: PCR-products of *KIF23* exon 21 digested by the *Hpy*CH4IV endonuclease. In case of *KIF23 c.2747C>G*, sequencing product is digested into two. Wt *KIF23* presenting as one band at 338 bp and *KIF23 c.2747C>G* resulting in two bands at 175bp and 163 bp. Consequently, CDA III cases with heterozygous mutation in *KIF23* will present three bands and unaffected persons just one.

KIF23 expression

Primers specific for exons 16 and 19 detected 2 bands (391 bp and 211 bp) that correspond to the earlier described **Δ**18 transcript and a novel transcript lacking both exons 17 and 18 (**Δ**17+18) (Figure 6). We also expected a band of 703 bp, corresponding to the full-length transcript, but we failed to visualise this on the gel, although the presence of this transcript was evident by the primers specific for the exons 17 and 18, resulting in a band of 474 bp.

Expression of Δ18 was detected in all examined tissues including peripheral CDA III lymphocytes, while expression of 17+18 (corresponding to full-length) and Δ17+18 was found to be tissue dependent (Figure 7).

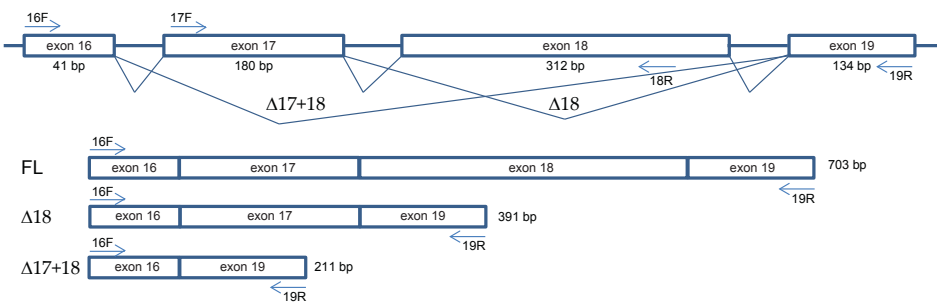

Figure 6. Transcripts of *KIF23* using primers of 16F, 17F, 18R and 19R. Full-length, Δ18 and Δ17+18 illustrated with the expected sizes of the RT-PCR products.
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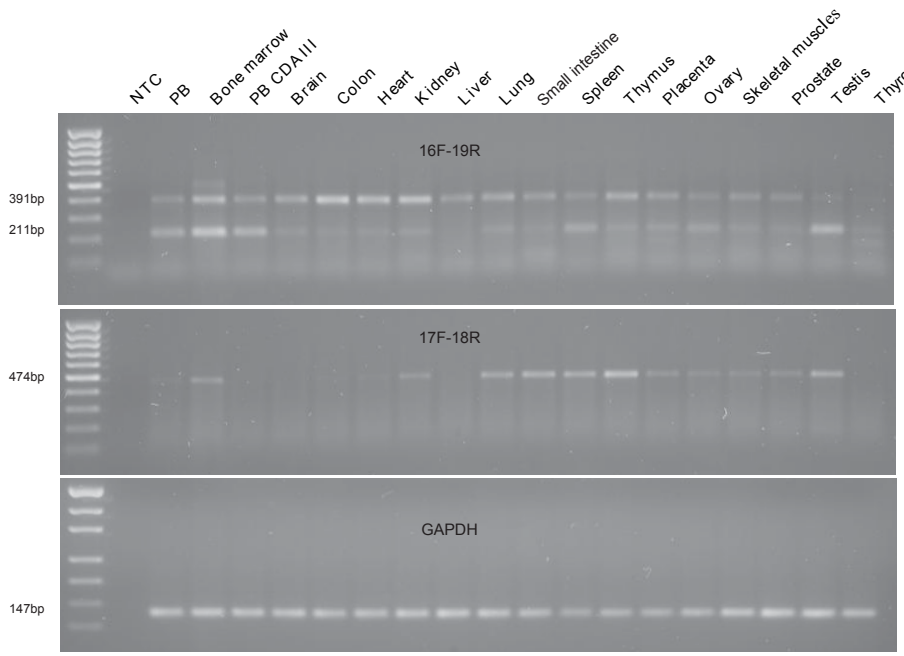

Figure 7. Detection of *KIF23* transcripts and control (*GAPDH*) transcript in a broad range of tissues. RT-PCR with primers 16F and 19R detected the **Δ**18 and **Δ**17+18 transcripts (upper). Full-length was not detected but its presence was confirmed with primers 17F and 18R (middle).
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KIF23 functional analysis

In anaphase the central spindle microtubule bundle forms between segregating chromosomes, with KIF23 highly accumulating to the central antiparallel overlap zone. The cleavage furrow forms and matures into the midbody. The intracellular bridge is maintained several hours until the final abscission of the cells (Figure 8).

Depletion of endogenous KIF23 caused failure of cytokinesis, due to premature breakdown of the intracellular bridge. This failure was efficiently rescued by expression of wt KIF23.

However, cells expressing KIF23 p.P916R could complete constriction of the cleavage furrow and form the midbody in a similar time course with the cells

expressing wt KIF23, but regressed to form binucleate cells about 2 hours after midbody formation (Figure 8). These data indicate that the p.P916R mutation impairs the function of KIF23, essential for the completion of cytokinesis.

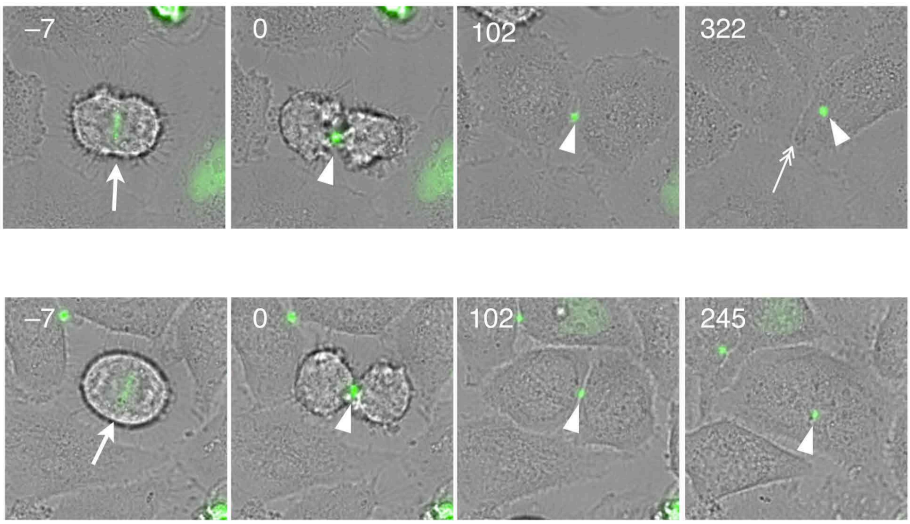

Figure 8. Upper images showing complete cell division with wt KIF23.
KIF23 p.P916R mutant (lower) was localized to the spindle midzone (white arrow) and condensed to form the midbody (arrowheads) in a similar manner to the wt KIF23 (upper white arrow and arrowheads). However, the cell membrane of the intercellular bridge was detached from the midbody approx. 3,5 hour after its formation, creating a binucleate cell (lower). The remnant of the midbody (arrowhead) was incorporated to the cell on the right after proper cell division (upper), and incorporated into the binucleate cell after cytokinesis failure (lower).
Reprinted with permission from Blood, paper I; CDA III is caused by mutation in *KIF23*.

Paper II

Flow cytometry in CDA III

Mean EMA MCF was higher (1.08) in the CDA III positive group according to the controls in each analysis (1.0) but did not differ between CDA III negative siblings (0.99) and their normal controls (1.0). The slightly increased EMA fluorescence in CDA III patients was significant ($p<0.001$) (Table 3). The expression of CD55 and CD59 did not differ between the CDA III positive patients and their controls, nor between the CDA III negative siblings and their normal controls (Table 3). There was a significant correlation between EMA-MCF and MCV ($r=0.72$, $p=0.01$).

Table 3. Flow cytometry with EMA, anti CD55 and anti CD59 on erythrocytes in CDA III and healthy siblings in the Västerbotten CDA III family.

		CDA III pos./controls	**CDA III neg. relatives/controls**	
	n	**median ratios and (range)**	**n**	**median ratios and (range)**
EMA MCF	16	1.08 *(1.01-1.17)	14	0.99 (0.89-1.08)
CD55	11	0.99 (0.96-1.0)	7	1.04 (0.99-1.32)
CD59	11	1.01 (0.96-1.15)	7	0.99 (0.97-1.02)

* $p< 0.001$

Paper III

HFE status

HFE genotyping was successfully performed in all 58 cases. *HFE* mutation was found in 24 out of 37 CDA III patients (65 %) and 13 out of 21 CDA III negative siblings (62 %). Among the 50 cases with available iron status, 21 out of 32 CDA III positive (66 %) and 11 out of 18 CDA III negative siblings (61 %) were found to have *HFE* mutations. 16 of the CDA III patients with *HFE*-mutation were heterozygous either for C282Y or H63D, and five were compound heterozygotes. Seven of the 18 CDA III negative siblings were heterozygous carriers of *HFE* mutations, two were compound heterozygotes, and two had homozygous *HFE* mutations. In individuals with heterozygous C282Y (9 CDA III positive and 6 CDA III negative cases), ferritin levels were significantly higher in CDA III patients, p=0.036.

Clinical appearance

The group of patients with CDA III and available iron status and hematological parameters (Hb and/or LD), consisted of 16 males and 16 females with a median age of 53 years, and the CDA III negative group consisted of 10 males and 8 females, with a median age of 44 years. Median Hb among CDA III patients was 116 g/L (94-157) and LD 6.2 ukat/L (4.2-10). In the CDA III negative group median Hb was 141 g/L and LD 2.7 ukat/L. Ferritin was higher (108 umol/L) in the CDA III positive group than in the CDA III negative group (76 umol/L), and TSAT was also higher among CDA III patients, 46 % vs. 34 %, but the differences were not significant (p=0.055 for both comparisons).

Laboratory evidence of iron overload was noted in four patients, one CDA III negative case with homozygous C282Y, and three CDA III positive individuals with heterozygous *HFE* mutations (Table 4). Magnetic resonance imaging (MRI) of the liver and S-hepcidin analysis was performed in the CDA III positive patient with heterozygous H63D mutation. Liver iron was 6700 ug/g dry tissue (normal range 300-1500) and hepcidin was 46 ug/L (normal range 8-76). To normalize ferritin and TSAT, treatment with phlebotomy was started in four patients; the CDA III negative sibling who was homozygous for C282Y and three CDA III patients, one compound heterozygote and two with heterozygous mutations, one C282Y, and one H63D.

A total of 18 individuals, 11 CDA III cases and 7 CDA III negative siblings, had wt *HFE*. Ferritin levels did not differ between these groups (p=0.86) One of these CDA III positive males, suffering from hypertension and hyperlipidemia,

had elevated ferritin (642 ug/L) but TSAT was not elevated, 40%. In another CDA III positive male without *HFE* mutation, TSAT was elevated (73%) due to subnormal transferrin 1.68 (reference interval 1.87-3.19 g/L). Ferritin was not elevated in this patient, 191 ug/L.

Table 4. Subjects who have started treatment with phlebotomy due to signs of iron overload.

CDA status	**Gender and age (years)**	***HFE* status**	**Ferritin (ug/L)**	**TSAT (%)**	**Hb (g/L)**	**LD (ukat/L)**
Wt	F 49	C282Y +/+	229	79	153	2.1
CDA III +	M 60	C282Y +/-	987	100	143	5.2
CDA III +	F 61	C282Y+/- H63D +/-	700	55	111	6.1
CDA III +	M 60	H63D +/-	1084	80	135	6.3

Discussion

In this project we found a genetic cause of familial CDA III. Furthermore we investigated the flow cytometric profile of CDA III erythrocytes from a clinical perspective and conclude that mutations of the *HFE* gene, even in heterozygous form, can cause iron overload in CDA III patients.

Paper I

In paper I, stepwise laboratory work using targeted sequencing, prioritization of gene sequences with the help of bioinformatics tools, Sanger sequencing and segregation analyses finally enabled us to identify *KIF23* as the causative gene in CDA III. The mutation *KIF23* c.2747C>G causes cytokinesis failure as the encoded motor protein, kinesin-like protein KIF23, also called mitotic kinesin-like protein 1 (MKLP1), is a crucial player in the formation of the midbody and intercellular bridge. Together with the GTPase regulating protein CYK4, KIF23 forms a heterotetramer, which builds up the protein centralspindlin (92). This protein enables assembly of microtubules in the midbody and through the C-terminus of CYK4, adheres the plasma membrane to the intercellular bridge. This connection makes the intracellular bridge reside several hours before final abscission (63, 93). Several proteins regulate the clustering and adherence capacity of centralspindlin. By binding a 14-3-3 protein, centralspindlin is inactivated, unable to carry out clustering or anchoring activity. At the central spindle, Aurora B disconnects the 14-3-3 protein, switching centralspindlin into an active state. As Aurora B activity peaks between segregating chromosomes in late anaphase and early telophase, centralspindlin is activated as the cell finalizes the last steps of mitosis and enters cytokinesis. This enables centralspindlin to perform the clustering activity that will finally form the midbody. The activity of Aurora B then rapidly declines. Thereafter the activated state is withheld by the GTPase ARF6, which by competitive binding prevents 14-3-3 to bind to, and inactivate, centralspindlin. ARF6 localizes to the midbody and makes sure that centralspindlin remains active until the final abscission of the cells (94).

Thus, as centralspindlin plays a crucial role in cytokinesis, dysfunction of one of its components, KIF23, results in cytokinesis failure and the multinucleated cells, which are the hallmark of CDA III (Figure 9) (95).

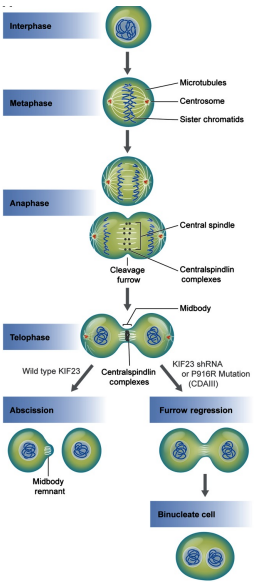

Figure 9. Failure of cytokinesis, due to mutation in *KIF23*.
This figure was originally published in Blood (95), reprinted with permission from Blood
Traxler E. Congenital dyserythropoietic anemias: III's a charm.
Blood 2013;121(23):4614-5.

Failure of controlling and regulating the steps of the cell cycle and mitosis are hallmarks of cancer development. The high proliferative ability of cancer cells also requires an excess of proteins, active in the mitotic processes. The last possibility in the cell cycle to slow down or stop proliferation is the step of cytokinesis. As *KIF23* is a key regulator of this final process, knowledge about its involvement in cancer is an area of interest.

KIF23 expression is cell cycle dependent, being suppressed in interphase and upregulated during S-phase. Interestingly, the tumor suppressor gene *p53* has been shown to reduce expression of *KIF23*. This repression is due to downregulation of *KIF23* promoter activity. Thus, mutations of the promoter region of *KIF23* or mutations of *p53* can result in uncontrolled proliferation (96).

KIF23 has recently been shown to be overexpressed in lung-, breast-, gastric- and hepatocellular cancer as well as in glioma (97-100). In addition, down regulation of *KIF23* suppresses cell division in glioma, breast cancer, and lung cancer cell lines as well as in a glioma mouse model (97, 99, 100). In gastric cancer *KIF23* was found to be upregulated in cell lines with resistance to the

cytotoxic drug paclitaxel, indicating poorer prognosis (98). In lung cancer patients, high expression of *KIF23* was associated with poor overall survival as well as poor recurrence-free survival five years after initial treatment (97). These studies indicate that knowledge of *KIF23* expression could be a tool regarding treatment strategy and possibly a future therapeutic target.

Little is known about involvement of *KIF23* in hematological malignancies, but CDA III patients have a higher incidence of myeloma and its precancerous condition MGUS (47). Thromobopoiesis and granulopoiesis seem to be unaffected by the mutation in CDA III patients, in spite the fact that *KIF23* is expressed in all cells, also myeloid and lymphatic cells (12, 48). Cell morphology in organs other than the bone marrow has not been studied in CDA III patients.

It is becoming clear that centralspindlin works in the context of a large protein-protein interaction network, which includes other microtubule-bundling proteins whose functions and activities seem to be able to overlap (60). It is possible, that cells make this protein-protein network adapt to their specific requirements, depending on developmental and physiological conditions. The erythropoiesis-specific cytokinesis failure in CDA III might reflect a lower tolerance of the cells in the erythropoietic lineage to the defect in *KIF23* than that of other cells. Cytokinesis failure caused by *KIF23* mutation in the lymphoid lineage might explain the higher incidence of myeloma and monoclonal gammopathy in CDA III patients.

Paper II

In paper II, we analyse the erythrocyte membrane in CDA III with flow cytometry, commonly used in clinical practice when investigating patients with DAT-negative hemolysis. Earlier studies have indicated some abnormalities of band 3 (48), but our study shows no major abnormality of the red cell membrane in CDA III. Furthermore, flow cytometry with EMA and anti CD59 cannot be used to distinguish CDA III from normal controls. Neither can CDA III be misdiagnosed as HS or PNH when using these methods. In CDA II and HPP, flow cytometry with EMA shows reduced fluorescence, which can lead to an incorrect diagnosis of HS (74, 101).

The affinity of EMA to the membrane protein band 3 is high, which is the main cause of lower fluorescence in HS. To a minor extent EMA also interacts with CD47 and Rh-antigen, also reduced in HS, contributing to the reduced fluorescence after EMA staining in this disease (78). The reduced fluorescence in CDA II is probably not due to a quantitative reduction of band 3 in the

erythrocyte membrane but rather an affected configuration, leading to a decreased affinity to EMA (79).

The subtle increase in EMA fluorescence in CDA III is probably due to the increase in erythrocyte volume (MCV) exposing a larger surface per cell to binding of EMA to band 3, Rhesus-antigen and CD47. Earlier data on the correlation of MCV and MCF in flow cytometry with EMA is a bit contradictory. Correlation has been confirmed in individuals with high MCV due to overconsumption of alcohol, but in DAT-positive hemolytic anemia no correlation between MCV and MCF has been observed (80). Low MCV due to iron deficiency does not seem to affect EMA MCF (79-80).

Since methods analysing erythrocyte fragility can also be misleading for differentiating between HS and other DAT-negative hemolytic anemias, caution should be taken in the diagnostic work up of these diseases. If there is no family history of CDA, the diagnosis is easy to overlook in the absence of a bone marrow investigation. When a bone marrow smear raises the suspicion of CDA, further subclassification should be carried out by molecular genetics, searching for causative genes, which are now defined in the three major forms of CDA**.**

Paper III

In paper III, we show that mutations of the *HFE* gene are present in the Västerbotten CDA III family. 65 % of CDA III positive cases and 62 % of their CDA III negative siblings carried C282Y and/or H63, the two major *HFE* mutations present in a European population. Two CDA III patients with heterozygous *HFE* mutations, one CDA III patient who was compound heterozygous, and one CDA III negative case with homozygous C282Y, needed treatment with phlebotomy due to iron overload.

One of the heterozygous cases carried H63D, which is not reported to lead to iron overload by itself in otherwise healthy individuals. MRI of the liver confirmed iron overload in this patient. Hepcidin was in the normal range, indicating dysfunction of the feedback system, which in a healthy individual would stimulate hepcidin expression due to iron overload. We suggest that ineffective erythropoiesis suppresses hepcidin through the ERFE-pathway and that *HFE* mutation contributes to further reduction of hepcidin, leading to iron overload.

All CDA III patients with *HFE* mutations do not develop iron overload. It is also known, even in healthy individuals, that the penetrance of *HFE* mutations is

relatively low, with about only 10-30 % of homozygous C282Y carriers developing hemochromatosis (89). Thus, there are other factors protecting the individual from accumulating iron. In addition it is also a question about time and gender. In the case of CDA III, as earlier stated, the continuous loss of iron in the urine may contribute to keep the balance in iron homeostasis (47).

None of the family members with wt *HFE*, nor healthy siblings carrying heterozygous or compound heterozygous *HFE* mutations, had laboratory signs of iron overload. One CDA III positive individual with wt *HFE* had elevated ferritin and one had elevated TSAT. Further investigation showed that these patients did not fulfil the criteria of iron overload.

This study indicates that heterozygous *HFE* mutation, even H63D, can cause iron overload when coexisting with a hemolytic disorder such as CDA III. Earlier studies on *HFE* mutations in patients with hemolysis where iron overload can be a problem, such as thalassemia and myelodysplastic syndrome (MDS), are contradictory. Some studies show iron overload in thalassemia patients with co-existing *HFE* mutations, even in heterozygous forms (102-104). Recently, non-transfused MDS patients with heterozygous *HFE* mutations were found to have higher ferritin level and inferior overall survival compared to MDS patients with wt *HFE* (105). However, other studies have not verified that heterozygous mutation affects iron overload in thalassemia (106, 107).

So far, phlebotomies have been performed without problems in spite of the continuous hemolysis in our CDA III patients, and phlebotomy alone is sufficient to control ferritin and TSAT levels.

Conclusions

- CDA III is caused by mutation in *KIF23*. The mutation causes dysfunction of KIF23 (MKLP1), which plays a central role in the last step of cytokinesis. Failure of this process results in the multinucleated erythroblasts, which are the hallmark of CDA III.

- Flow cytometry with EMA and anti CD59 cannot be used to distinguish CDA III from normal controls. Nor can CDA III be misdiagnosed as HS or PNH when using flow cytometric methods, which are commonly used in the investigation of DAT-negative hemolysis.

- Heterozygous *HFE* mutation, C282Y or even H63D, is sufficient to cause pathologic iron overload when occurring concomitantly with CDA III. Therefore, in order to prevent organ damage in these patients, screening for hereditary hemochromatosis should be performed in all patients with CDA III.

Future perspectives

Rare anemias constitute a small health problem from a global perspective. Nevertheless, patients with these disorders have the right to find help and knowledge when they turn to health care. Furthermore, taken together as a group, patients suffering from either of these rare anemias are quite numerous. Thus, to gather and spread knowledge on rare diseases and to enable good research, collaboration and networking between centres involved in this field is essential.

The European Network for Rare and Congenital Anaemias (ENERCA) is such a collaboration, attempting to offer improved health service to professional medical practitioners and patients in every aspect of rare anemias. The project is cofounded by the European Commission through its Executive Agency for Health and Consumers (EAHC), being a part of the EU Health Programme, which focuses on reducing health inequalities across the European Union. The project is now working on a registry with the attempt to cover every patient who gets a diagnosis of a rare anemia. A good registry would enable good research and hopefully lead to new insights in how these patients should be followed and treated. As *KIF23* now has been found to be mutated in CDA III in one family in Västerbotten as well as in one American family, it would be of great interest to investigate sporadic CDA III to search for mutations in *KIF23*. Hopefully, the collaboration of ENERCA could facilitate such projects.

Moreover, it is important to look at the results of this project from a broader perspective, where CDA III can serve as a model for both cytokinesis failure and DAT-negative hemolysis.

KIF23 and its involvement in cancer development and the opportunity to use *KIF23* expression for risk stratification or treatment options in different cancers, should be further looked into. Since we know that plasma cell neoplasia, myeloma and MGUS, is overrepresented in CDA III, it would be of interest to look at *KIF23* expression and its correlation with clinical parameters in myeloma patients.

Furthermore it would be interesting to look at the connection between *KIF23* and *p53* from a clinical point of view, since we know that many hematological malignancies with mutation in or loss of *p53* have very bad outcome. For example, *KIF23* expression in chronic lymphatic leukemia (CLL) with deletion of 17_p or mutations in *p53* could be further looked into.

Iron overload is a great problem in many hemolytic diseases, such as thalassemia and other hemoglobinopathies. In addition, secondary iron overload is a frequent problem in hematologic disorders and malignancies requiring regular transfusions. Knowledge on iron metabolism and its regulation has been expanding in the last decade. It is of further interest to look at these regulation mechanisms and how they work together. Since *HFE* mutations are frequent in Europe, knowledge on how these mutations can influence development of iron overload in patients already at risk of iron accumulation could be valuable. If mutations lead to a faster iron accumulation this could influence how these patients should be followed and when treatment should be considered.

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I

Regular Article

RED CELLS, IRON, AND ERYTHROPOIESIS

Congenital dyserythropoietic anemia type III (CDA III) is caused by a mutation in kinesin family member, *KIF23*

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Key Points

- *KIF23*/MKLP1 mutation found in the CDA III patients causes cytokinesis failure.

Haplotype analysis and targeted next-generation resequencing allowed us to identify a mutation in the *KIF23* gene and to show its association with an autosomal dominant form of congenital dyserythropoietic anemia type III (CDA III). The region at 15q23 where CDA III was mapped in a large Swedish family was targeted by array-based sequence capture in a female diagnosed with CDA III and her healthy sister. Prioritization of all detected sequence changes revealed 10 variants unique for the CDA III patient. Among those variants, a novel mutation c.2747C>G (p.P916R) was found in *KIF23*, which encodes mitotic kinesin-like protein 1 (MKLP1). This variant segregates with CDA III in the Swedish and American families but was not found in 356 control individuals. RNA expression of the 2 known splice isoforms of *KIF23* as well as a novel one lacking the exons 17 and 18 was detected in a broad range of human tissues. RNA interference–based knock-down and rescue experiments demonstrated that the p.P916R mutation causes cytokinesis failure in HeLa cells, consistent with appearance of large multinucleated erythroblasts in CDA III patients. We conclude that CDA III is caused by a mutation in *KIF23*/MKLP1, a conserved mitotic kinesin crucial for cytokinesis. (*Blood*. 2013;121(23):4791-4799)

Introduction

Congenital dyserythropoietic anemia (CDA) is a group of rare hereditary disorders with ineffective erythropoiesis and distinct dyserythropoietic changes in the bone marrow. Three major types (I, II, and III) and several subtypes have been described.[1-3] CDA III is the rarest form of the 3 classical CDAs, with about 60 cases described globally, the majority belonging to a family in Sweden. The disease is characterized by intravascular hemolysis in combination with dyserythropoiesis with large multinucleated erythroblasts (gigantoblasts) in the bone marrow. The anemia is mild to moderate and red blood cell transfusions are rarely needed. In the Swedish family, retinal angioid streaks, monoclonal gammopathy of undetermined significance, and myeloma have also developed in a substantial number of patients.[4] The first family with CDA III identified and described by Wolff and von Hofe in 1951 was an American family consisting of a mother and her 3 affected children. The anemia was named "familial erythroid multinuclearity."[5] Later, a family, with church records dating back to the 18th century was described in the Swedish County Västerbotten and the condition was named benign hereditary erythroreticulosis.[6] A dominant pattern of inheritance was noted in both the American and the Swedish families. A small Argentinean family with autosomal dominant CDA III has also been described.[7]

The few reported sporadic cases of CDA III have shown considerable differences in clinical presentation, with severe erythroid hyperplasia associated with skeletal disorders, mental retardation, and hepatosplenomegaly. The pattern of inheritance seems to be autosomal recessive in these cases, suggesting a different genetic alteration.[8,9]

CDA III, as it appears in the Västerbotten family, has the characteristic morphology in bone marrow smears with large multinucleated erythroblasts. When a bone marrow smear is not available, elevated thymidine kinase or laboratory data indicating intravascular hemolysis, with elevated lactate dehydrogenase (LDH) and undetectable haptoglobin, confirm the CDA III diagnosis, provided that 1 of the parents is affected.[4,10] By linkage analysis the genetic location of CDA III (MIM 105600) was mapped to an 11-cM interval on chromosome 15q21-q25, but the disease gene was not identified.[11]

The genes responsible for recessive forms of CDA, CDA I (MIM 224120), and CDA II (MIM 224100) have been reported. CDA I is caused by mutations in codanin I (*CDAN1*; MIM 607465), also located on chromosome 15, whereas CDA II depends on mutations in a component of coat protein complex II–coated vesicles (*SEC23B*, MIM 610512) situated on chromosome 20.[12-14] Recently, a fourth type of CDA with an autosomal-dominant inheritance pattern (CDA IV, MIM 613673) and genetic defect in a transcriptional activator on chromosome 19p13 (*KLF*, 600599) was identified.[15] In the present study, we aimed at identification of the gene responsible for CDA III.

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Materials and methods

Patients and DNA samples

DNA was available from 39 affected and 21 unaffected members of the Swedish family (see supplemental Materials and supplemental Figure 1 on the *Blood* website), and 4 affected and 2 unaffected members of the American family. Clinical and laboratory information for each member of the Swedish family is provided in Table 1. Ethical approval was obtained from the Regional Ethics Committee in Umeå, Sweden, and informed consent was obtained from all patients. The study followed the tenants of the Declaration of Helsinki.

Targeted sequencing and data analysis

DNA samples from 2 sisters, 1 affected and 1 unaffected, from the Swedish family (supplemental Materials, supplemental Figure 1) were selected for targeted sequencing of the candidate region at 15q23 at Ambry Genetics (Aliso Viejo, CA) (http://www.ambrygen.com). Library preparation and indexing were performed using Roche NimbleGen 385k sequence capture array target enrichment, and 54-bp paired-end processing was done using the Illumina GAIIx (San Diego, CA). Initial data processing and base calling, including extraction of cluster intensities, was done using RTA1.8 (SCS version 2.8). Sequence quality filtering script was executed in the Illumina CASAVA software (version 1.7.0). The reads were mapped against University of California San Cruz hg19.

For bidirectional sequencing of *KIF23* (MIM 605064, ENSG00000137807), intronic sequences adjacent to exon 21 were amplified from genomic DNA. Sequences for *KIF23* primers designed with Primer3 software were 21F: 5′gctcattttggaggaacagaa; and 21R: 5′ gggagttcctgatgaagtgg. Polymerase chain reaction (PCR) amplification and the sequencing reactions were performed as described elsewhere.[16] The products of sequencing reactions were analyzed on ABI 3500xL Dx Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were aligned and evaluated using Sequencher software version 4.9 (Gene Codes Corporation, Ann Arbor, MI). All identified variants were denoted using accepted nomenclature recommended by the Human Genome Variation Society. To predict the impact of sequence variants on protein function, missense mutations were analyzed by Sorting Intolerant from Tolerant (http://sift.jcvi.org) and Polymorphism Phenotyping (http://genetics.bwh.harvard.edu/pph). Variants detected in intronic sequences were analyzed with the splice site prediction programs GeneSplicer (http://www.cbcb.umd.edu/software/GeneSplicer) and Splice Site Finder (www.genet.sickkids.on.ca/ali/splicesitefinder). All bioinformatics tools were available via the Alamut software version 2.0 (Interactive Biosoftware, Rouen, France).

Sequences of *MYO9A* and *TLE3* specific primers are listed in supplemental Table 1.

KIF23 expression

Reverse transcription (RT)-PCR was performed on First Choice Human Total RNA Survey Panel (Ambion Life Technologies, Carlsbad, CA). We used RNA isolated from the following tissues: brain, colon, heart, kidney, liver, lung, small intestine, spleen, thymus, placenta, ovary, skeletal muscles, prostate, testis, and thyroid. Furthermore, RNA was extracted from the lymphocytes of whole blood of a CDA III patient and a control case. Two sets of *KIF23*-specific primers were used along with *GAPDH* primers. Primer sequences and RT-PCR protocol are given in supplemental Table 2. RT-PCR products were then separated on agarose gel and visualized under ultraviolet light after staining with ethidium bromide.

KIF23 functional analysis

Knock-down and rescue analyses in HeLa cells synchronized by double-thymidine block were performed as previously reported,[17,18] using small interfering RNA (siRNA) targeted to the 3′-untranslated region of *KIF23* and a rescue complementary DNA construct of the KIF23 Δ18 splice isoform (ENST00000559279) tagged with a green fluorescent protein (GFP) at the N terminus. P916R mutation was introduced by site-directed mutagenesis with primers 5′-cttccacagtagcacGtgcccaaccagatgg-3′ and 5′-ccatctggttgggcaCgtgctactgtggaag-3′ and confirmed by sequencing. Time-lapse observation by differential interference contrast and GFP fluorescence microscopy was performed with a DeltaVision system (Applied Precision) equipped with a UPlanFL N 40×/1.30 objective lens (Olympus) and a CoolSNAP HQ2 cooled CCD camera (Photometrics) using softWoRx software (Applied Precision). During imaging, cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a microscope stage incubator (Tokai Hit) at 37°C and 5% carbon dioxide. Data on cytokinesis failure were analyzed with generalized linear model for binomial data using R (http://www.r-project.org/).

Results

Clinical findings

The diagnosis of CDA III was confirmed in 26 bone marrow smears and excluded in 13 cases (Table 1, Figure 1). When bone marrow smears were not available, thymidine kinase and laboratory data revealing hemolysis were used (Table 1). In the group, without available bone marrow samples, the diagnosis of CDA III was based on elevated thymidine kinase, LDH, and/or undetectable haptoglobin in 11 cases. In 1 case, the diagnosis was based on elevated LDH and haptoglobin alone. CDA III was excluded by normal thymidine kinase and normal LDH and haptoglobin in 6 cases.

Genetic findings

Haplotype and candidate gene analyses. CDA III disease was mapped to 15q21-q25 in 11-cM interval.[11] Analysis of some genes (eg, *CENP8*, *HEXA*, *ARIH1*) in the linkage region by direct sequencing or by denaturing high performance liquid chromatography did not reveal any pathogenic mutations. To find the minimal shared haplotype, genotyping with microsatellite markers was performed 3 times between 1993 and 2004 (supplemental Materials, supplemental Figure 2). Because CDA III patients of younger generation were diagnosed during these years, we expected to find a new recombination event that would allow refining of the disease locus. As a result of haplotype reevaluation, the disease-causative gene was expected to reside in an interval of 2.41 Mb between markers D15S100 and D15S1050 (69571161-71981252) based on a current version of genome sequence (hg 19, BUILD 37.2).

Gene identification. To identify the disease gene within the refined disease locus, DNA from 2 females of the Swedish CDA III family, 1 affected (V: 12) (supplemental Materials, supplemental Figure 1) and her unaffected (V: 13) sister (supplemental Materials, supplemental Figure 1) were selected for targeted sequencing of the candidate region. For this purpose, we decided to cover the region of 3.54 Mb between markers D15S100 and D15S980 (69571161-73113373) (supplemental Materials, supplemental Figure 2).

In total, 29 sequence variants in 13 genes were detected in the affected CDA III individual compared with 23 sequence variants in 12 genes in the healthy sister (supplemental Materials, supplemental Table 3). Ten sequence variants absent in the control sample (V: 13) and unique for the CDA III patient (V: 12) are summarized in Table 2. Because of autosomal dominant inheritance in the CDA III family, 6 homozygous sequence variants in *THAP10, LRRC49*, *GRAMD2*, *HEXA*, and *ADPGK* were excluded from further analysis. Furthermore, all these variants reported previously would not affect protein function according to the bioinformatics tools (Table 2). Two

Table 1. Diagnostic characteristics in the CDA III Swedish family

	Sex	Bone marrow morphology*	Hb, g/L†	Haptoglobin, g/L‡	LDH, μkat/L	Thymidine kinase§	CDA IIIII
III:6	M	+	143	<0.1	5.2	52.5	+
III:9	M	−	ND	ND	ND	1.5	−
III:10	M	+	107	<0.1	5.8	36.5	+
III:12	F	−	ND	ND	ND	1.4	−
III:14	M	+	116	0.1	4.3	18.1	+
III:18	M	−	162	0.6	2.7	2.6	−
III:20	F	+	128	<0.1	6.3	36.2	+
IV:2	F	+	80	<0.1	5.3	27.9	+
IV:4	F	+	126	<0.1	8.5	250.0	+
IV:5	M	−	149	1.6	3.1	1.9	−
IV:7	F	+	105	<0.1	8.0	39.1	+
IV:8	M	+	157	<0.1	6.8	37.7	+
IV:10	M	+	107	<0.1	6.2	46.5	+
IV:12IV:12	M	+	ND	ND	ND	56.8	+
IV:14	M	−	153	1.8	2.0	2.2	−
IV:15	M	+	146	<0.1	6.2	28.9	+
IV:17	M	+	129	<0.1	6.2	59.1	+
IV:20	F	+	120	<0.1	4.8	53.3	+
IV:21	F	−	135	1.1	1.9	2.7	−
IV:22	M	−	154	0.6	2	4.5	−
IV:24	F	ND	105	<0.1	ND	65.0	+
IV:26	F	+	114	<0,1	6.1	62.3	+
V:1	M	+	118	<0.1	5.7	43.6	+
V:3	M	+	127	<0.1	7.1	75.0	+
V:4	M	−	145	0.5	2.8	ND	−
V:5	M	+	106	<0.1	6.8	64.3	+
V:7	F	+	94	<0.1	7.7	149.0	+
V:8	F	−	107	0.5	2.4	1.7	−
V:9	M	+	135	<0.1	6.3	65,6	+
V:12	F	ND	114	<0.1	6.7	184.0	+
V:13	F	−	145	1.9	2.0	2.6	−
V:14	M	+	113	<0.1	5.7	41.9	+
V:17	M	−	130	0.6	2.9	1.0	−
V:18	F	−	136	1.0	2.0	3.4	−
V:19	F	ND	153	0.7	2.1	4.0	−
V:20	M	ND	158	1.0	ND	3.3	−
V:22	F	+	121	<0.1	6.1	89.0	+
V:23	M	+	135	<0.1	6.4	139.0	+
V:24	M	ND	105	<0.1	ND	52.3	+
V:26	M	ND	120	<0.1	ND	108.0	+
V:27	M	ND	147	0.6	2.7	5.0	−
V:28	M	ND	145	0.3	3.1	8.0	−
V:30	F	ND	109	<0.1	4.1	120.0	+
V:31	M	ND	118	0.8	ND	3.8	−
V:32	M	ND	127	ND	3.4	7.0	−
V:33	F	+	119	0.1	4.3	18.5	+
V:35	M	ND	ND	<0.1	ND	69.0	+
V:36	F	+	124	<0.1	8.3	167.0	+
V:38	F	−	138	2,6	2.7	6.4	−
VI:1	F	ND	102	<0.1	7.5	279.0	+
VI:2	F	ND	133	1.22	2.2	30.0	−
VI:3	F	ND	114	1.87	2.6	ND	−
VI:4	M	+	123	<0.1	10.0	ND	+
VI:5	M	ND	149	0.53	3.3	ND	−
VI:6	F	ND	116	<0.1	5.9	116	+
VI:7	F	ND	109	<0.1	6.6	120	+
VI:8	M	ND	125	ND	4.6	134	+
VI:10	M	+	108	<0.1	5.8	286	+
VI:11	F	ND	115	ND	5.3	102	+
VI:14	F	ND	107	<0.1	8.8	ND	+

F, female; CDA III Hb, hemoglobin; LDH, lactate dehydrogenase; M, male; ND, no data.
*Consistent with CDA III.
†Normal range for males is 134-170 and for females 117-153.
‡Normal range 0.24-1.9.
§Normal range <3.4, adjusted according to Nordic Reference Interval Project.
‖CDA III final diagnosis.

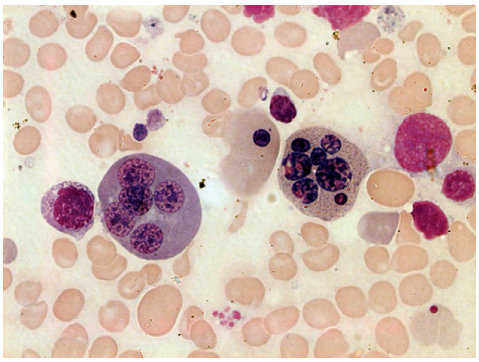

Figure 1. Bone marrow smear from a patient with CDA III. May-Grünwald-Giemsa staining was used and the image was taken by bright field microscopy with 100× objective (Leica DM3000, Leica Microsystems CMS GmbH, Wetzlar, Germany). Two polychromatic erythroblasts, 1 with 7 nuclei (right) and 1 with 5 nuclei (left) are shown. Anisocytic and hypochromatic erythrocytes are also evident.

sequence variants in the *TLE3* gene could also be excluded because c.615G>C resulted in a synonymous amino acid (p.205S>S) and IVS207+3T>C was a common variant present in 23% of the general population (Table 2); however, they were considered for further testing in segregation analysis. Sequence changes in 2 genes, *KIF23* and *MYO9A*, were predicted to be not tolerated and deleterious for protein function. The variant in *MYO9A*, c.4892T>A (p.N1631I) (rs80283650), was previously reported as a rare variant. The most promising gene associated with CDA III was *KIF23*. Novel sequence variant c.2747C>G in exon 21 was confirmed by Sanger sequencing (Figure 2) and resulted in amino acid substitution p.P916R (Table 2).

Segregation analysis. The 2 *TLE3* variants c.615G>C (p.205S>S) and IVS207+3T>C as well as the *MYO9A* variant c.4892T>A (p.N1631I) were excluded as a potential cause of CDA III because they did not segregate with the disease in the Swedish family (data not shown). Furthermore, the c.4892T>A variant in the *MYO9A* gene was detected at a higher frequency in the Swedish population (0.065) compared with the 1000 Genome project (0.011; Table 2).

Segregation analysis of the *KIF23* c.2747C>G variant was done by restriction fragment length polymorphism analysis using the *Hpy*CH4IV endonuclease for digestion of *KIF23* exon 21 in 2 CDA III families of Swedish (data not shown) and American origin (Figure 3). The c.2747C>G (p.P916R) mutation was present in heterozygous form only in affected CDA III patients. Furthermore, the mutation was absent in 356 control individuals from a geographically matched Swedish population. The p.P916R mutation has not been described in the literature previously and is thus novel to both the Swedish and the American families. These data imply that the *KIF23* is a strong candidate gene for causing the disease.

KIF23 expression. *KIF23* encodes mitotic-kinesin-like protein 1 (MKLP1), a highly conserved factor crucial for formation of the central spindle and midbody and thus in the completion of cytokinesis. RNA expression of *KIF23* in 15 different tissues and whole blood of a CDA III patient was analyzed by RT-PCR. Specific primers were designed for detection of the 2 known isoforms generated by alternative splicing of exon 18,[19] ENST00000260363, a full-length (FL) transcript containing exon 18 and encoding a 960AA protein, and ENST00000559279, which lacks exon 18 (Δ18) and encoding a protein of 856AA. Primers specific for exons 16 and 19 detected 2 bands (391 bp and 211 bp) that correspond to the Δ18 transcript and a novel transcript lacking both exons 17 and 18 (Δ17+18), respectively (Figure 4A-B). The identity of these transcripts was confirmed by direct sequencing of the PCR products (supplemental Materials, supplemental Figure 3). However, under our conditions, these primers failed to detect a 703-bp band that corresponds to the FL transcript, although the presence of this transcript was confirmed with the primers specific for the exons 17 and 18. Although expression of the FL and Δ17+18 transcripts was tissue-dependent, the Δ18 transcript was detected in all the tissues examined, including the in peripheral blood of the CDA III patient. Our results were in agreement with the data available via http://biogps.org in which broad *KIF23* expression with the highest level in erythroid precursors is observed.

Functional analysis. To evaluate the effect of the *KIF23* p.P916R mutation on the function of its product, MKLP1, in cytokinesis, a knock-down and rescue assay with the most ubiquitously expressed MKLP1 Δ18 isoform was performed in HeLa cells (Figure 5). In this assay, endogenous MKLP1 was depleted with an siRNA against its 3′ untranslated region (Figure 5A) and RNA interference–resistant MKLP1 constructs tagged with GFP-MKLP1 (Figure 5B) were expressed during synchronization of the cell cycle (supplemental Figure 4). Cytokinesis was monitored by time-lapse imaging. During normal cell division in HeLa cells, anaphase onset triggers formation of the central spindle microtubule bundle between segregating chromosomes (Figure 5D, yellow arrows), with MKLP1 highly accumulating to the central antiparallel overlap zone (white arrow). This induces cleavage furrow ingression at the cell equator. Compacted by the ingressing furrow, the central spindle matures into the midbody (arrowhead), which maintains the intercellular bridge up to several hours until the daughter cells are finally separated by

Table 2. Sequence variants in CDA III patient detected by targeted resequencing

Gene	Nucleotide change	Mutation form	Amino acid change	dbSNP	SIFT	PolyPhen	MAF
KIF23	c.2747C>G	Heterozygous	p.916P>R	New variant	Not tolerated	Deleterious	
MYO9A	c.4892T>A	Heterozygous	p.1631N>I	rs80283650	Not tolerated	Possibly damaging	A = 0.011/24
TLE3	c.615G>C	Heterozygous	p.205S>S	rs17759219	Not predictable	Not predictable	NA
TLE3	IVS297+3T>CT	Heterozygous		rs2291986	Not predictable	Not predictable	C = 0.228/497
THAP10	c.117A>G	Homozygous	p.39G>G	rs2955035	Not predictable	Not predictable	A = 0.486/1061
LRRC49	c.1061_1063delAAC	Homozygous	p.Q354del	rs56720495	Not predictable	Not predictable	NA
GRAMD2	IVS134+23A>C	Homozygous		rs11072348	Not predictable	Not predictable	A = 0.05/109
HEXA	c.1306T>C	Homozygous	p.436I>V	rs1800431	Tolerated	Benign	T = 0.101/220
ADPGK	c.551T>C	Homozygous	p.184K>R	rs8024644	Tolerated	Possibly damaging	C = 0.195/426
ADPGK	c.546A>G	Homozygous	p.182G>G	rs8023358	Not predictable	Not predictable	G = 0.184/402

dbSNP, Single Nucleotide Polymorphism Database; MAF, minor allele frequency (established in 1000 Genome project); NA, not available; PolyPhen, Polymorphism Phenotyping; SIFT, Sorting Intolerant from Tolerant.

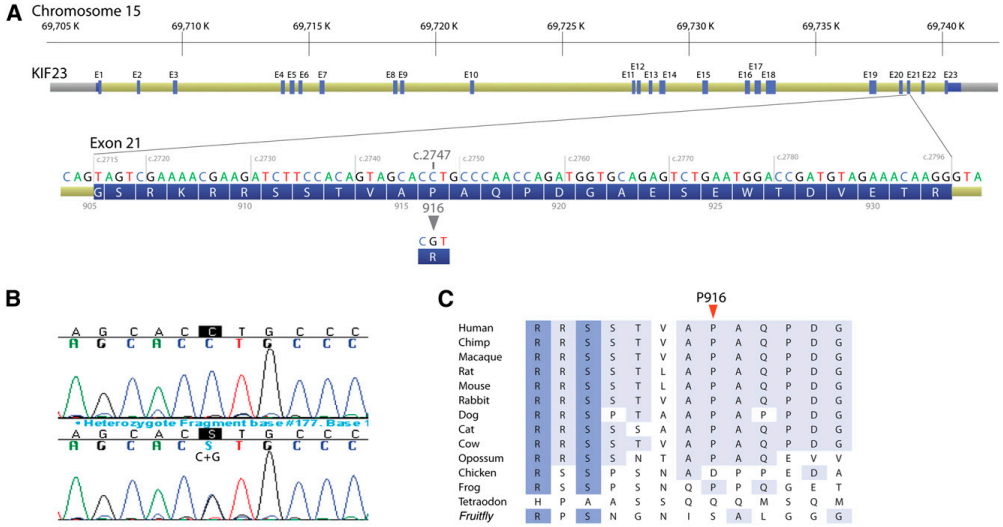

Figure 2. A novel c.2747C>G (p.P916R) mutation in the *KIF23* gene. (A) Exon structure of the *KIF23* gene with DNA and protein sequence of the exon 21 where the mutation resides. (B) DNA sequence showing c.2747C>G mutation. (Upper) Wild type and (lower) c.2747C>G heterozygous mutations, with mutation position marked in black. (C) Phylogenetic alignment of partial *KIF23* protein sequence.

abscission (double-headed arrow). MKLP1 plays essential roles in the formation of the central spindle and the stable maintenance of the midbody. Failure of cytokinesis by depletion of the endogenous MKLP1 was efficiently rescued by expression of wild-type GFP-MKLP1 (Figure 5C). In contrast, GFP-MKLP1 with the p.P916R mutation (P812R mutation in this isoform) showed little or no rescue activity above the level of the control empty vector (Figure 5C, P916R vs GFP). Cells expressing GFP-MKLP1-P916R could complete constriction of the cleavage furrow and form the midbody in a similar time course with the cells expressing the wild-type GFP-MKLP1, but regressed to form binucleate cells about 2 hours after midbody formation (180 ± 148 nub [mean ± standard deviation.], mode at 105 min, n = 225) (Figure 5D-E and supplemental Videos 1 and 2). These data indicate that the p.P916R mutation impairs the function of MKLP1 essential for the completion of cytokinesis.

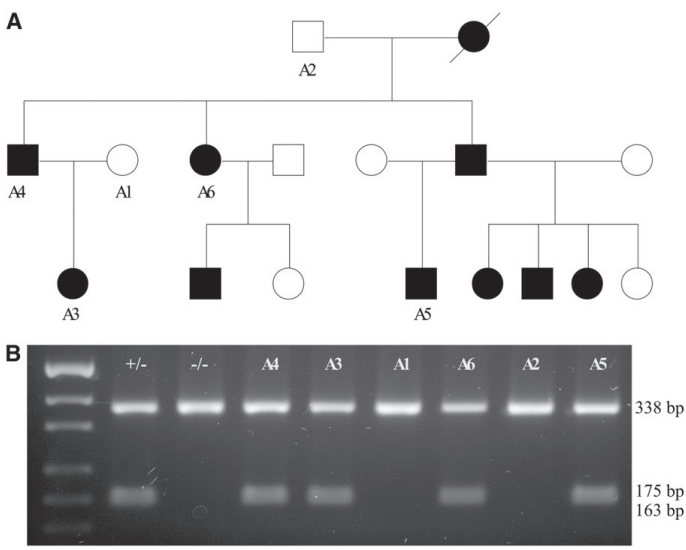

Figure 3. c.2747C>G segregates CDA III in the American family. (A) Pedigree of the family with affected individuals shown in black symbols and healthy individuals shown as unfilled symbols. DNA was available only for cases marked with A1–A6. (B) Restriction length fragment polymorphism assay. Amplification of exon 21 of *KIF23* was done as described in "Materials and Methods." PCR products were digested using the *Hpy*CH4IV endonuclease. c.2747C>G creates a restriction site; therefore, all mutation carriers show 3 bands—338, 175, and 163 bp (small fragments are poorly resolved on agarose gel)—whereas all unaffected individuals show only 1 band (338 bp).

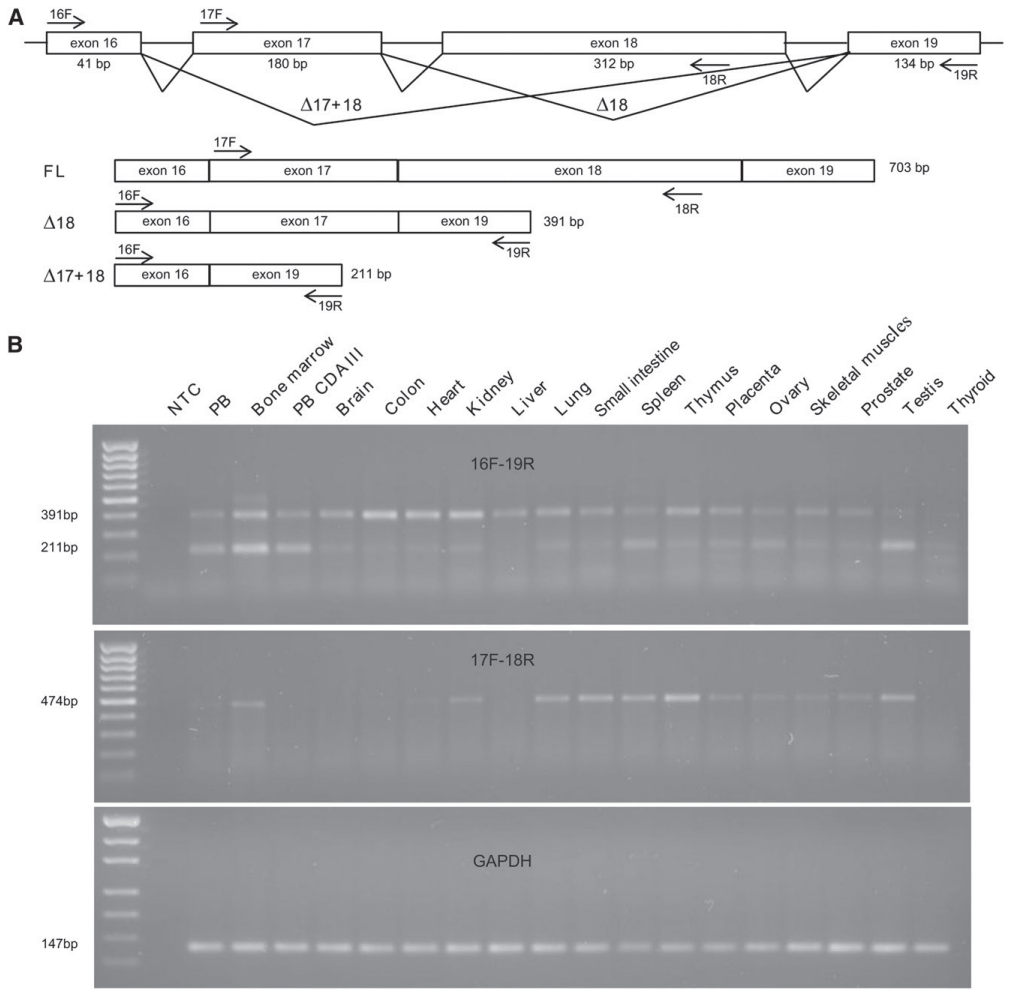

Figure 4. RNA expression of *KIF23*. (A) Schematic representation of alternative splicing of the exons 17 and 18 of *KIF23*. The primers used for RT-PCR are indicated by arrows. Two previously reported transcripts (FL and Δ18) as well as a novel one (Δ17+18) are illustrated with the expected sizes of the RT-PCR products. (B) Detection of *KIF23* transcripts and a control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript in a broad range of tissues by RT-PCR with primers 16F and 19R (top), 17F and 18R (middle), and GAPDH primers (bottom). Under these conditions, the primers 16F and 19R detected the Δ18 and Δ17+18 transcripts but not the FL transcript, although its expression was observed with the primers 17F and 18R (FL). MassRuler Low Range DNA Ladder (80-1031 bp) and pUC19DNA/*MspI* (*HpaII*) Marker, 23 (Thermo Fisher Scientific, Inc., Waltham, MA) were used as a size standard for *KIF23* and *GAPDH*, respectively.

Discussion

The genetic cause of the rarest form of CDA, CDA type III, was identified in this study almost 2 decades after its chromosomal localization was detected. At the time of DNA and laboratory sampling, 10 of the CDA III–positive individuals and 6 of the CDA III–negative individuals were younger than age 18, explaining why bone marrow investigation was not done in all cases. Moreover, it was difficult to motivate all individuals in the family to undergo a bone marrow aspiration because the diagnosis in this family can be determined by laboratory sampling of peripheral blood. A previously shown correlation of CDA III with elevated LDH and undetectable haptoglobin in this family was confirmed in this study. Analysis of these parameters in 25 of the 26 CDA III–positive individuals, diagnosed by bone marrow morphology, showed elevated LDH and undetectable haptoglobin in all cases. Twelve CDA III negative individuals, without signs of the disease in the bone marrow, showed normal levels of haptoglobin and LDH. Thymidine kinase analyzed in 25 out of 26 morphology-based CDA III–positive cases spanned from 18.1 to 289 U/L. This is far higher than the normal range in adults, which is set to less than 5 U/L. However, in 1 16-year-old female in the CDA III–negative group diagnosed by bone marrow examination, the thymidine kinase was 6.4 U/L. As discussed in an

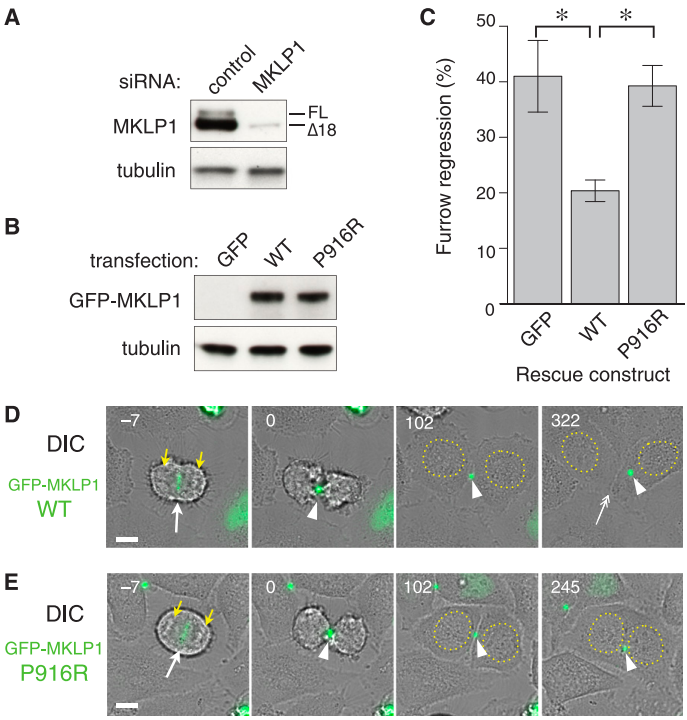

Figure 5. Cytokinesis failure caused by the *KIF23* c.2747C>G (MKLP1 p.P916R) mutation. Efficacy of depletion of endogenous MKLP1 (A) and equal expression of wild-type (WT) and P916R GFP-MKLP1 (B) constructs was confirmed by western blotting of the whole cell lysates with anti-MKLP1 (A) or anti-GFP (B) antibody. Tubulin was also blotted as a loading control. (C) Quantitation of cytokinesis failures from live cell recordings of HeLa cells treated with MKLP1 siRNA and transfected with GFP vector or the MKLP1 variants as indicated. The graph shows the percentage of cells that failed cytokinesis averaged from 3 independent experiments in which at least 80 GFP-MKLP1 or GFP-expressing cells were analyzed (supplemental Table 4). *$P < .001$ after Turkey correction for multiple comparisons. Error bars indicate the standard deviation. (D-E) Stills from the live recordings (A-C) by differential interference contrast (DIC) and GFP fluorescence microscopy. Number indicates the time after midbody formation. GFP-MKLP1 P916R mutant (E) was localized to the spindle midzone (−7 min, arrow) and condensed to form the midbody (0 and 102 min, arrowheads) in a similar manner to the WT GFP-MKLP1 (D). However, the cell membrane of the intercellular bridge was detached from the midbody 217 min after its formation, creating a binucleate cell (245 min). Note that in the WT GFP-MKLP1 cell, abscission happened at 252 min and the 2 daughter cells were separated (D) at 322 min, double arrow. The remnant of the midbody (arrowhead) was incorporated to the cell on the right. Bar, 10 μm. Yellow arrowheads and dotted circles indicate segregating chromosomes and reformed nuclei, respectively.

earlier study, thymidine kinase levels are higher in children and normal ranges for different ages have not been established.[10] In the CDA III–negative group with no available bone marrow smears, none of the patients showed any sign of hemolysis. One 4-year-old child with a thymidine kinase of 30 U/L was considered CDA III–negative because LDH and haptoglobin levels were normal.

For identification of the CDA III causative gene, we used targeted resequencing of the region where the disease was previously mapped. We established a minimal haplotype shared by all affected individuals in the family. This region covered 2.41 Mb and contained 10 genes; however, for practical reasons a 3.54-Mb region with 24 genes was sequenced. To exclude all population-specific normal sequence variants, we compared sequences of a CDA III patient and her unaffected sister. To identify a potential disease mutation, we prioritized sequence variants that are unique for a CDA III–positive patient, are heterozygous, segregate with the phenotype, and are novel or have a frequency <1% in the Single Nucleotide Polymorphism Database. As a result, a novel heterozygous sequence variant in the *KIF23* gene, c.2747C>G (p.P916R), was the most promising candidate as a cause of the disease among 10 CDA III–unique single nucleotide polymorphisms. This mutation was predicted to be damaging for protein function via bioinformatics tools and this point mutation was found only in individuals diagnosed with CDA III and not only in the Swedish family but also in the American family. The Swedish and American CDA III families are, to the best of our knowledge, not related.

KIF23 encodes the kinesin superfamily molecule MKLP1 that plays critical roles in cytokinesis.[19,20] It has a kinesin motor domain on its N terminus and a domain predicted to form a coiled coil in the middle of the molecule. In proliferating cells, it primarily exists as centralspindlin, an evolutionarily conserved stable heterotetrameric complex with CYK4/MgcRacGAP encoded by *RACGAP1*, which binds to the neck region of MKLP1 connecting the motor domain to the coiled coil domain.[21] Both components of centralspindlin are required for proper formation of the central spindle and the midbody. Centralspindlin accumulates to the central antiparallel overlap zone of these microtubule-based structures and recruits various downstream cytokinesis factors to the site of division.[22,23] In our knockdown and rescue experiments, the P916R mutant MKLP1 failed to rescue the cytokinesis failure caused by depletion of the endogenous wild-type molecule, indicating that the p.P916R mutation affects the function of MKLP1 in cytokinesis. This accounts for the characteristic pathological condition of the CDA III (ie, the large multinucleated erythroblasts found in bone marrows of the patients).

Centralspindlin heterotetramers further oligomerize into higher order clusters in vivo enhancing its interaction with microtubules. This plays a crucial role in its sharp accumulation into the midbody,[24] which is important for the stable maintenance of the intercellular bridge through anchorage of the plasma membrane to the midbody microtubule bundles until the final separation of the daughter cells through abscission.[18,25] This unique clustering activity is regulated through the C terminal tail domain of MKLP1. The 14-3-3 proteins, which bind to a short motif containing a phosphorylated serine (S814 in the longest splicing isoform, same hereafter), sequester centralspindlin into an unclustered, inactive state.[17] Phosphorylation of the second serine (S812) within the 14-3-3 binding motif by Aurora B kinase, which is active at the spindle midzone, releases centralspindlin from the sequestration by 14-3-3. ARF6 GTPase, which binds the

MKLP1 C terminal domain in a competitive manner against 14-3-3, colocalizes with centralspindlin on the late midbody and thus prevents premature collapse of the intercellular bridge.[18,26] The p.P916R mutation might affect the stability of the midbody by interfering with these or unknown regulations of centralspindlin clustering. Stable postmitotic maintenance of the midbody is also controlled by phosphorylation of S911 by Aurora B kinase, which prevents collapse of the midbody from premature onset of the nuclear import of centralspindlin driven by a bipartite nuclear localization signal in the tail of MKLP1.[27,28] Thus, interference with this regulation of nuclear import might provide an alternative route to the cytokinesis defect caused by the p.P916R mutation. Future studies with more detailed biochemical and cell biological analyses would be required for testing these possibilities.

The question of why we see multinucleated cells only in the erythropoiesis, but not in other cell lineages in the CDA III patients, remains to be investigated. Although centralspindlin components are essential for cytokinesis in all metazoan cells so far examined, it is becoming clear that it works in the context of a large protein–protein interaction network, which includes other microtubule-bundling proteins whose activities seem to be partially redundant with those of centralspindlin.[29] Indeed, there is a significant difference in expression levels of 1 such microtubule-bundling protein, PRC1, between late epidermal and early neural cells during embryogenesis.[30] We speculate that cells within a living organism might modify the conserved protein–protein network to adapt to the specific requirements from their developmental and physiological conditions. The erythropoiesis-specific cytokinesis failure in CDA III might reflect the lower tolerance of the cells in the erythropoietic lineage to the defect in KIF23/MKLP1 than that of the other cells. Transient cytokinesis failure generates unstable tetraploid cells, which can be transformed into malignant tumors via aneuploidy.[31] Less frequent cytokinesis failure in lymphoid lineage might explain the myeloma and monoclonal gammopathy in CDA III. The mutations in CDAN1 and SEC23B responsible for CDA I and II, respectively, might also be involved in cytokinesis, or more broadly cell division, in the erythropoietic lineage. We believe that deeper knowledge of the molecular mechanism of cytokinesis would contribute to better understanding of these diseases and, ultimately, to improvement of its medical treatments.

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Authorship

Contribution: M.L., A.W., M.M., and I.G. designed the study; M.L., A.W., S.M., and H.S. collected clinical data; A.-L.V. and M.L. performed mutation analyses and expression studies; A.F.I. did functional assays; A.-L.V., A.F.I., A.N., M.M., and I.G. analyzed and interpreted the data; M.L., M.M., and I.G. provided financial support; all authors contributed to the writing process and approved the final manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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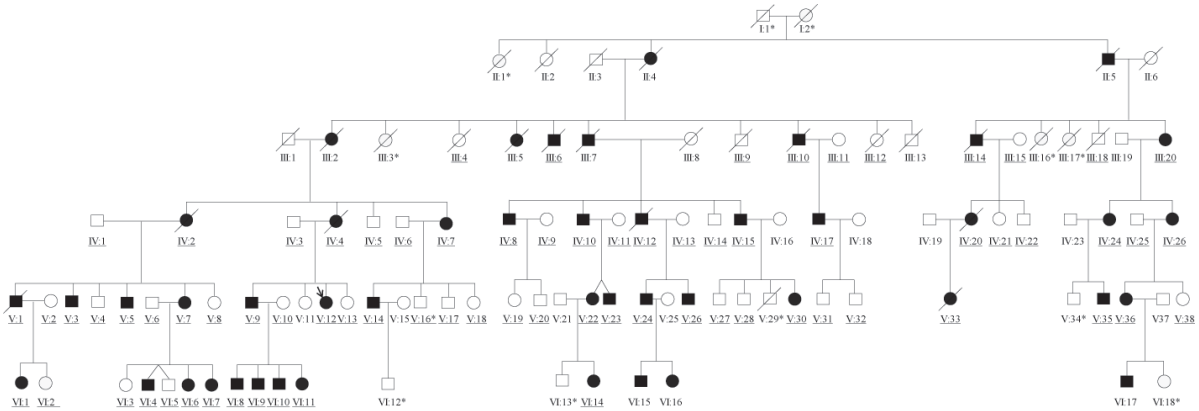
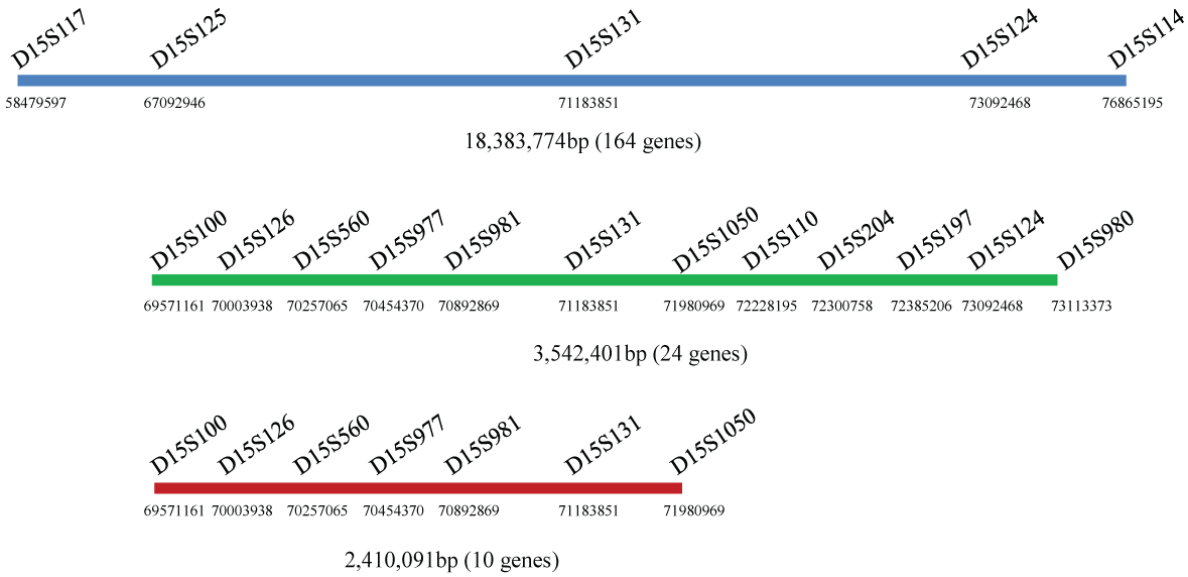
Figure S1



Pedigree of CDA III Swedish family. Filled symbols indicate affected individuals, while unfilled symbols indicate unaffected. Underlined symbols represent individuals where DNA was available and tested for *KIF23* mutation in the segregation analysis. Symblos with asterisk represent individuals not examined clinically. Individual V:12 marked with a black arrow was analyzed by targeted re-sequencing.

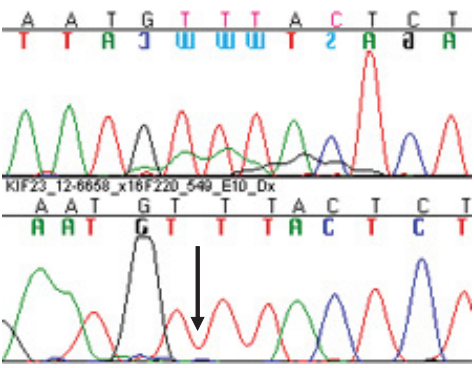
Figure S2



Haplotypes shared by affected individuals in CDA III Västerbotten family. Haplotype analysis was done three times using microsatellite markers shown above colour bar while their start positions are indicated under the bars. The minimal region where CDA III gene could reside was 2.4 Mb including flanking markers. For targeted re-re-sequencing region of 3.5 Mb was chosen based on equal laboratory work.

Figure S3

Sequence of a novel *KIF23* isoform lacking exons 17 and 18



Arrow shows boundary between end of exon 16 and start of exon 19. Sequence was done on 211 bp PCR fragment amplified with primers 16F and 19R

Figure S4

Design of the knock-down and rescue experiments in Figure 6.

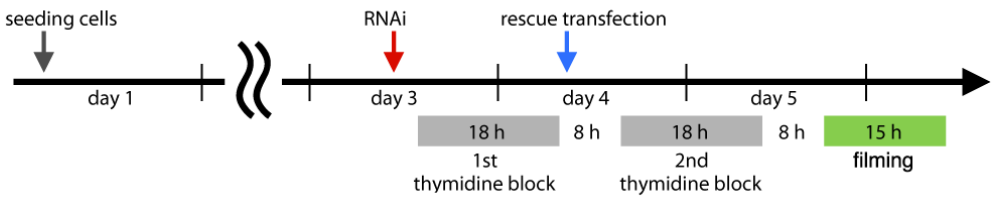


Table S1

Primers for segregation analysis

Primer	Sequence 5'-3'
MYO9A 25F	TTAATGTACTGGGGTCCCTATCA
MYO9A 25R	TGAAGAAAATGGGCCTAGCA
TLE3 5F	TCCTGGTAGGAGGCTGACTG
TLE3 5R	TATGAATGGACAGGGGCTGA
TLE3 9F	GCAGAAAGTTTTAACCCCGAAC
TLE3 9R	TATCGCCAACCTGTGCTTCT

Table S2

***KIF23*-specific primers for analysis of RNA expression**

Primer	Sequence 5'-3'
KIF23 16F	GCATGGTGACAGAAACGACA
KIF23 17F	GAGCGTAGAGTGGCAGCCAA
KIF23 18R	ATTTCTGAATGTAGGAACCAC
KIF23 19R	ACATGTGGCTGCATGACTGT

Total RNA was extracted using TRIzol reagent (Invitrogen®Life Technologies, Carlsbad, CA, USA) according to the manufacturer'instructions. Total RNA was then reverse-transcribed with SuperScript III RT (Invitrogen®Life Technologies, Carlsbad, CA, USA) and the resulting cDNA was used as template for PCR analysis. All PCR reactions were performed with Amplitaq Gold (Applied Biosystems) as recommended with an annealing temperature of 57°C (16F-19R), 47°C (17F-18R), 54°C (GAPDH) and an extension time of 30 s for a total of 39 cycles.

Table S3

Number of sequence changes detected by targeted next generation sequencing in CDA III patient and her healthy sister

	ADPGK	***BBS4***	***HEXA***	***GRAMD2***	***SENP8***	***MYO9A***	***NR2E3***	***THSD4***	***LARP6***	***LRRC49***	***THAP10***	***UACA***	***TLE3***	***KIF23***	**Total number**
CDA III-	1	4	1	1	1	2	1	4	1	-	-	2	1	4	23
CDA III+	3	3	2	2	1	3	1	1	-	3	1	2	2	5	29

CDA III+ - CDA III positive patient (V:12) and CDA III- - CDA III negative patient, a healthy sister to V:12 (V:13).

Table S4

Summary of scoring of the time-lapse movies in the knock-down and rescue experiments in Figure 6. s.d., standard deviation

rescue construct	biological replicates	total cells counted	cytokinesis failure	% failure	s.d. (%)
GFP	3	334	137	41.0	6.5
GFP-MKLP1 WT	3	368	74	20.4	1.9
GFP-MKLP1 P916R	3	574	225	39.3	3.7

Legends to Supplementary Movies 1 and 2

Time lapse movies of HeLa cells expressing wild-type GFP-MKLP1 (Movie 1) or P916R mutant GFP-MKLP1 (Movie 2) as rescue construct in an RNAi-based knock-down and rescue assay. Fluorescence image of GFP-MKLP1 (green) was merged on DIC image (grayscale). Bar, 10 µm. Time, hour:min.

II

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Research article Open Access

Erythrocyte Flow Cytometric Analysis in Congenital Dyserythropoietic Anemia Type III-Evaluation of Eosin-5´-Maleimide, CD55, and CD59

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Abstract

Introduction: Flow cytometry with eosin-5´-maleimide (EMA), anti-CD55 and anti-CD59 is commonly used when investigating non-autoimmune hemolytic anemias. Reduced fluorescence of EMA, typically detected in hereditary spherocytosis is also seen in congenital dyserythropoietic anemia type II (CDA II). Reduction of CD55 and CD59 characterizes paroxysmal nocturnal hemoglobinuria (PNH). We studied the flow cytometric profile of EMA, CD55 and CD59 on erythrocytes in congenital dyserythropoietic anemia type III (CDA III).

Methods: Erythrocytes from 16 CDA III positive individuals, 14 CDA III negative relatives and three normal controls per assay were studied with flow cytometry after EMA staining. Flow cytometry after anti-CD55 and anti-CD59 was performed on erythrocytes from 12 CDA III positive and 7 CDA III negative relatives with one normal control per assay.

Results: CDA III - erythrocytes exhibited marginally stronger fluorescence after EMA-staining than normal controls. Correlation between EMA fluorescence and erythrocyte volume was confirmed. CDA III subjects did not differ from normal controls concerning CD55 and CD59.

Conclusion: The results of the present study indicate no abnormality of the erythrocyte membrane in CDA III and show that standard flow cytometry cannot be used to discriminate between CDA III and normal controls.

Keywords: Congenital dyserythropoietic anemia type III; Flow cytometry; Eosin-5´-maleimide; CD55; CD59

Introduction

Flow cytometry with recording of mean cellular fluorescence (MCF) after eosin-5'-maleimide (EMA) staining is a valuable tool in the work-up of patients with direct antiglobulin test (DAT) negative anemia [1,2]. EMA binds predominantly to band 3, but also to CD47 and Rhesus (Rh)-related glycoproteins, together leading to the markedly reduced EMA binding in hereditary spherocytosis [3,4]. EMA binding appears to be normal in most common anemias such as DAT positive hemolytic anemia and iron deficiency as well as in hemoglobinopathies such as thalassemia [5]. Normal MCF after EMA staining has also been reported in anemias due to enzymopathies such as glucose-6 phosphate dehydrogenase deficiency and pyruvate kinase deficiency [6]. Reduced binding of monoclonal antibodies against the glycosylphosphatidylinositol anchor proteins CD55 and CD59 to erythrocytes and myeloid cells forms the basis of the flow cytometric diagnosis of paroxysmal nocturnal hemoglobinuria (PNH) [7].

Congenital dyserythropoietic anemias (CDA) constitute a group of rare anemias with DAT-negative hemolysis, dysplastic and ineffective erythropoiesis. Three major types, I, II, and III, and several subgroups have been identified [8]. MCF after EMA staining is reduced in CDA II and pyropoikilocytosis [2,9]. CD59 also seems to be slightly reduced in pyropoikilocytosis, albeit only studied in a few cases [10].

CDA III is the rarest form of CDA. At least two forms of CDA III exist, one familial which is inherited in an autosomal dominant manner, and one sporadic that might be inherited as an autosomal recessive trait or arising as de novo spontaneous mutation [11-13]. The largest known family with CDA III originates from the Swedish county of Vasterbotten [11]. The detection of the mutated gene linked to CDA I, Codanin-1, and the mutated gene SEC23B in CDA II, has facilitated the diagnosis in these two subtypes of CDA [14-16]. We have recently found that a mutation in KIF23 is associated with CDA III in two unrelated families, so a genetic diagnostic approach is now available for the three major types of CDA [17].

We have previously studied the erythrocyte membrane in two patients with CDA III [18]. The study, which was based on monoclonal antibodies and gel electrophoresis, did not reveal any gross alteration of the erythrocyte membrane concerning CD44, CD47, CD59 and Rh-related proteins, but there was a slight reduction of glycosylation of band 3. The osmotic fragility test appeared normal in 10 CDA III patients from the Västerbotten family [11].

The aim of this study was to examine the erythrocyte membrane in CDA III with flow cytometry using EMA-binding test and monoclonal antibodies against CD55 and CD59. These assays are routinely performed in the diagnostic workup of DAT-negative hemolytic anemia. It is unknown if flow cytometry with EMA and monoclonal antibodies against CD55 and CD59 can be used to identify samples from CDA III patients.

The study was approved by the Regional Ethical Review Board in Umea, Sweden, dnr: 2010-8-31.

Materials and Methods

To our knowledge, there are 59 known persons alive with CDA III or with one parent diagnosed with CDA III, in the Västerbotten family. Seven affected and one unaffected person, living abroad, was not contacted for the study. Because of mental retardation, two adults

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with unknown CDA-status were also excluded. The remaining 49 family members were contacted and informed of the study by mail. A few days after mail correspondence the family members were contacted by phone and asked if they wanted to participate. Concerning children under the age of 18, their parents were contacted. Children between 15 to 18 years of age were given information specially designed for this age group. 30 out of 43 adults but none of the 6 children wanted to participate. The p.P916R mutation of KIF23 was confirmed in all CDA III positive cases and absent in all CDA III negative cases. Study sample characteristics are shown in (Table 1).

Ten ml peripheral blood, divided into 2 tubes, was obtained at the nearest hospital or in the nearest district health care centre. One sample was sent to the nearest hospital to be analysed the same day concerning hemoglobin (Hb) and erythrocyte cellular volume (MCV). The other tube was sent to the Hematopathology Laboratory in Umeå for flow cytometric assays. Flow cytometry was performed within 24 hours.

Flow cytometry

Flow cytometric assays were performed on a BD Biosciences FACS Calibur flow cytometer. Mean channel fluorescence (MCF) levels were determined by Cellquest software. All assays were performed by one and the same biomedical scientist.

EMA-labelling of red cells and flow cytometry was performed as previously described by King et al. [3]. 16 CDA III positive and 14 CDA III negative individuals were analyzed. In each assay, the study sample from a subject of the Västerbotten family was set up with three normal controls, randomly selected from the hospital routine laboratory, provided that they had hemoglobin, MCV, leukocytes, and platelets within the normal range for our laboratory. Fluorescence intensity in MCF units was determined for 30000 events. Mean-MCF was determined for the study sample as for the three normal controls.

The expression of CD55 and CD59 on erythrocytes was analysed in 12 CDA III positive subjects and 7 CDA III negative siblings. Erythrocytes were prepared and analysed for CD55 and CD59 as described by Sutherland et al. [19]. In each assay the study sample was set up with one normal control. 15000 events were recorded. A threshold for populations of erythrocytes expressing CD55 and CD59 in the normal controls was identified and set at the same level in the study sample.

Statistical analysis

Ratios of MCF in study samples and control(s) in each assay were calculated. Study sample MCF and control MCF values were normally distributed according to Kolmogorov-Smirnov test. Results are presented as MCF ratios, standard deviation, and range. The Sign test was used to compare MCF of study samples and their normal controls. $p<0.05$ was regarded as significant. Pearson correlation test was used for analysis of correlation between EMA fluorescence and MCV.

	CDA III positive subjects	CDA negative relatives
No.	16	14
Males/Females	8/8	6/8
Age, years	55 (21-72)	51 (27-82)
Ery-MCV[1] (fL)	99 (94-109)	88,5 (70-94)
Hb[2] (g/L)	119 (105-157)	145,5 (94-150)

Values are presented as median and range
[1]Erythrocyte mean corpuscular volume
[2]Hemoglobin

Table 1: Study sample characteristics.

Results

Mean EMA MCF was higher (1.08) in the CDA III positive group than in their normal controls (1.0) but did not differ between CDA III negative siblings (0.99) and their normal controls (1.0) (Table 2 and Figure 1). The slightly increased EMA fluorescence in CDA III patients was significant ($p<0.001$). There was a significant correlation between EMA-MCF and MCV ($r=0.72$, $p=0.01$) (Figure 2). The expression of CD55 and CD59 did not differ between the CDA III positive patients and their controls (0.99 and 1.01, respectively), nor between the CDA III negative siblings and their normal controls (1.04 and 0.99, respectively) (Table 2 and Figure 3). Correlation analysis between MCV and CD55 or CD59 could not be performed due to the small study sample.

	n	CDA III pos./controls ratios and (range)	n	CDA III neg. relatives/ controls ratios and (range)
EMA MCF	16	1.08*** (1.01-1.17)	14	0.99 (0.89-1.08)
CD55	11	0.99 (0.96-1.00)	7	1.04 (0.99-1.32)
CD59	11	1.01 (0.96-1.15)	7	0.99 (0.97-1.02)

*** $p<0.001$

Table 2: Summary of flow cytometry results.

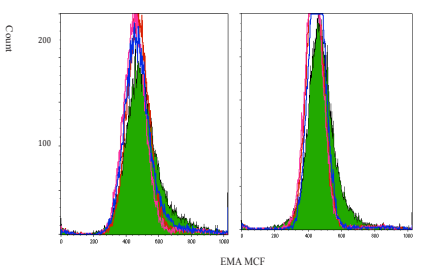

Figure 1: Fluorescence histograms for two patients with CDA III. Three normal controls in each assay. Erythrocytes incubated with EMA. Green areas constitute the CDA III patients. Flourescence is slightly increased in CDA III compared to normal controls.

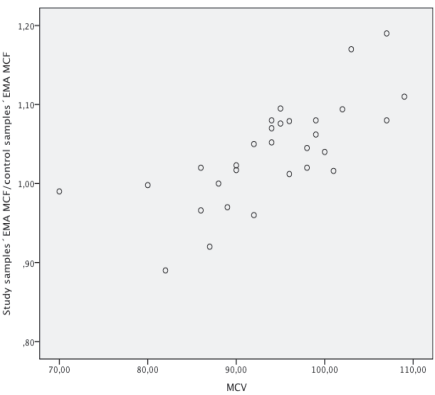

Figure 2: Positive correlation between MCV and EMA MCF was established, $r=0.715$, $p=0.01$.

Discussion

Reduced fluorescence intensity of intact red cells after incubation with EMA has been reported in patients with CDA II by King et al., and we have previously found indications of an abnormality in band 3 in patients with CDA III [3,18]. In the present study, we performed flow cytometry of erythrocytes from members of a family with CDA III. Unexpectedly, we found that the fluorescence intensity of red blood cells after EMA incubation is higher in samples from patients with CDA III than in normal controls, but this difference was correlated to higher MCV in the patients compared to the normal controls. This finding motivated us to perform an analysis of EMA fluorescence and MCV in 22 random hospital patient samples that were analysed by us at the same time period. We found a significant (r=0.5, p=0.02) correlation between EMA fluorescence and MCV also in this cohort.

EMA MCF was normal in samples from normal siblings of the CDA III patients.

The flow cytometric profiles of CD55 and CD59 did not differ between patients with CDA III and normal controls.

The affinity of EMA to the membrane protein band 3 is high, which is the main cause of lower fluorescence in hereditary spherocytosis. To a minor extent EMA also interacts with CD47 and Rh-antigen, also reduced in hereditary spherocytosis, contributing to the reduced fluorescence after EMA staining in this disease [4]. The reduced fluorescence in CDA II is probably not due to a quantitative reduction of band 3 in the erythrocyte membrane but rather an affected configuration leading to a decreased affinity to EMA [6]. The increase in EMA fluorescence in CDA III is probably due to the slight increase of erythrocyte volume (MCV) exposing a larger surface per cell to binding of EMA to band 3, Rhesus-antigen and CD47 on the erythrocyte membrane. A correlation has been confirmed in individuals with high MCV due to overconsumption of alcohol, but in DAT-positive hemolytic anemia no correlation between MCV and MCF was observed [3]. Low MCV due to iron deficiency does not affect EMA MCF [3,6,20]. The results of the present study indicate no abnormality of the erythrocyte membrane in CDA III and show that standard flow cytometry cannot be used to discriminate between CDA III and normal controls.

Acknowledgements

We thank all members of the CDA III family in Västerbotten and acknowledge collaboration with Göran Roos, Professor at the Department of Medical Biosciences, Umeå University and Hematopathology Laboratory at Umeå University hospital.

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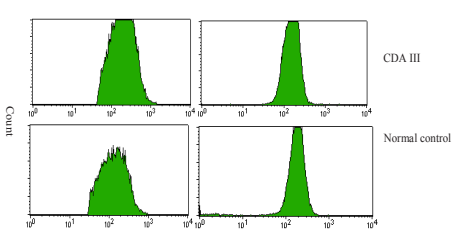

Figure 3: Similar expression of CD 55 and CD 59 in one patient with CDA III and one normal control.

Authorship

Contribution: M.L., A.W., and E.G. designed the study; M.L. collected clinical data and samples from the CDA III family; E.G. collected control samples and performed flow cytometry analyses. M.L., and A.W. analyzed an interpreted flow cytometry data. M.L. and A.W. wrote the paper and all authors contributed to the writing process and approved the final manuscript.

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III

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Congenital Dyserythropoietic Anemia Type III and Primary Hemochromatosis; Coexistence of Mutations in *KIF23* and *HFE*

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Abstract

Background: Congenital dyserythropoietic anemia type III (CDA III) can be caused by mutation in KIF23. CDA III differs from CDA I and II in the sense that secondary hemochromatosis has not been reported. However, we have observed elevated serum ferritin in a CDA III family. Since primary hemochromatosis is common in Northern Europe we decided to screen the family for *HFE* mutations.

Aim: Study clinical appearance and prevalence of *HFE* gene mutations, C282Y and H63D, in a CDA III family.

Methods: DNA from 37 CDA III patients and 21 non-affected siblings was genotyped. Iron status from EDTA plasma was measured in 32 of the CDA III patients and 18 of the non-affected siblings.

Results: Out of 37 CDA III patients, 18 carried heterozygous *HFE* mutations and six were compound heterozygotes. Out of 21 CDA III negative siblings, nine had heterozygous *HFE* mutations, two were homozygous (one H63D and one C282Y), and two were compound heterozygous. None of the patients with *wt HFE*, regardless of CDA III status, suffered from iron overload. Four patients with *HFE* mutations needed treatment with phlebotomy to normalize ferritin and transferrin iron saturation; one CDA III negative patient with homozygous C282Y, two CDA III patients with heterozygous *HFE* mutations and one CDA III case with compound heterozygosity.

Conclusion: *HFE* mutations were found in 65 % of CDA III patients and in 62 % of their CDA III negative siblings. Heterozygous *HFE* mutation, C282Y and even H63D, can cause iron overload when occurring concomitantly with CDA III.

Keywords: Congenital dyserythropoietic anemia; Hereditary hemochromatosis; Iron overload; *HFE* gene; *KIF23* gene

Introduction

Congenital dyserythropoietic anemias (CDA) is a group of rare hereditary hemolytic disorders. Three major subtypes (I, II, III) and some minor entities (IV-VII) have been described [1]. The CDAs present with laboratory signs of increased hemoglobin (Hb) turnover, such as low or absent haptoglobin, elevated lactate dehydrogenase (LDH) and bilirubinemia. The bone marrow is hypercellular with a predominance of large bi- to multi-nucleated erythroblasts. Erythroblast morphology differs between CDAs, but the diagnosis of CDA I, II and III is best set by genetic testing [2,3].

CDA I and II are the most common forms, both with autosomal recessive inheritance [4-6]. Symptoms and signs of CDA I and II are mainly mild to moderate anemia, splenomegaly, and iron overload. The risk of secondary hemochromatosis increases when transfusions are needed [1]. Overexpression of Growth Differentiation Factor 15 (GDF15) in CDA I and II has, as in thalassemia, been shown to limit hepcidin expression thereby increasing iron absorption [7,8].

CDA III is the rarest form of the major CDA subtypes with only about 60 reported cases worldwide. A familial form with dominant inheritance has been described in two families, one American family with four cases and one family in Västerbotten County, Sweden, with the majority of reported cases [9,10]. One family in Argentina with eight cases has been described, although the pattern of inheritance in this family has not been confirmed [10]. A few cases of CDA III with recessive inheritance have been described in the literature [11,12]. One of these patients developed iron overload after erythrocyte transfusions [12].

In the Västerbotten CDA III family, the disease is caused by a mutation in c.2747C>G, p.P916R in *KIF23*. The mutation results in a dysfunctional *KIF23* protein, also known as Mitotic Kinesin Like Protein 1 (MKLP1), leading to failure of cytokinesis, the final stage of mitosis. As a result, the bone marrow in CDA III is characterized by gigantoblasts, large erythroblasts with up to 12 nuclei in the same cell [13].

The Swedish CDA III family has been mapped through six generations back to the middle of the 19th century [14,15]. The disease presents with mild to moderate anemia, rarely in the need of red cell transfusions. Hemolysis is extra- as well as intravascular, leading to hemosiderinuria. Since CDA III is a disorder of ineffective erythropoiesis, hepcidin is expected to be suppressed, leading to iron overload, but ferritin levels were not found to be elevated in CDA III patients in earlier studies [15]. Intravascular hemolysis with hemosiderinuria and loss of iron in the urine has been suggested to protect patients against iron overload [15].

However, we have recently observed elevated ferritin levels in some CDA III positive and negative members of the Västerbotten family. Further investigations revealed mutations of the hemochromatosis gene (*HFE*), the gene involved in the development of hereditary hemochromatosis (HH). *HFE* mutations (C282Y and H63D) are frequently found in Northern Europe and the prevalence of HH in Sweden is 0.5% [16]. Homozygous mutation of C282Y or compound heterozygosity of C282Y and H63D reduces hepcidin, thereby increasing iron absorption, and may result in clinical signs of hemochromatosis, but the penetrance is relatively low, estimated in the literature to 10-30% [17]. Heterozygous C282Y is found in 7% of the Swedish population and 0.5% carry the mutation on both alleles [16]. Heterozygous H63D is frequently found all over the world with a prevalence of about 10-20% but does not by itself lead to significant iron overload [17,18]. Treatment of iron overload in patients with primary hemochromatosis is based on phlebotomy.

We have studied the clinical appearance and presence of mutations in *KIF23* and *HFE* in the Västerbotten CDA III family.

Patients and Methods

Study population

All patients belonged to the "Västerbotten CDA III family" (Figure 1). None of the participants in the study was transfused.

DNA samples and genotyping – detection of *HFE* mutations

DNA was available from 37 individuals with CDA III and 21 non-affected siblings. The status of CDA III was confirmed by detection of *KIF23* c.2747C>G as earlier described [13].

Sequencing of *KIF23* was performed by polymerase chain reaction (PCR). The products of sequencing reactions were analysed on ABI 3500xL Dx Genetic Analyzer (Applied Biosystems, Foster City, CA). PCR - products were digested by endonuclease, separated by electrophoresis on an Agarose gel and visualized after staining with ethidium bromide [13].

HFE analysis was done with TaqMan® SNP Genotyping Analysis. Assay for detecting c.187C>G, p.H63D, rs1799945 was done using predesigned assay C1085600_10 (Applied Biosystems). Assay used for detecting c.845G>A, p.C282Y, rs1800562 was designed using Custom TaqMan® Assay Design Tool.

Hepcidin was analysed using a commercially available Competetive ELISA test (Bachem, Peninsula Laboratories, LLC, CA, USA).

Clinical appearance

Blood samples in EDTA-tubes were used for hematological analyses including iron status (ferritin and TSAT) and hematological parameters (Hb and LDH). Reference intervals for ferritin were 30-400 ug/L for males and 13-150 ug/L for females. Upper limit of normal for TSAT was < 60% for males and < 50% for females. From 1997 to 2007 ferritin was analysed by Boehringer Mannheims Tina-Quant kit 1661400. From 2007 analyses were done using Roche Modular E170 with electrochemiluminescence immunoassay (ECLIA). Correlation between methods was 1.0. Serum-Fe was analysed with Hitachi 717/Hitachi 911, Boehringer Mannheims Ferrozine 1997-2007 and thereafter with Hitachi 911, delta, till Vitros 5.1 FS. Transferrin analysed with Vitros 5,1. TSAT was calculated by the following formula: S-Fe [µmol/L] x 100 / (S-Transferrin [g/L] x 25,1).

All individuals with *HFE* mutation and elevated TSAT were offered clinical and laboratory investigation to confirm or reject iron overload. Phlebotomy was initiated in individuals with iron overload and in patients with homozygous C282Y plus TSAT above the upper limit of normal.

Statistics

Ferritin, TSAT, Hb and LDH are presented as median values and range. Two-sided Mann-Whitney U-test was used for comparison between the CDA III positive and negative group. $P < 0.05$ was regarded as significant.

Ethical approval was obtained from the Regional Ethics Committee in Umeå, Sweden, and informed consent was obtained from all patients.

Results

The group of patients with CDA III and available iron status and hematological parameters (Hb and/or LDH), consisted of 16 males and 16 females with a median age of 53 years, and the CDA III negative group consisted of 10 males and 8 females, with a median age of 44 years. The median hemoglobin value among CDA III patients was 116 g/L (94-157), LDH 6.2 ukat/L (4.2-10), and bilirubin 18 umol/L (7-86).

Iron status

Ferritin was higher (108 umol/L) in the CDA III positive group than in the CDA III negative group (76 umol/L), and TSAT was also higher among CDA III patients, 46% vs. 34%, but the differences were not significant (p=0.055 for both comparisons). The two groups did not differ concerning age or gender.

HFE status

HFE genotyping was successfully performed in all 58 cases. *HFE* mutation was found in 24 out of 37 CDA III patients (65%) and 13 out of 21 CDA III negative siblings (62%), (Figure 1). Among the 50 cases with available iron status, 21 out of 32 CDA III positive (66%) and 11 out of 18 CDA III negative siblings (61%) were found to have *HFE* mutations. 16 of the CDA III patients with *HFE*-mutation were heterozygous either for C282Y or H63D, and five were compound heterozygotes. Seven of the 18 CDA III negative siblings were heterozygous carriers of *HFE* mutations, two were compound heterozygotes, and two had homozygous *HFE*-mutations (Table 1). In individuals with heterozygous C282Y (9 CDA III positive and 6 CDA III negative cases), ferritin levels were significantly higher in CDA III patients, p=0.036.

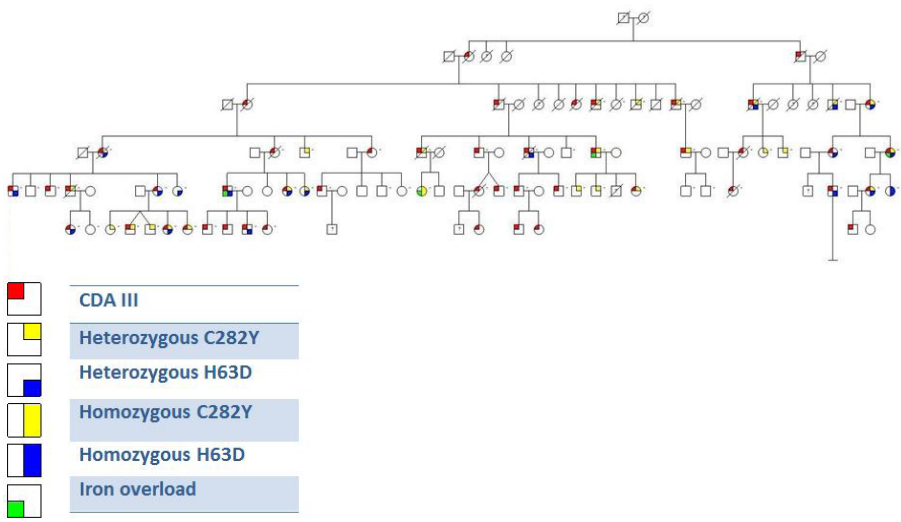

Figure 1: Pedigree of the Västerbotten CDA III family. Participants with available DNA are indicated with dash (–) to the right of the individual symbol

Laboratory evidence of iron overload was noted in four patients, one CDA III negative case with homozygous C282Y, and three CDA III positive individuals with heterozygous *HFE* mutations (Table 2). Magnetic resonance imaging (MRI) of the liver and S-hepcidin analysis was performed only in the CDA III positive patient with heterozygous H63D mutation. Liver iron was 6700 ug/g dry tissue (normal range 300 -1500) and hepcidin was 46 ug/L (normal range 8-76). To normalize ferritin and TSAT, treatment with phlebotomy was started in four patients; the CDA III negative sibling who was homozygous for C282Y and three CDA III patients, one compound heterozygote and two with heterozygous mutations, one C282Y, one H63D.

A total of 18 individuals, 11 CDA III cases and 7 CDA III negative siblings, had *wt HFE*. Ferritin levels did not differ between these groups (p=0.86, Table 1). One of these CDA III positive patients, suffering from hypertension and hyperlipidemia, had elevated ferritin (642 ug/L) but TSAT was not elevated, 40%. In another CDA III positive case without *HFE* gene mutation TSAT was elevated (73%) due to subnormal transferrin 1.68 (reference interval 1.87-3.19 g/L). Ferritin was not elevated in this patient, 191 ug/L.

Discussion

We found that 65% of CDA III patients and 62% of their CDA III negative siblings in the Västerbotten family carried mutations in the *HFE* gene. Two CDA III patients with heterozygous *HFE*-mutations and one CDA III patient who was compound heterozygous for the *HFE*- mutations needed treatment with phlebotomy due to iron overload.

HFE status	Ferritin ug/L CDA III +	Ferritin ug/L CDA III –	TSAT % CDA III +	TSAT % CDA III –	Hb g/L CDA III +	Hb g/L CDA III –	LDH ukat/L CDA III +	LDH ukat/L CDA III –
***HFE* wt**	116 (28-642)* n=11	90 (28-392) n=7	45 (26-73) ** n=11	32 *** (27-44) n=7	119 (105-135) n=11	135 (118-153) n=7	6.1 (4.2-8.0) n=11	2.8 (1.9-4.5) n=7
C282Y +/-	89 (71-987) n=9	73 (13-154) n=6	45 (37-100) n=9	33 (21-46) n=6	123 (107-157) n=9	145 (114-154) n=6	6.0*** (5.2-10.0) n=9	2.8 (1.9-3.1) n=6
C282Y +/+		229 n=1		79 n=1		153 n=1		2.1 n=1
C282Y +/- H63D +/-	66 (48-700) n=5	75 (56-94) n=2	55 (28-80) n=5	49 (42-56) n=2	116 (114-124) n=5	154 (145-162) n=2	6.4*** (5.9-8.3) n=5	2.4 (2.0-2.7) n=2
H63D +/-	139 (41-1084) n=7	3 n=1	39 (24-80) n=7	4 n=1	105 (80-135) n=7	94	6.7*** (5.8-7.7) n=7	2.4
H63D +/+		20 n=1		49 n=1		138 n=1		2.7 n=1

+/- heterozygous mutation, +/+ homozygous mutation.
* elevated ferritin in one patient with hyperlipidemia and normal TSAT.
** elevated TSAT in one patient with subnormal Tf and normal ferritin.
*** sample missing in one case.
Table 1: Iron status, Hb and LDH in individuals of the Västerbotten CDA III family, with and without HFE mutation. Medians and (range)

CDA status	Gender and age (years)	*HFE* status	Ferritin (ug/L)	TSAT (%)	Hb (g/L)	LDH (ukat/L)
wt	F 49	C282Y +/+	229	79	153	2.1
CDA III +	M 60	C282Y +/-	987	100	143	5.2
CDA III +	F 61	C282Y +/- H63D +-	700	55	111	6.1
CDA III +	M 60	H63D +/-	1084	80	135	6.3

Table 2: Individuals in whom phlebotomy has been initiated due to iron overload

One of the heterozygous cases carried H63D, which is not reported to lead to iron overload by itself. MRI of the liver confirmed iron overload in this patient. Hepcidin was in the normal range, but we believe that the level was inappropriate considering the iron status, indicating disruption of the feedback system. We hypothesise that increased erythropoiesis produces larger amounts of erythroferrone suppressing hepcidin that may be further suppressed by *HFE* mutation.

None of the healthy siblings carrying *HFE*-mutations with heterozygous or compound heterozygosity nor CDA III positive cases with *wt HFE* had laboratory signs of iron overload. One CDA III positive individual with *wt HFE* had elevated ferritin and one had elevated TSAT. After further investigation none of these patients fulfilled the criteria of iron overload. Ferritin levels were higher among CDA III positive vs CDA III negative individuals with heterozygous C282Y, though study population is limited. Earlier studies have shown that homozygous mutation of *HFE* C282Y or, eventually, compound heterozygosity of C282Y and H63D is needed to develop clinical signs of hemochromatosis in healthy individuals, though penetrance is relatively low [17]. In this study we found that heterozygous mutation of the *HFE*- gene, either C282Y or H63D can be sufficient to cause iron overload in patients with CDA III. All patients were screened for diabetes since this is a well-known metabolic factor causing elevated ferritin, although TSAT is not elevated in diabetes [19]. One male CDA III patient, heterozygous for H63D, was diagnosed with type II diabetes mellitus. Except for elevated ferritin (1084 ug/L), TSAT was also elevated (80%) in this patient, indicating iron overload.

Earlier studies on *HFE*-mutations in patients with hematologic disorders where iron overload can be a problem, such as thalassemia and myelodysplastic syndromes (MDS), are contradictory. Some studies show iron overload in thalassemia patients with co-existing *HFE*-mutations, even in heterozygous forms [20-22]. Recently, non-transfused MDS-patients, carriers of *HFE*-mutations, were found to have higher ferritin level and inferior overall survival compared to non-mutated MDS-patients [23]. However, other studies have not verified that heterozygous mutation affects iron overload in thalassemia [24,25].

So far, phlebotomies have been performed without problems in spite of the continuous hemolysis in our CDA III patients and phlebotomy alone is sufficient to control ferritin and TSAT-levels.

Conclusion

This study indicates that heterozygous *HFE* mutation, C282Y or H63D, is sufficient to cause pathologic iron overload when occurring concomitantly with CDA III. Therefore, in order to prevent organ damage in these patients, screening for primary hemochromatosis should be performed in all patients with congenital dyserythropoietic anemia type III.

Conflict of Interest and Sources of Funding

Financial support was obtained through regional agreement between Umeå University and Västerbotten County Council on cooperation in the field of Medicine, Odontology and Health (ALF), and unrestricted grants from Cancerforskningsfonden Norrland. None of the authors has any conflict of interest to declare.

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