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Characterisation of weak and null phenotypes in the KEL and JK blood group systems

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Abstract

The focus of this thesis is on KEL and JK, two clinically important blood group systems, and the investigations of blood samples with aberrant antigen expression as in null and weak phenotypes, or with a phenotype that does not correspond to genotype.

With the increasing use of DNA assays based on single nucleotide polymorphisms for blood group prediction, it is important to characterize the blood-group-encoding genes. Otherwise, when using these assays for phenotype prediction, tests risk giving rise to false results if null or otherwise altered genes are present and the test are not designed to take alterations into consideration. This is especially applicable for foetal blood group prediction, finding matching blood donors to transfusion-dependent patients or to correctly type multi-transfused patients where serology is inadequate.

For each blood group system investigated here, four novel null alleles and one allele giving rise to weak antigen expression were characterised. Most importantly, a novel Jk(a+^w) phenotype was described and associated with a novel *JK*01M* allele. A control cohort was screened for the associated polymorphism and allele frequencies calculated.

A simple PCR-ASP method for screening of five null alleles giving rise to the Jk(a-b-) phenotype was devised, utilised and proven useful.

In summary, this thesis contributes to the overall understanding of the molecular genetic basis of human blood group diversity in the two investigated systems and the results will improve the prediction of blood group phenotypes from DNA-based assays.

Key words: Blood group, KEL, Kell, JK, Kidd, SNP, null, weak

There's nothing you can do that can't be done
Lennon/McCartney

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List of papers

This thesis is based on the following papers, which are referred to in the text by the Roman numerals.

- I **Wester ES**, Storry JR, Schneider K, Nilsson Sojka B, Poole J, Olsson ML.
Genetic basis of the K₀ phenotype in the Swedish population.
Transfusion, 2005;45(4):545-549.
- II **Wester ES**, Johnson S, Copeland T, Malde R, Lee E, Storry JR, Olsson ML
Erythroid urea transporter deficiency due to novel JK^{null} alleles
Transfusion, 2008;48(2):365-72.
- III **Wester ES**, Gustafsson J, Snell B, Spruell P, Hellberg Å, Olsson ML, Storry JR.
A simple screening assay for the most common JK*O alleles revealed compound heterozygosity in Jk(a-b-) probands from Guam.
Immunohematology, in press.
- IV **Wester ES**, Steffensen R, Ligthart PC, Vad J, de Haas M, Storry JR, Olsson ML.
KEL*02 alleles with alterations in and around exon 8 in individuals with apparent KEL:1,-2 phenotypes.
Vox Sanguinis, in press.
- V **Wester ES**, Storry JR, Olsson ML.
Characterisation of Jk(a+^{weak}): a new blood group phenotype associated with an altered JK*01 allele.
Submitted to *Transfusion*.

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Populärvetenskaplig sammanfattning

På samma sätt som vi människor ser olika ut, har även våra blodceller olika utseende s.k. fenotyp. Denna mångfald av utseenden benämns polymorfism och beror på att våra gener finns i olika versioner, s.k. alleler.

På och invävda i den röda blodkroppens cellyta sitter de molekyler som bär våra blodgrupper (Figur 1). Våra olika blodgruppsantigener uppstår på grund av variationer i molekylerna, vilket i sin tur beror på olika alleler. Idag finns mer än 300 blodgruppsantigener beskrivna, inordnade i 30 olika blodgruppssystem, där ABO- och RH- systemen är det mest kända. Via samarbetet i International Society of Blood Transfusion (ISBT) har man fastställt vad som krävs för klassificering av ett blodgruppssystem: antigenen måste vara ärftliga, uttryckta på den röda blodkroppen, ha gett upphov till en antikropp och vara kodade av en känd gen med en känd plats i våra kromosomer. Man har även fastställt den terminologi som ska användas: blodgruppssystem (kortnamn på två-fyra bokstäver), blodgruppsgen (skrivs kursivt), allel (kursivt följt av numrering), blodgruppsantigen (systemets namn följt av numrering).

Många av våra blodgrupper finns även på andra celler än på den röda blodkroppen och vi känner idag till flera av de funktioner som bärarmolekylerna har i cellen. Utifrån detta kan blodgrupperna indelas i funktionella grupperingar som transportproteiner, kolhydrater, receptorer etc. (Figur 2).

Blodgruppsgenerna finns spridda över våra kromosomer och uttrycks codominant d.v.s. både genen från mamma och från pappa uttrycks. Det innebär att vi i vår genuppsättning, s.k. genotyp, kan vara homozygota med identiska kopior av samma allel, eller heterozygota med två olika alleler. Vissa gener är tystade på grund att de har en defekt som gör att de inte uttrycks. Inom blodgruppsvärlden kallas de tysta generna ibland för null-gener och inom ABO-systemet är allelen *O* en sådan tyst gen. Den individ som är blodgrupp *O* (noll) har alltså ärvt en *O*-allel från vardera föräldern, medan individen med blodgrupp *A* kan ha ärvt antingen *A*- och *A*-allel eller *A*- och *O*-allel.

När en person transfunderas med blodkroppar från en annan person kan immunförsvaret känna igen och reagera på främmande blodgruppsantigener genom att bilda antikroppar, sk. alloantikroppar. På samma sätt kan blodkroppar hos ett foster, som har ärvt hälften av sina blodgruppsantigener från pappa, uppfattas som främmande av mammans immunförsvaret. Mot blodgruppsantigen som består av kolhydrat kan vi spontant bilda antikroppar d.v.s. utan transfusion eller graviditet och slutligen kan s.k. autoantikroppar (antikropp mot den egna

blodgruppen) förekomma i sällsynta fall. Vid blodtransfusion eller under graviditet kan de olika typerna av antikroppar förstöra den röda blodkroppen med blodbrist som följd. Om antigenet, som antikroppen är riktad mot, även finns på våra blodplättar kan även dessa förstöras och koagulationsrubbing uppstå. Antikroppar mot ABO-, RH- och KEL-systemen är vanligast vid graviditetsimmunisering.

Vid blodtransfusion väljs alltid ABO-förenligt blod (för att undvika reaktion med de naturligt förekommande antikropparna) och som om möjligt är grupplikt avseende RhD (för att undvika antikropps bildning). Om patienten har bildat antikroppar mot ytterligare blodgruppsantigen väljs dessutom matchande blod avseende detta. För att undvika KEL1-immunisering transfunderas kvinnor under 50 års ålder, i Sverige och många andra länder, med KEL1-negativt blod. Till patienter som behöver livslångt stöd av blodtransfusioner förebygger man ytterligare antikropps bildning genom att försöka hitta blodgivare med en blodgruppsuppsättning som liknar patientens så mycket som möjligt.

Analys av blodgrupper och antikroppar mot blodgrupper sker idag rutinmässigt med serologisk metodik och med detta fastställs individens fenotyp och antikroppsstatus. För att uppnå korrekta resultat krävs bra testreagens, d.v.s. både bra testantikroppar för att identifiera antigener och bra testblodkroppar för att identifiera antikroppar.

Under senare år har genetisk analys utvecklats och med god kännedom om blodgruppsgenerna kan dessa utvecklas vidare till att ge säkra underlag för att förutsäga den faktiska blodgruppen dvs. beskriva fenotypen ur den funna genotypen. Genetiska analyser går bra att automatisera och en framtidsbild kan vara att blodgivare genotypas, resultaten lagras i en databas och används när matchande blod behövs. En annan användning för genetisk blodgruppsanalys är vid graviditetsimmunisering där fostrets blodgrupp kan undersökas via fritt foster-DNA i ett blodprov från mamman. Dessutom används genteknik när en individs blodgrupp kan vara svår att fastställa. Detta gäller t.ex. efter en blodtransfusion då blodprovet innehåller röda blodkroppar från både mottagare och blodgivare.

Om gentekniken endast innebär undersökning av en eller flera specifika punkter i gensekvensen, s.k. single nucleotide polymorphisms (SNPs) som skiljer en allel från en annan, blir tolkningen felaktig om den undersökta allelen innehåller förändringar i andra delar, om inte även dessa förändrade delar undersöks.

De två kliniskt viktiga blodgruppsystemen KEL och JK är i fokus för denna avhandling och de variationer på gennivå (polymorfismer om de är vanligt förekommande och mutationer om de är sällsynta) som ger upphov till null-fenotyp eller ett väldigt svagt blodgruppsuttryck är studerade.

Med antikroppsbaseade metoder har blodgruppsvarianter och blodgruppsmolekyler undersökts och med DNA-baserade metoder undersöktes blodgruppsgenerna.

Det provmaterial som ingår i studierna kommer från laboratorier i olika delar av världen där man har noterat någon form av avvikelse i samband med blodgruppsbestämning. Kontrollprover är hämtade från friska blodgivare i Lund.

KEL-systemet: Artikel I och IV

KEL-genen studerades i **artikel I** hos tre svenska individer (samt familjemedlemmar till en av individerna) som alla var *KEL*-null, d.v.s. saknade Kell-proteinet och därmed alla *KEL*-antigener. Två av individerna hade bildat en antikropp som reagerade med alla *KEL*-positiva röda blodkroppar. Hos alla tre identifierades SNPs som orsakar att allelerna tystas. **Artikel IV** beskriver fler *KEL* utredningar, denna gång undersöktes både uttrycket av blodgruppsvarianterna *KEL1*, *KEL2* och bärarmolekylen Kell med olika metoder samt *KEL*-genen hos danska och holländska individer. Unika SNPs som gav upphov till två *KEL*-null alleler och en allel som orsakar ett svagt *KEL2*-antigen identifierades.

Totalt identifierades inom *KEL* systemet fyra nya *KEL*02* null-alleler och en *KEL*2* svag allel inom *KEL* systemet.

JK-systemet: Artikel II, III och V

JK systemets gen, *JK*, undersöktes i **artikel II** hos nio individer av olika ursprung, som alla var *Jk(a-b-)* d.v.s. saknade Kidd-proteinet som bär antigenen *Jk^a*, *Jk^b* och *Jk3*. Fyra nya *JK*-alleler med SNPs som ger upphov till tystat genuttryck identifierades. Hos övriga undersökta individer identifierades alleler som beskrivits tidigare i litteraturen, men i flera fall hos andra befolkningsgrupper än de nu undersökta. För att bekräfta att en av de funna mutationerna verkligen gav upphov till en *JK*-null allel undersöktes röda blodkroppar från denna individ med annan metod än vid vanlig blodgruppering. Eftersom inget Kidd-protein återfanns på cellytan kopplades den funna mutationen till förklaringen.

I **artikel III** presenteras en ny genanalys som enkelt identifierar mutationer i fem utvalda *JK*-null alleler. Med denna metod undersöktes en familj med mamma och två söner där alla saknade *JK* uttryck. Resultaten visade att mamman hade två olika *JK*-null alleler som gick i arv till sönerna och även via pappan (ej undersökt) ärvde sönerna en *JK*-null allel.

Observationer av svagt Jk^a-uttryck inledde analyserna som gav underlag till **artikel V**. Här undersöktes sex individer där det lokala laboratoriet hade svårighet att bestämma om de röda blodkropparna hade eller saknade antigenet Jk^a. Återigen studerades *JK*-genen och en ny allel, *JK*01M* kartlades. Denna hade samma polymorfism som ger upphov till Jk^a via *JK*01*-allelen, men innehöll även andra variationer. Samma metod för att undersöka proteinet på cellytan som i artikel II användes även här tillsammans med andra analysmetoder. De bekräftade att röda blodkroppar från individer med *JK*01M* innehåller mindre mängd Kidd-protein och Jk^a-antigen på cellytan. Att denna allel ärvs, visas i en ännu inte publicerad studie (Figur 9), där en familj med mamma, pappa och sex barn beskrivs och där arvet av *JK*01M* med ett svagt Jk^a-uttryck går att följa tack vare släktutredningen. Denna intressanta familj kan dessutom ha en eventuell null-variant av samma gen, vars defekt dock inte identifierats ännu.

Totalt identifierades inom JK systemet en ny *JK*01* null-allel, tre nya *JK*02* null-alleler och för första gången en allel, *JK*01M*, som kan kopplas ett svagt Jk^a uttryck. Den senare finns inte beskriven i läroböckerna och därför betraktar vi detta fynd som en ny fenotyp in JK-systemet.

Sammanfattning

Ett flertal *KEL*- och *JK*-alleler som ger upphov till avsaknad alternativt svagt blodgruppsuttryck på den röda blodkroppen är karakteriserade.

Förutom de individer som beskrivs ovan och i de publicerade artiklarna undersöktes ytterligare ett antal individer. I ett par fall identifierades inga null-alleler trots att de enligt preliminära DNA-tester borde finnas. Detta kan förklaras av antingen fel vid de preliminära testerna eller att avsaknad av blodgruppsuttryck orsakas av någon annan faktor än variation i den undersökta genen.

Denna avhandling bidrar till den övergripande förståelsen för mångfalden i human genvariation inom de undersökta blodgruppsystemen och ger därmed underlag till att bättre fastställa fenotyp ur genotyp.

Abbreviations

aa	Amino acid
ACD	Acid citrate dextrose
ASP	Allele-specific primer
bp	Base pairs
CD	Cluster of differentiation
CPD	Citrate phosphate dextrose
CPD-A	Citrate phosphate dextrose adenine
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
HDFN	Haemolytic disease of the foetus and newborn
HTR	Haemolytic transfusion reaction
ISBT	International Society of Blood Transfusion
kb	Kilobase
kDa	Kilodalton
MAB	Monoclonal antibody(ies)
NAITP	Neonatal alloimmune thrombocytopenic purpura
nt	Nucleotide
PAb	Polyclonal antibody(ies)
PE	R-phycoerythrin
PCR	Polymerase chain reaction
PTM	Post-translational modification
RBC	Red blood cell
RFLP	Restriction-fragment-length polymorphism
PVDF	Polyvinylidene difluoride
SNP	Single nucleotide polymorphism
WBC	White blood cell

Introduction

Blood groups

The first blood group system described was ABO by Karl Landsteiner in 1901 [1]. Since then, a total of 30 blood group systems have been defined by the International Society of Blood transfusion (ISBT), comprising >300 antigens (www.blood.co.uk/ibgri). To be recognized as a blood group system, certain criteria have to be met; the antigens are to be inherited and encoded by an identified, sequenced gene with a known chromosome location. Furthermore antigens are to be expressed on the red blood cell (RBC) surface (Figure 1) and to be able to trigger the production of alloantibodies. Antigens where the molecular and/or biochemical background are not yet clarified are gathered into collections or series of high- and low-frequency antigens thus waiting to achieve blood group system status [2]. The majority of systems are not only restricted to RBCs, but are found on other cells as well.

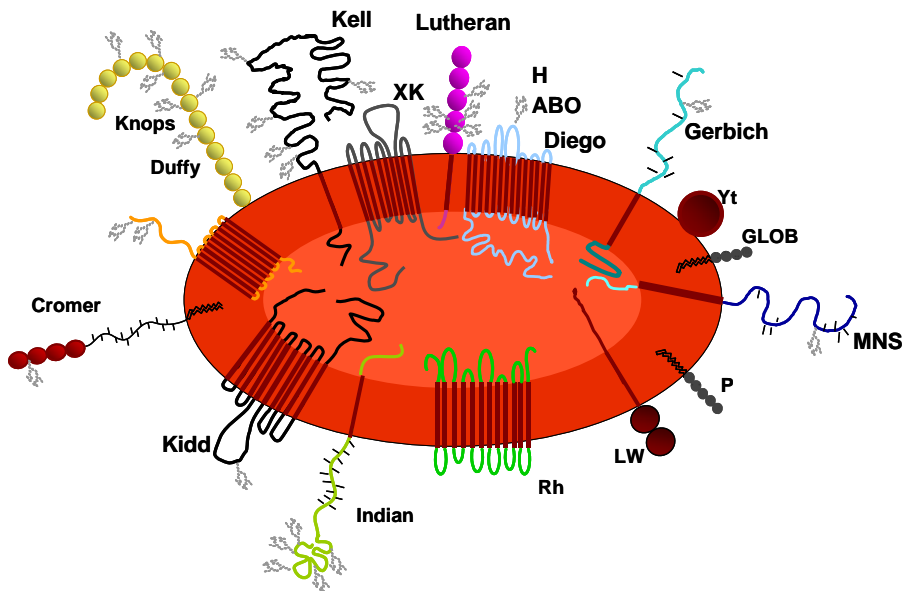


Figure 1. Cartoon of a RBC with examples of some of the blood-group-antigen carrying molecules. Many of the proteins are glycosylated and thus carry carbohydrate blood group antigens as well.

The formation and development of blood cells is called haematopoiesis, a constant process giving rise to billions of cells daily. Haematopoiesis is traditionally described to occur in two major lineages; the lymphoid line producing lymphocytes and the myeloid line producing granulocytes, monocytes, thrombocytes (platelets) and erythrocytes (red blood cells RBC). During haematopoiesis cell surface molecules, including glycoproteins, other proteins and glycolipids, are assembled in the cell membrane. Many of these, but not all, carry blood group antigens. RBCs, the blood-group-carrying cells of particular interest in this thesis are produced through erythropoiesis (Figure 2).

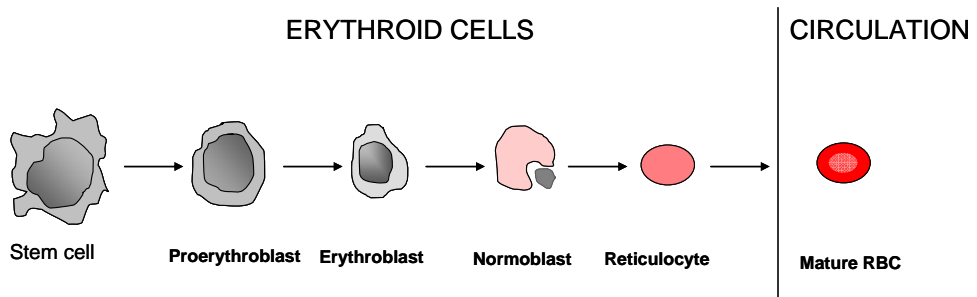


Figure 2. Erythropoiesis, the differentiation of RBCs that starts with the multipotent haematopoietic stem cell and finishes with the RBC in circulation.

By cell culture studies, the time-course expression of blood-group-carrying molecules during erythropoiesis has been described. The Kell glycoprotein was determined to be the first blood group protein arising, closely followed by Rh-associated glycoprotein and Glycophorin A, commonly used as an erythroid cell marker. The other blood group carrying molecules followed thereafter [3-5].

Blood group genetics

Genes encoding blood groups are typically inherited in a co-dominant fashion, meaning that the products of both genes present are equally expressed. If more than one antigen, or a null phenotype, is found within the blood group system this reflects alleles i.e variants of the gene or the occurrence of closely linked genes. As in genes coding for other inherited variations, blood group alleles differ from each other by single nucleotide polymorphisms (SNPs) or by other gene alterations such as partial or whole gene deletions or hybrid formations between genes [6,7].

Table 1. Three types of single nucleotide polymorphisms (SNPs) that change a DNA sequence

Silent (synonymous)	Missense (nonsynonymous)	Nonsense (altered reading frame)
Resulting in an alternative codon that encodes the same amino acid. Can affect splice site recognition and possibly translation efficacy and/or protein folding [8]	Changing the codon which thus encodes a different amino acid.	Changing the sequence to a stop codon. May also affect splice site recognition and correct transcription.

The most common SNPs are caused by transitions, the exchange of purines (A↔G) or pyrimidines (C↔T). Less common are transversions, which exchange a purine for a pyrimidine or a pyrimidine for a purine.

Polymorphisms are hereby described as variations in the genetic sequence that generally affects only a single nucleotide (nt.) and occurs in more than 1% of a population. A mutation, on the other hand, is either a variation occurring in less than 1% of a population and/or causing a phenotype with an abnormal function. Non-lethal polymorphisms/mutations accumulate within the gene pool if they are neutral to, or enhance survival value to the individual/population and thereby increase the amount of genetic variation. Thus, variation including blood group variation is driven by natural selection (the value of having certain characteristics) as well as occurrences such as assortative mating or a small population pool, due to the so called bottle neck or founder gene effect, where only a small part of the population are involved in reproduction [9].

SNPs in blood group genes follow the same order as in other genes and may thus be found within the gene, including regulatory areas [10,11], as well as in other genes responsible for regulation and modification [12].

All steps of gene expression are regulated; from DNA transcription via RNA to post-translational modifications (PTM) of the protein. RNAs with pre-mature stop codons are cleared by the nonsense mediated decay system (NMD) [13] and aberrant proteins are degraded in proteosomes or may be accumulated in aggresomes [14] (Figure 3).

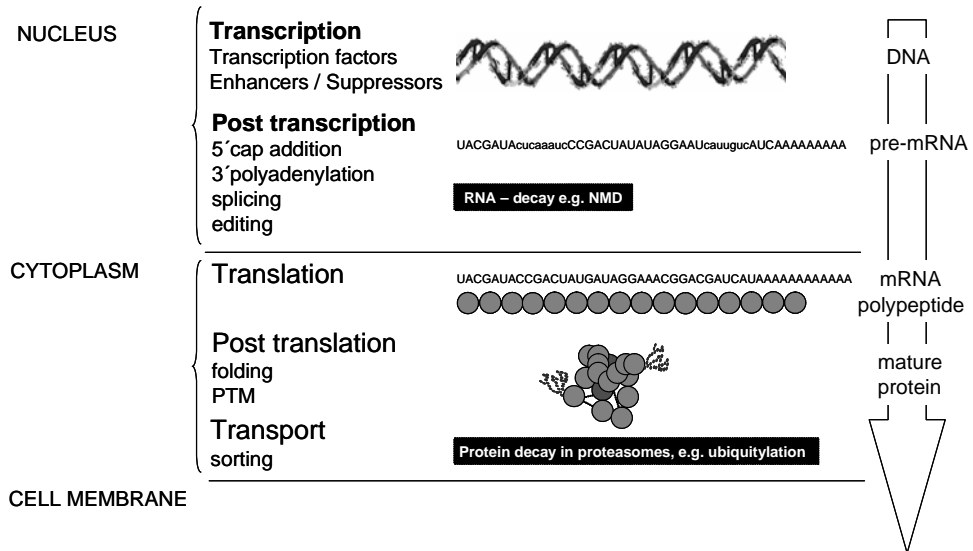


Figure 3. The progress from translation to a mature protein comprise a stepwise process with control mechanisms for degrading non-functional RNA and polypeptides.

Structure and function of blood group carrying molecules

Blood-group-carrying molecules include glycoproteins, proteins or glycolipids situated in the RBC membrane. The list of known or speculative functions is growing and blood groups may be assigned into functional assemblies including channel/transporter, receptor/adhesion, glycocalyx, enzyme and membrane structure molecules (Figure 4) [7]. Some molecules, as the Rh family, are essential for maintaining stability of the RBC membrane and a cell lacking these molecules will have an altered morphology and shortened life span [15].

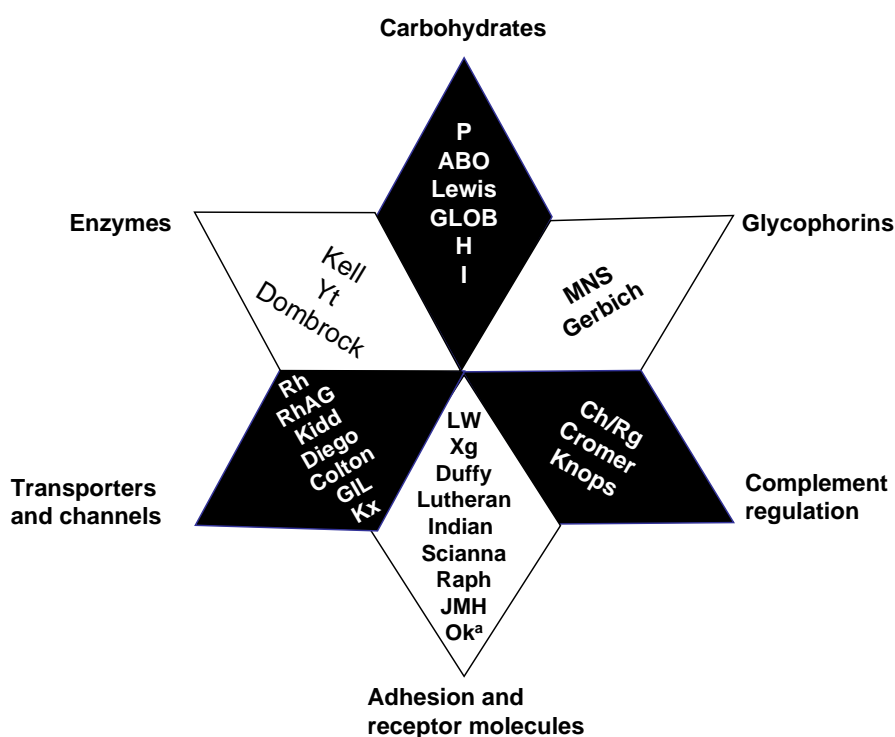


Figure 4. In this illustration the 30 blood group systems are arranged after the proven or hypothesised function of their carrier molecule.

The majority of proteins and glycoproteins (gp) are either single- or multi-pass transmembrane structures of type I, II or III although some a few are linked to the cell surface by a glycosylphosphatidylinositol (GPI) anchor.

Blood group antigens and antibodies

Even if the ISBT-based definition of blood group antigen is based on human antibodies, blood group phenotypes are defined today by using either monoclonal (MAb) or polyclonal antibodies (PAb) [16]. Most antigens are the product of a gene expressed in erythroid tissues but a few (LE and CH/RG) are produced by other cells and later adsorbed to the RBC membrane.

Some antigens e.g. KEL1 carried on the Kell-gp and S on Glycophorin B are more immunogenic than their antithetical antigen (in this case KEL2 and s respectively), which may reflect the number of antigen copies (antigen density) and/or how the antigens are exposed on the carrier molecules.

Antibodies to blood group antigens follow the normal immunization pattern, demonstrating a primary IgM response that undergoes class-switching to IgG [17]. Alloantibodies are the result of the host being exposed to a non-self antigen by transfusion, transplantation or pregnancy. Naturally-occurring antibodies e.g. anti-A and anti-B in the ABO system, are mostly IgM and thought to be produced without this exposure. The current explanation is that they arise from immunization by microbial homologues to blood group antigens [18,19]. In some cases, virtually all people have them (e.g. anti-I) but otherwise they are often associated with pathological states (e.g. autoimmune haemolytic anemia of the warm or cold type) [20]. In general, the Rh proteins are the most common targets for human autoantibodies.

Blood group antibodies are involved in two types of haemolytic transfusion reactions (HTR) which may be immediate or delayed but both lead to the destruction of transfused RBCs. In the most severe form, mainly associated with the ABO, H, P/GLOB and JK blood group systems, intravascular hemolysis may occur if incompatible blood is transfused and the RBCs are thought to be lysed by complement-activating antibodies. Extravascular clearance by antibody-coated RBCs mainly takes place in the spleen via Fc-receptors and may be severe but often with a slower turnover and a milder clinical manifestations involving fatigue and jaundice [17].

To prevent transfusion reactions when a clinically significant antibody has been detected, or to limit antibody production, specifically in a multi-transfused patient, antigen phenotyping is performed prior to transfusion and antigen-negative blood is selected for transfusion following crossmatching, which originate from laboratory procedures described in 1908 [21].

Another severe complication, haemolytic disease of the foetus and newborn (HDFN) is caused by IgG antibodies, produced by the immunised mother to antigens that her own RBCs lack, but which are present on the foetal RBCs. The antibodies are transported to the foetus over the placental barrier. There they may cause RBC sensitization and subsequent destruction of the RBCs by liver and spleen macrophages. In some cases where the blood group molecule targeted by the maternal antibodies is expressed on early cells during erythropoiesis, anaemia and neonatal alloimmune thrombocytopenia (NAITP) or leukopenia may occur [22].

With the aim of monitoring antibody formation during pregnancies, blood samples are drawn from the expecting mother for antibody screening, and identification, if needed. The foetal blood type can be predicted from the same blood sample, by analysis of free foetal DNA in the maternal blood circulation [23]. Often a sample from the prospective father is analysed as well, to ensure accuracy of the test. Depending on antibody specificity and the antibody titre, the foetus may be supported during pregnancy by maternal plasma exchange (to reduce the titre) and with intra-uterine transfusions.

Null and weak blood group phenotypes

Null phenotypes are characterized by the total absence of all antigens within a blood group system, sometimes in combination with antibodies that react with all RBCs carrying antigens within the same blood group system. Individuals who lack a whole blood group carrier molecule may, when immunised, produce antibodies not only against the missing polymorphic blood group antigen(s), but to the whole molecule as well, i.e. against high-frequency antigens. These antibodies present a clinical challenge when compatible blood is required for transfusions and often only individuals of the same null phenotype are suitable donors for the patient's transfusion needs.

Null phenotypes arise from critical gene alterations for instance deletions of a single nucleotide that alters the reading frame or even deletion of a whole gene, insertions or conversions of nucleotides resulting in splice site-defects, nonsense or missense mutations within the coding or regulatory regions. Both alleles must be affected with either the same mutation (homozygosity) or with different mutations (compound heterozygosity) for the null phenotype to occur.

Depending on which functional group the missing molecule is assigned to, lack of function may accompany the null phenotype, although in some cases there seems to be redundancy between molecules so that one can partially take over the function of another one that is not expressed properly. The Rh system is such an example with the two proteins RhD and RhCE [24].

It can be a benefit to carry a null phenotype as some blood group molecules are used as receptors for microorganisms, as in for instance with Duffy and malaria infection [25] (Table 2). On the other hand, lacking a molecule might cause a shortened survival of RBCs as some blood group molecules are integrated parts of the RBCs membrane [26,15].

In summary, null blood group phenotypes are of interest in the context of:

1. *Alloantibodies*

The antibodies produced may be useful tools for biochemical analyses of the carrier molecule and subsequently the identification of its genetic basis. In addition these antibodies may be used to screen donors for compatible blood to a null recipient.

2. *Function*

The effect of the absent blood-group-carrying molecule may help explaining its function. In this sense, the null donors can be considered equivalent to knock-out mice, and cells and tissues can be studied to understand the effect of a certain molecule by observing function in its absence.

3. *Genetic studies*

Once the carrier molecule of the blood group antigen(s) is discovered, the genetic basis underlying the phenotype(s) of interest can be investigated and population studies undertaken to determine allele frequencies etc. The genetic basis for a certain blood group antigen or phenotype can subsequently be used for DNA-based blood group determination.

In addition, there is often a fine line between null phenotypes and weak expression of the antigen(s) in question. By studying the null phenotypes, useful tools for defining a possible weak expression might be established.

Weak antigen expression can arise from many molecular bases. The most common genetic event appears to be SNPs within the gene affecting the transmembrane or intracellular portions of the proteins, at least in multimembrane proteins [11,27]. However, it may also result from an overall depression of antigens within the same blood group system as in KEL where a weak expression occurs by absence of the co-expressed Kx protein. This is further explained in the KEL-section of this thesis. Other examples of the latter principle also applies to the dominant form of Rh_{null} where the *RHAG* gene that encodes for Rh-associated glycoprotein is altered [28]. The Rh proteins will then be suppressed to very low levels. A completely different principle applies to the In(Lu) phenotype, in which heterozygosity for mutations in the erythroid transcription factor gene *EKLF* [12] suppresses not only the Lutheran blood group antigens but also several others [16].

Table 2. Null phenotypes of the blood group systems: cause of the phenotype, relative prevalence and consequence [26,29,30,6,12]

System symbol	Gene	Phenotype	Null Mutation*	Prevalence†	Examples of consequence
ABO	<i>ABO</i>	O	SNPs	1	None identified although altered susceptibility e.g. to malaria, thrombosis, bleeding, cancer etc.
CH/RG	<i>C4A, C4B</i>	Ch/Rg-null	Part/whole deletion of <i>C4A, C4B</i>	5	Systemic lupus erythematosus
CO	<i>AQP1</i>	Co(a-b-)	SNPs, exon 1 deleted	5	Reduced ability to concentrate urine
CROM	<i>CD55</i>	Inab	SNPs	5	Possible intestinal disorders
DI	<i>SLC4A1</i>	Di(a-b-)	SNPs	5	Spherocytosis. Severe haemolytic anaemia; distal renal tubular acidosis
DO	<i>ART4</i>	Gy(a-)	SNPs, deletion of 8 nt. in exon 2	4	None identified
FY	<i>DARC</i>	Fy(a-b-)	SNP in <i>GATA-1</i>	3	Resistance to <i>Plasmodium vivax</i>
GE	<i>GYPC</i>	Yus	Deletion of exon 2	4	Resistance to some <i>Plasmodium falciparum</i>
GIL	<i>AQP3</i>	Gerbich	Deletion of exon 3	2	Elliptocytosis None identified
		Leach	Deletion of exons 3 & 4	5	
		GIL-	SNPs	5	
GLOB	<i>B3GALT1</i>	P ₁ ^k or P ₂ ^k	SNPs	5	Recurrent spontaneous abortions, Resistance to Parvovirus B19 and reduced susceptibility to HIV-1 (at least <i>in vitro</i>)
H	<i>FUT1</i>	O _h (Bombay)	SNPs	4	None identified

System symbol	Gene	Phenotype	Null Mutation*	Prevalence†	Examples of consequence
I	<i>GCNT2</i>	I-	SNPs	4	Congenital cataracts
IN	<i>CD44</i>	In(a-b-)	None identified in <i>CD44</i> *	5	Not known
JK	<i>SLC14A1</i>	JK(a-b-)	SNP	5	Reduced ability to concentrate urine
JMH	<i>SEMA7A</i>	JMH-**	Not known	4	None identified
KEL	<i>KEL</i>	K ₀	SNP	4	None identified
KN	<i>CRI</i>	Helgeson	SNPs	4	Autoimmune disease e.g. systemic lupus erythematosus
XK	<i>XK</i>	McLeod	SNP, partial/whole deletion of gene	5	McLeod syndrome (further explained in the KEL section)
LE	<i>FUT3</i>	Le(a-b-)	SNPs	1	Resistance to <i>Helicobacter pylori</i>
LU	<i>LU</i>	Lu(a-b-)	SNP in <i>LU</i>	4	None identified
			SNPs in <i>EKLF</i>	5	In(Lu) phenotype (depression of LU; IN, KN and PI antigens); dyserythropoietic anemia
LW	<i>ICAM4</i>	LW(a-b-)	Unidentified X-linked suppressor gene del. 10 nt. in exon 1	4	None identified
MNS	<i>GYPA</i> , <i>GYPB</i>	M ^k M ^k	Deletion of both <i>GYPA</i> and <i>GYPB</i>	5	Resistance to some <i>Plasmodium falciparum</i>
		En(a-)	SNPs; deletion of <i>GYPA</i>	5	Resistance to some <i>Plasmodium falciparum</i>

System symbol	Gene	Phenotype	Null Mutation*	Prevalence†	Examples of consequence
		S-s-U-	Deletion of <i>GYPB</i>	2	Resistance to some <i>Plasmodium falciparum</i>
OK	<i>BSG</i>	Ok(a-)**	Not known	4	None identified
P	<i>A4GALT</i>	P2	SNPs	1	None identified although altered susceptibility to P-fimbriated <i>E.coli</i>
RAPH	<i>CDI5I</i>	MER2-	SNPs	5	Basement membrane disorders
RH	<i>RHD</i> , <i>RHCE</i>	Rh _{null}	Amorph Rh _{null} <i>RHD</i> deleted and SNPs in <i>RHCE</i>	5	Compensated haemolytic anaemia
RHAG			SNPs in <i>RHAG</i> ‡		
SC	<i>ERMAP</i>	SC:-1,-2,-3	SNPs	5	None identified
YT	<i>ACHE</i>	Yt(a-b-)	Not known	5	None identified
XG	<i>XG</i> , <i>MIC2</i>	Xg(a-)	None identified	1	None identified

* SNP indicates one SNP and SNPs that more than one SNP causing the null phenotype is identified.

† 1. Frequent albeit with a varying prevalence in different populations.

2. Frequent in populations within areas with *Plasmodium falciparum*.

3. Frequent in populations within areas with *Plasmodium vivax*.

4. Rare but with a higher prevalence in certain populations.

5. Very rare

• The In(a-b-) phenotype has been reported as a consequence of a mutation in EKLf.

** The antigen-negative phenotype is known but the null phenotype (of the same name) has not been described.

‡ RhAG is essential for expression of Rh-antigens, but does not carry blood group antigens.

Nomenclature

Historically, blood groups, antigens and their corresponding antibodies were named after the discoverer or the individual who had made the antibodies. This gave a variety of names without uniform logical rules and which sometimes can be both misleading and difficult to remember. With the aim of clarifying this, ISBT introduced the Working Party on Terminology for Red Cell Surface Antigens (www.blood.co.uk/ibgr1) in 1980 (later this group became an ISBT Committee and recently received its current name, the ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology). Antigens are now categorized into blood group systems, collections or series of high- and low-frequency according to the criteria described above [2].

To support a computerized description as well as genetic classification, a numerical terminology was also developed. All blood group antigens are now assigned a six-digit identification number, with the first three digits describing the system and the following three the antigen. In addition, each system has a two- to four-letter symbol (NN, NNN, NNNN) as shown in Table 2. To describe a phenotype, the blood group symbol is used followed by a colon, with the present antigens listed, as well as missing antigens described with a minus prior to the antigen (e.g. NN:1,-2). Blood group genes are written as *NN*, alleles as *NN*01* and *NN*02* while genotypes as *NN*01/02*. Information about blood group systems is today easily accessed by using the Blood Group Antigen Gene Mutation (dbRBC at www.ncbi.nlm.nih.gov) [6].

In the papers included in this thesis, the aim was to follow the current terminology in general but also to use the terminology with the addition regarding blood group alleles that was recently proposed by a subcommittee of the ISBT Working Party mentioned above. This addition can now be accessed in draft format (www.blood.co.uk/ibgr1) for most blood group systems but it should be noted that this has inevitably resulted in a variation between papers in this thesis as the terminology has been refined.

Blood group SNPs for genotyping

Knowing the blood group genes and the allelic variants provides means for predicting phenotype from genotype and SNPs can thus be used for different allelic discrimination assays. Examples of these are the restriction-fragment-length polymorphism (RFLP) where the SNP generates/abolishes a restriction enzyme site that gives a base for analysis, allele-specific primer PCR (PCR-ASP) where primers are designed to match the SNP, or fluorophore probes and the 5' nuclease assay (as in the TaqMan assay[®]) where the probe is designed to match the SNP. In the last decade several medium- or high-throughput systems for genotyping have been developed which in one way or another are likely to make their way into the traditional blood bank setting [31-34]. One such microarray-based product has already been CE-labelled and is thereby possible to use for blood group diagnostic work in Europe [35].

When using SNP assays for phenotype prediction, all tests will give a false result if a null or otherwise altered gene is present and the alterations are not considered in the test. This is especially applicable for foetal blood group prediction, finding matching blood donors to transfusion-dependent patients or to type correctly multi-transfused patients where serology is inadequate, e.g. due to positive direct antiglobulin test or mixed-field agglutination. As RBCs are used as typing reagents for identification of antibodies national recommendations from the Swedish Society of Transfusion Medicine are given, which state that reagent RBCs used for the screening/detection of antibodies should have a double dose of the appropriate antigen, i. e. the donors should be homozygous for the allele of interest, with the aim to detect also antibodies of low titre. In some blood group systems, homo- versus heterozygosity may readily be deduced by results from routine serology by a dosage effect, but in others genotyping by SNP testing or sequencing is the only way to obtain the correct answer [36].

The Kell blood group system, KEL, ISBT 006

The first description of this blood group system was in 1946 when Mrs Kelleher gave birth to a child affected by antibodies in her plasma [37]. The antibodies which reacted with RBCs from her husband and their older children was named anti-Kell (later anti-K, anti-KEL1). The antithetical anti-Cellano (anti-k, anti-KEL2) was described the years later and named after the proband [38]. Anti-Ku, produced by an individual lacking all KEL antigens tested for was described in 1957 [39].

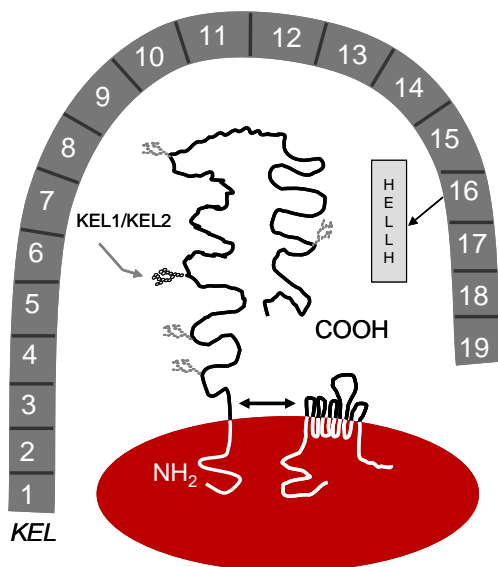


Figure 5. Schematic representation of the Kell glycoprotein, the exons of the KEL gene and the adjacent Kx protein. The KEL1/KEL2 polymorphism is encoded by exon 6 and HELLH refers to the characteristic zinc-binding catalytic site encoded by exon 16 [40].

The *KEL* gene and its alleles

The *KEL* gene comprises 19 exons distributed over ~21.5 kbp on chromosome 7q33 (Figure 5) [40]. All Kell blood group antigens arise from SNPs in the coding region [6]. The two major alleles *KEL**01 and *KEL**02 which encode all high-incidence antigens differ at nt. 578 (previously designated nt. 698) in exon 6 where *KEL**01 carries 578T and *KEL**02 carries 578C [41]. The start codon, ATG, is located 120 bp downstream of the first nucleotide in exon 1 which include SP1 and GATA-1 sequences [40]. In the early reports the first nucleotide of exon 1 was

numbered as nt. 1 but according to current practice, nt. 1 indicates the first nucleotide of the translation-initiating start codon [42].

*KEL*02* is the reference allele and encodes KEL2 and all the other KEL antigens of high prevalence (<http://ibgrl.blood.co.uk/>).

A null phenotype is caused by either homozygosity for a silenced *KEL* gene, or by two alleles carrying different silencing mutations. Silencing SNPs causing missense, nonsense mutations and exon skipping due to splice-site alterations are described, but so far no partial or whole deletion of the gene is reported. Silenced alleles carrying mutations on a *KEL*01* or *KEL*02* backbone are by ISBT referred to as *KEL*01N* or *KEL*02N* and alleles responsible for weak or modified antigen expression described as *KEL*01M* and *KEL*02M* (<http://ibgrl.blood.co.uk/>). A total of 22 *KEL*02N* but so far no *KEL*01N* has been reported and nine *KEL*02M* and two *KEL*01M* are described as well [6].

Weak antigens may be the result of mutations within *KEL* or absence of Kx (discussed below) or by the influence of the KEL3 antigen encoded by an allele *in cis* [43].

The *KEL* gene is conserved in chimpanzee, dog, cow, mouse, rat, and chicken (www.ensembl.org, www.ncbi.nlm.nih.gov).

Kell glycoprotein

The Kell antigens are carried on Kell (CD238), a 93kDa transmembrane type II glycoprotein of 732 amino acids (Figure 5) [44]. Kell gp has six potential sites for N-linked glycans and 15 cysteine residues responsible for folding and conformation of the molecule [44]. The transmembrane region is encoded by exon 3 and the *KEL1/KEL2* polymorphism arises from Met193Thr encoded by exon 6. The presence of Met193 abolishes one of glycosylation sites on Kell gp and this is thought to have an impact on the secondary structure and and/or the degree of exposure of the underlying peptide backbone. Thus, this has been hypothesized to explain why the *KEL1* antigen is more immunogenetic than *KEL2*.

By function Kell gp is included in the M13 endopeptidase family as an endothelin-3-converting enzyme, which cleaves "big" endothelin-3 to produce an active form that is a potent vasoconstrictor [45]. A homology model of the Kell gp was obtained by using the crystal structure of neutral endopeptidase 24.11 as a template, featuring a type II protein with an N-terminal intracellular domain and the C-terminus in one of two extracellular domains [46]. The second extracellular domain includes the HELLH motif which is the zinc-binding catalytic site encoded by exon 16. Kell gp is attached to Kx, described below, and to the erythrocyte cytoskeleton through an association with protein 4.1 and glycophorin C [47].

Kell gp is expressed in haematopoietic and lymphoid tissues and testis [48,49] but mRNA transcripts are found in a wider range of tissues [50,51]. The antigen density is deduced to range from 3,500-18,000 copies per RBC [52]. By cell culture and flow cytometric analysis Kell gp was shown to be the earliest blood group molecule expressed during erythropoiesis [3-5].

Kell antigens and antibodies

A total of 31 antigens are assigned to KEL with a prevalence that varies between ethnic groups. In all populations KEL2 (k, ISBT 006002) is of high prevalence together with the other antigens encoded by the same allele [16,30]. The KEL1 (K, ISBT 006001) antigen has a lower prevalence and is found, for instance in 9% of Caucasians and 2% in Blacks.

Five sets of antithetical antigens have been described;

- KEL1/KEL2
- KEL3/KEL4/KEL21 of which KEL3 (Kp^a) and KEL21 are of low prevalence in all populations
- KEL6/KEL7 where KEL6 (Js^a) is very rare in Caucasians and more common in Blacks
- KEL11/KEL17 where KEL17 is of low prevalence in all populations
- KEL14/KEL24 where KEL 14 is of low prevalence in all populations.

All other KEL antigens are of high prevalence.

The generation of antibodies toward KEL antigens is generally triggered by transfusion or pregnancy but rare cases of IgM anti-KEL1 has been described in KEL1-negative male individuals with no prior blood transfusion [17]. The mechanism is either unexplained or considered to be a response to microbial infection as for other naturally-occurring antibodies. Autoanti-KEL1 has been detected in a few samples of KEL1-positive individuals.

KEL antibodies are all clinically important and may be involved in both transfusion reactions and foetal complications during pregnancies. The antibodies mostly consist of IgG, do not activate complement and are eliminated through extravascular clearance. During pregnancy these antibodies may cause suppression of fetal erythro- and myelopoiesis followed by anaemia and thrombocytopenia and neutropenia [53,54,22] To avoid KEL1-immunization KEL1-negative blood is transfused to females below gestational age according to recommendations in many countries.

The prevalence of K₀ is unknown and no disadvantage regarding functional aspects has been reported [16,55]. K₀ individuals may produce anti-KEL5, also known as anti-Ku, antibodies reacting with all KEL-positive RBCs and must be transfused with K₀ RBCs to avoid HTR.

The interaction between Kell glycoprotein and Kx protein

Kell gp is co-expressed with Kx, a non-glycosylated protein belonging to the Kx blood group system (ISBT symbol XK, ISBT number 019) and carries the Kx antigen [44]. The Kx protein is encoded by the three exons of the *XK* gene on the X chromosome and is predicted to have ten membrane spanning domains [56]. Cys72 in Kx is linked by a disulphide bond to Cys 347 in the Kell gp. Although the function of Kx is unknown the effect of the null phenotype is apparent in that Kx null males may develop the McLeod syndrome, which is characterized by acanthocytosis and reduced RBC survival as well as muscular and neurological defects [57]. Mutations in the *XK* gene include SNPs in the coding region, promoter region or at splice site junctions as well as deletions ranging from one nucleotide to include the whole gene or even adjacent genes [6]. By the lack of Kx less Kell gp is expressed as shown by the weakening of KEL antigens, conversely RBCs lacking Kell show an enhanced expression of Kx antigen [58]. Two homologues (*XPLAC* and *XTES*) to *XK* are described and included in the XK-family [59]. McLeod individuals may produce anti-Km and anti-Kx following transfusion with random RBC units.

Table 3. Comparison between the normal KEL-, K₀- and McLeod phenotypes

Phenotypes→ Features↓	Common KEL	K ₀	McLeod
Pathology	None	None	McLeod syndrome*
Creatine kinase	Normal	Normal	Elevated
RBC morphology	Discocytes	Discocytes	Acantocytes
Gene defect	None	SNPs in <i>KEL</i>	SNPs and partial or whole deletion of <i>XK</i>
KEL antigens	Present	Not present	Weak
Kx antigen	Present	Elevated	Not present
Km (KEL20) antigen	Present	Not present	Not present
Antibodies	Lacking antigens	Anti-Ku	Anti-Km (anti-Kx in rare cases)
RBCs for transfusion	Antigen negative for the corresponding antibody	K ₀	McLeod or K ₀

* The McLeod phenotype is further categorised as McLeod non-CGD and McLeod CGD. This reflects the deletion of the CDG gene causing chronic granulomatous disease, adjacent to XK.

The Kidd blood group system, JK, ISBT 009

The three antigens and corresponding antibodies in the Kidd (JK) blood group system were all described during the 1950s. The system was named after Mrs. Kidd who in 1951 during pregnancy produced anti-Jk^a, which caused HDFN in her child [60]. Two years later, in 1953, the second antibody, anti-Jk^b was described, [61] and in 1959 was anti-Jk³ detected in an individual from Hawaii with the null phenotype Jk(a-b-) [62].

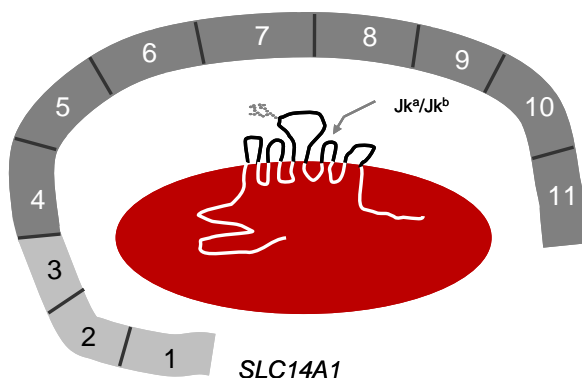


Figure 6. Schematic representation of UT-B, Kidd glycoprotein and exons of the *JK* gene with the first three untranslated exons in light gray. The Jk^a/Jk^b polymorphism is encoded by exon 9. Kidd is glycosylated at the 4th extra cellular loop.

The JK gene and its alleles

The chromosomal location of the *JK* or *UT-B* gene, also known as *SLC14A1* (Solute carrier family 14, member 1), is at 18q12 and it consists of 11 exons distributed over 30 kb (Figure 6). The translation start codon is in exon 4 and the 5'-end contains TATA, inverted CAAT boxes and GATA1/SP1 erythroid-specific *cis*-acting regulatory elements [63]. *SLC14A1* is carried on the (+) strand and lies in tandem to *SLC14A2* encoding the kidney homologue *UT-A*.

The two major alleles, *JK*01* and *JK*02*, differ in the coding sequence at nt. 588 and 838 where *JK*01* carries 588A and 838G in exons 7 and 9 whilst *JK*02* carries 588G and 838A [64]. 588A>G is a silent SNP while 838G>A is a missense SNP responsible for the amino acid shift Asp280Asn, differentiating Jk^a from Jk^b. *JK*01* and *JK*02* are equally distributed in the Caucasian population. *JK*01* is the reference allele and encodes Jk^a and JK3 antigens (<http://ibgrl.blood.co.uk/>).

The Jk(a-b-) phenotype results primarily from a recessive trait meaning either homozygosity or compound heterozygosity for a silent *JK* gene. Whole deletion of the gene has not been reported but partial deletion (exons 4-5) [65,66] as well as silencing SNPs of the missense [67,68,69], nonsense [66,68] and exon skipping [63,67] types have been described as responsible for Jk(a-b-). Silenced alleles carrying mutations on a *JK*01* or *JK*02* sequence backbone are described as *JK*01N* or *JK*02N* by the ISBT and in total, five *JK*01N* and eight *JK*02N* alleles have been described. Another extremely rare cause of apparent Jk(a-b-) status, described only in few individuals in the Japanese population is referred to as *In(JK)* where a dominant but hitherto unknown inhibitor gene is proposed to account for this phenotype [70].

The *SLC14A1* gene is conserved in chimpanzee, dog, cow, mouse, and rat (www.ensembl.org, www.ncbi.nlm.nih.gov).

UT-B the Kidd glycoprotein

The Kidd glycoprotein is not only expressed on RBCs but also in kidney endothelial cells of the vasa recta in the inner and outer medulla, together with its homologue, the kidney urea transporter UT-A [71]. In humans UT-B is also expressed in colon [72], brain, testes and spleen [73]. UT-B is predicted to consist of two hydrophobic domains that span the membrane five times each, and are linked by a large glycosylated extracellular loop (Figure 6) [74]. The Asn211 on this third loop carries an N-glycan with ABO antigens as terminal epitopes and the Jk^a/Jk^b polymorphism is described to be situated on the neighbouring 4th extracellular loop.

Urea is produced as the end product of protein catabolism and functions both as a carrier of nitrogen excretion and an osmolyte, which by high concentration in the kidneys provide the osmotic gradient essential for water re-absorption [75]. As the RBC urea transporter, UT-B is thought to play a role in preserving this osmotic stability by taking up urea when RBCs flow through the descending vasa recta and releasing urea when passing the ascending vasa recta [76,77].

Members of the UT family have been found in many species ranging from bacteria to mammals [78,79]. All UT sequences have two homologous halves that probably arose from the duplication of an ancient gene, and are predicted to contain ten transmembrane helices. Recently the first crystal structure of a urea transporter was reported [80] visualising the UT of the bacterium *Desulfovibrio vulgaris* (dvUT). The structure of dvUT is described as a homotrimer with a channel-like mechanism.

Persons who lack UT-B have the Jk(a-b-) phenotype and thus suffer from a mild urea concentration disorder [81]. Jk(a-b-) RBCs can be screened for and identified by exposure of the RBCs to 2 M Urea [82]. While Jk(a-b-) RBCs resist urea for at least 15 minutes, normal RBCs will haemolyse immediately [83].

Kidd antigens and antibodies

Three antigens, Jk^a, Jk^b and JK3 combined into four different phenotypes; Jk(a+b-), Jk(a+b+), Jk(a-b+) and Jk(a-b-) are described within the Kidd blood group system. Jk^a is represented by Asp 280 and Jk^b by Asn280. The distribution of Jk^a and Jk^b antigens is relatively balanced across different populations [16,55]. The Jk(a-b-) phenotype is rare in many populations except in Polynesians (2,7%) [84] and Finns 0,3% [85]. Since this phenotype has the highest prevalence in Oriental populations, screening has been undertaken and resulting in descriptions not only of Polynesians in total but in different Polynesian ethnic groups as well [84]. By using the simple and cost-effective urea lysis test, mass screening in the Japanese [70] and Thai [86] was performed which showed that the Jk(a-b-) phenotype is indeed rare in these populations.

Complement-activating antibodies of IgM and IgG type are produced following immunization to JK system antigens. They constitute an obstacle in that they may be difficult to detect in a routine blood banking analysis because of a fast-falling titre. On the other hand, these low titres are rapidly boosted if incompatible blood is transfused.

JK-antibodies are a significant cause of acute and delayed HTR, and also cause HDFN as well albeit the severity of the disease varies from mild to lethal.

JK-antibodies following transfusion are described in association with acute kidney transplant rejection [87].

Summary and discussion of the present investigation

The focus of this thesis is on KEL and JK, two clinically important blood group systems, and the investigations of index blood samples with aberrant antigen expression as in null and weak phenotypes, or where phenotype does not correspond to genotype.

The index samples investigated included individuals of many different ethnic/geographic origins, referred by national or international laboratories to the Nordic Reference Laboratory for Genetic Blood Group Typing in Lund (see details regarding sample origins in paper I-V). If inconclusive results were obtained, further investigation by non-routine analyses were performed at research laboratories within the Division of Hematology and Transfusion Medicine, Department of Laboratory Medicine, Lund University. Sample materials varied between whole blood no more than a week old, to frozen and thawed RBCs or ready-prepared DNA. This means that all index samples could not be included in all investigations (Table 4).

Normal controls were acquired at the local Blood Centre and included healthy blood donors. All blood samples were drawn after informed consent.

Table 4. Summary of areas explored and methods used in paper I-V.

Focus	Sample source	Method	Presented in	
			KEL paper	JK paper
Antigen	RBC	Haemagglutination	I, IV	V
	RBC	Flow cytometry	IV	V
Protein	RBC	Flow cytometry	IV	V
	RBC	Western blot		II, V
mRNA	Reticulocytes	Real-time PCR		V
	WBC			
Alleles	WBC, DNA	Direct sequencing	I, IV	II, V
SNPs	WBC, DNA	PCR-ASP	I, IV	II, III, V
		Database search		V
Function	RBC	Urea haemolysis test		II, V

KEL

Paper I	Genetic basis of the K ₀ phenotype in the Swedish population
Paper IV	<i>KEL*02</i> alleles with alterations in and around exon 8 in individuals with apparent KEL:1,-2 phenotypes
Additional and continuing investigations – included here.	

In **paper I** three K₀ samples of Swedish origin; Uppsala, Umeå and Linköping, including family members to the Uppsala sample, were investigated. These rare samples were found over a 20-year period by standard serology either by antigen typing or antibody identification followed by antigen typing. By PCR amplification followed by direct DNA sequencing, the whole *KEL* coding region, 19 exons including part of the surrounding introns were analysed in 13 segments. DNA from the family members was only sequenced in selected regions of interest. As explained in the introduction of KEL in this thesis; the first nucleotide of exon 1 was numbered as nt. 1 in early reports [40] of KEL but according to current practice, nucleotide 1 indicates the first nucleotide of the translation-initiating start codon [42]. To clarify this, nt. positions of mutations reported in paper I, are here written with the old position in brackets.

Homozygosity for two novel nonsense mutations was identified. The Umeå sample carried a 903delG (previous 1023delG), a frameshift of -1 nt. causing a premature stop codon in exon 9, twenty-eight codons downstream from the deletion.

Two samples from Uppsala, the index sample and his sister, carried 1420C>T (previous 1540C<T) in exon 13 changing Gln474Stop. Both parents were heterozygous for the same mutation. The Linköping sample carried intron 3 +1 g>a, the previously reported splice site mutation in the conserved gt sequence at the donor splice site, resulting in skipping of exon 3 which encodes the transmembrane region [88]. All alleles had the 578C SNP (previously 698) normally responsible for expression of KEL2 antigen. Therefore, these alleles will now be described here as *KEL*02N* followed by an allele number according to the recently proposed ISBT nomenclature for blood group alleles (Table 5).

RBCs from the three index samples lacked all KEL antigens tested for, by standard serology including adsorption and elution. Anti-KEL5 or -Ku was identified in plasmas from the Umeå and Linköping samples as a consequence of previous transfusions in both cases. No KEL antibodies were identified in plasma from the Uppsala index sample, which may be expected since he was a blood donor without prior transfusions. In the case of his sister, however, the lack of anti-KEL5 in her plasma was more of a surprise due to her multiparous history with five children.

In **paper IV** another three index samples, this time originating from Denmark and the Netherlands were investigated. They represent three clinically important sample types: blood donors (sample 1), test reagent donors (sample 2) and prospective fathers involved in allo-immunization during pregnancy (sample 3). They all had an apparent KEL:1,-2 phenotype that did not correspond to the initial SNP-derived *KEL*01/KEL*02* genotype, which together with phenotyping was performed at the referring laboratories. The three samples were further investigated by direct DNA sequencing performed as described in paper I. Again, DNA from family members (of sample 2) was included to follow the inheritance of alleles, and was only sequenced in regions of interest.

By direct sequencing, heterozygosity for single nucleotide substitutions in and around 8 was revealed. In sample 1, a novel missense mutation, 787G>A predicting Gly263Arg, was identified as the likely cause for the altered phenotype (Table 6). Although KEL2 expression was not detected by routine haemagglutination methods, adsorption/elution studies with polyclonal anti-KEL2 resulted in a positive signal, thus indicating that this sample expressed KEL2 weakly.

Sample 2 carried intron 8 +1g>t and sample 3 had -1g>c in intron 7. Whilst the former of these changes has been reported also in a case of K₀ by another research group [89], the latter is not previously described. Both mutations are predicted to cause splice-site defects by disrupting the invariant donor and acceptor motifs [90]. Since these three mutations were found in and around exon 8 we speculated that this may be a hot spot area for mutations in the *KEL* gene, but as reported in dbRBC [6] this is a highly polymorphic gene with alterations in almost all exons.

Flow cytometric analysis was performed to investigate expression of KEL1 and KEL2. Control RBCs followed the expected pattern with a higher level of expression in samples with a double dose of KEL1 or KEL2, respectively, while index samples 2 and 3 had a level of KEL1 closer to RBCs with a single dose of KEL1. By flow cytometric analysis using anti-Kell gp the two index samples demonstrated lower expression than the majority of control RBCs of the common phenotypes. One KEL:1,-2 control RBC typed as *KEL*01/01* by homozygosity for the KEL1-defining SNP 578T gave a weaker expression which may implicate the presence of an altered allele.

The very weak KEL2 antigen expression in sample 1 was neither detected by flow cytometry nor by standard haemagglutination; but only found by adsorption/elution. This failure to detect expression may be attributed to the chosen antibodies, but similar cases are previously described [89].

Summary of novel KEL alleles

Table 5. Summary of novel *KEL*02N* alleles identified in this study

	Allele‡	Nucleotide change	Intron / Exon	Alteration	Sample ID
Paper I	<i>KEL*02N.10</i>	1420C>T	Exon 13	Gln474X	Uppsala
	<i>KEL*02N.11</i>	903delG	Exon 8	fs, premature stop	Umeå
Paper IV	<i>KEL*02N.13</i>	IVS8 +1g>t	Intron 8	Alternative splicing	Sample 2
	<i>KEL*02N.22</i>	IVS7 -1g>c	Intron 7	Alternative splicing	Sample 3

‡ Allele numbers taken from the table of proposed null alleles to be found on the ISBT terminology website.

Table 6. Summary of novel *KEL*02M* alleles identified in this study

	Allele	Nucleotide change	Intron /Exon	Alteration	Sample ID
Paper IV	<i>KEL*02M10</i>	787G>A	Exon 8	Gly263Arg	Sample 3

‡ Allele numbers taken from the table of proposed modified alleles to be found on the ISBT terminology website.

DNA sequences carrying novel mutations were deposited at NCBI GenBank (<http://www.ncbi.nlm.nih.gov>)

Additional investigations in the KEL blood group system

1. Another case of allo-immunisation during pregnancy was examined. Similar to sample 3 in paper IV a KEL:1,-2 prospective father and partner to a woman with anti-KEL1 was typed in the diagnostic work-up. DNA from the father was analysed by PCR and direct sequencing of *KEL* performed as described above. The sample typed *KEL*01/02* by PCR-ASP which was confirmed by sequencing but in this case no alterations were detected in exons 1-19.

Similar results have been reported [89] in other *KEL*01/02* samples where KEL2 antigen was not expressed. This may be caused by alterations outside the investigated sequence. Direct sequencing of exon 1, which is described as carrying sequences recognised by transcription factors [40] was performed, but other 5' regulatory elements may be involved in *KEL* transcription as well.

2. Expression of KEL1, KEL2 and Kell gp by flow cytometric analysis is described in **paper IV**. Flow cytometry was applied again when evaluating antigen expression of the in-house test RBC reagent cells used for antibody identification (Figure 7). This time anti-KEL1 (Diagast) and anti-KEL2 (BioRad) as primary antibodies incubated with RBCs for 20 min. at 37°C, followed by incubation with the secondary antibody, which was a blend of FITC-conjugated rabbit anti-human kappa light chain and rabbit anti-human lambda light chain, both from Dako (Electra-Box Diagnostica AB, Stockholm, Sweden). Anti-Kell (Mima 24, a gift from Greg Halverson, New York Blood Center) was used as described in the paper.

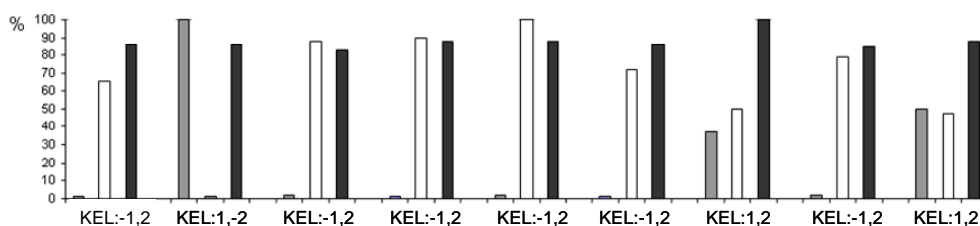


Figure 7. Flow cytometric analysis with anti-KEL1 (light grey bars), anti-KEL2 (white) and anti-Kell gp (dark grey) on nine in-house test reagent RBCs with the KEL phenotypes indicated below the diagram. Mean fluorescence intensity (MFI) values are given as the percentage of the maximum MFI detected in this cohort.

These results followed the same pattern as results in paper IV, with RBCs from KEL:1,2 samples expressing less KEL1 and KEL2 than homozygous RBCs with the phenotypes KEL:1,-2 and KEL: -1,2 samples, respectively. A similar amount of Kell gp was detected in all samples.

3. By the flow cytometric method discussed above, another rare case was studied in our research laboratory. A blood sample from a young Swedish male suspected to have the McLeod phenotype was investigated by serology and genetic analysis. RBCs were analysed for KEL1, KEL2 and Kell gp expression and while KEL:1,2 control RBCs readily gave a strongly positive reaction with anti-Kell, the McLeod RBCs were as non-reactive as the K₀ control RBCs (Figure 8).

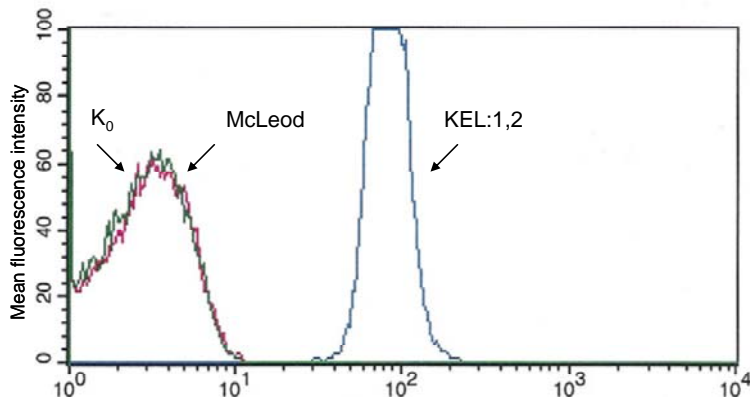


Figure 8. Flow cytometry with anti-Kell on RBCs from the McLeod sample and controls; K₀ and KEL:1,2.

The same pattern was identified with anti-KEL1 and KEL2, respectively (data not shown). By standard hemagglutination lack of KEL1, KEL3 and KEL4 was noted while typing with anti-KEL2 was weakly positive. The KEL2 expression would be expected to yield a positive reaction with anti-Kell but the difference in sensitivity between methods and also between antibody sources probably explains this apparent discrepancy. Kx typing performed with human polyclonal antibodies gave a negative result.

DNA from the McLeod sample was analysed by direct sequencing of the three exons of *XK*. For the PCR reaction 100 ng of DNA in a total volume of 10 μ L, using 0.2 mM dNTPs, 0.25 pmol of each primer (Table 7), 0.5 U TaqGold™ (Perkin Elmer/Roche Molecular systems, Branchburg, NJ, USA) in the buffer supplied, was used. The PCR was utilised as follows: 7 minutes at 96°C with by 35 cycles of: 30 seconds at 96°C, 30 seconds at 58°C, 60 seconds at 72°C; finishing with a final elongation of 7 minutes at 72°C.

Table 7. Primers used for PCR amplification and sequencing of the *XK* gene

Primer name	Nucleotide sequence (5'→3')
XK5'F	CGGGGCTGCGCAGCTCGC
XK-in1R	CGCGGTGTACGAAGAGAAGC
XK-in1F	GTGACTATAGTTCAGAGTAGCC
XK-in2R	GCCTAGAATGCAGAGTCATAAG
XK-in2F	ACAACCTGGAAGTCAGGCTGTG
XK-e3F	TTGTAGTCCTGGTCCTCTTTAC
XK-e3R	CAGGAGGAGGAGGATGGC
XK-3'R	GTTAACCTCCTTGTGAGCTGCTT

Primers designed to amplify exons 1 and 2 gave the expected products compared to normal control samples analysed in parallel. In contrast, exon 3 from the index sample did not give rise to an amplicon whilst controls did. This may indicate that this part of the gene is altered, for instance by a major insertion or deletion. Deletion of intron 2 and exon 3 has been mentioned as the possible cause of a Japanese case of McLeod phenotype previously, but without further clarifications [91]. Investigations to find the genetic background to McLeod in our index sample, i.e. a more detailed definition of a possible deletion breakpoint are yet to be completed. However, the lack of exon 3 explains the lack of Kx antigen in this case and also why KEL antigens are depressed to almost undetectable levels. It remains to be shown if the altered *XK* gene has a new genetic basis of McLeod or not.

JK

Paper II	Erythroid urea transporter deficiency due to novel <i>JK^{null}</i> alleles
Paper III	A simple screening assay for the most common <i>JK*0</i> alleles revealed compound heterozygosity in Jk(a-b-) probands from Guam
Paper V	Characterization of Jk(a+ ^{weak}): a new blood group phenotype associated with an altered <i>JK*01</i> allele
Continuing investigation – included here.	

The Jk(a-b-) cohort in **paper II** included nine samples from individuals of different ethnic groups, of which two were from siblings. Phenotyping and adsorption and elution, in most cases, were performed by the referring laboratories.

Investigation of the SNP 838G/A responsible for *JK*01/JK*02* was performed on DNA using an in-house PCR-ASP [92]. This was followed by screening for two known silencing *JK* mutations: 871T>C and intron 5 -1g>a, for which there was also an in-house PCR-ASP. Further resolution was carried out by direct sequencing of the promoter region and the 11 *JK* exons including the 5' and 3' ends of the surrounding introns. Four individuals homozygous for previously reported *JK*02N* alleles were identified; *JK*01N* with deletion of exon 4 and 5 [65,66] in a Bosnian sample, *JK*02N* with intron 5 -1g>a [63,67], in a Vietnamese sample, *JK*02N* with 582C>G [66] in a sample of Swiss origin and *JK*02N* with 871T>C [67,93] in a Swedish sample. DNA from a sister to the Vietnamese sample was amplified around exon 5 and sequenced. She was heterozygous for *JK*02N* intron 5 -1g>a.

Four novel *JK*01* and *JK*02* alleles were identified: homozygosity for *JK*01* 202C>T, a nonsense mutation, was found in American siblings of Caucasian origin. Another individual of Hispanic-American origin was compound heterozygous for a novel *JK*02N* allele that contained a nonsense mutation, 723delA together with *JK*02N* intron 5 -1g>a. The novel missense mutation 956C>T was identified in an African-American sample carried on a *JK*01*-like allele: while the allele also encoded Jk^a, it carried non-coding SNPs that are generally associated with the *JK*02* allele. Homozygosity for 956T was found as well in an individual of Indian origin but this time carried on a *JK*02* allele.

A phenotyping discrepancy between a mother and baby revealed the presence of the *JK*02N* 956T allele in another individual from the Indian subcontinent who

was heterozygous for this null allele in combination with a normal *JK*02*. The novel alleles are summarised in Table 8.

To evaluate the effect of this missense mutation 956C>T on JK glycoprotein expression, Western blotting was performed with an anti-rat UT-B1 [94] on RBCs from the African-American individual. This confirmed that no Kidd glycoprotein was present in the RBC membrane.

Table 8. Summary of novel *JK*01N* and *JK*02N* alleles identified in this study (paper II)

Allele	Nucleotide position							Alteration	Sample ID
	130	202	588	723	838	IVS9 – 46	956		
<i>JK*01N.02</i>	G	T	A	A	G	a	C	Gln68Stop	American
<i>JK*01N.04</i>	G	C	G‡	A	G	g‡	T	Thr319Met	African-American
<i>JK*02N.05</i>	G	C	G	delA	A	g	C	fs→ Ile262Stop	Hispanic-American
<i>JK*02N.08</i>	G	C	G	A	A	a	T	Thr319Met	Indian

Grey areas illustrate altered nucleotides in these alleles.

‡ Polymorphic nucleotides associated with the *JK*02* allele.

DNA sequences carrying novel mutations were deposited at the NCBI GenBank (<http://www.ncbi.nlm.nih.gov>)

The PCR-ASP described in **paper III** was refined from an in-house assay [67] to include the detection of five silent *JK* alleles: deletion of exon 4 and 5 (Δ exons 4+5) [65,66], intron 5 –1g>a [63,67], 582C>G [66], 871T>C [67,93] and 956C>T [68]. By using this simple screening assay, in combination with the standard *JK*01/JK*02* PCR-ASP [92], on samples from a family of three Jk(a–b–) individuals originating from Guam, two different nucleotide substitutions were identified. All three samples were homozygous for 838A encoding Jk^b however, the mother and one son were compound heterozygotes for intron 5 –1g/a and 956C/T, while the second son was homozygous for intron 5 –1g>a. The detected alleles were previously found in samples of Polynesian (intron 5 –1g>a) and Indian origin (956C>T) and can now be assigned to a broader geographic area. These samples demonstrated the utility of the new screening approach.

The observation of weak or varying Jk^a antigen expression the Jk(a+^w) phenotype) was the activator of investigations in **paper V**. The Jk(a+^w) cohort included six individuals given sample numbers S1 – S6 (Table 1 in paper V). Again initial serology was performed at the referring laboratories but in contrast to investigations in paper I-IV, both RBC and DNA were available for testing, with the exception of S1 and S3 where only DNA was referred. By PCR and direct sequencing as described in **paper II**, a novel allele was unravelled and named *JK*01M* due to its association with the modified Jk^a expression. The *JK*01M* allele carried the missense mutation 130G>A, which encodes an amino acid change of Glu44Lys, but was further characterised by carrying the silent SNPs 588G and intron 9 –46g, both of which are associated with the *JK*02* allele (Table 9). Thus *JK*01M* carried the same features found in the first cloned allele [95], which later was abandoned for another clone [96].

Table 9. Comparison of the novel *JK*01M* with *JK*01* and *JK*02*

Allele	Nucleotide position			
	130	588	838	IVS9 –46
<i>JK*01M</i>	A	G	G	g
<i>JK*01</i>	G	A	G	a/g‡
<i>JK*02</i>	G	G	A	g

‡ Polymorphic position where adenosine is more strongly associated with JK*01.

To investigate if 130A was a common SNP in the Swedish population, screening for 130A/G in normal controls (n=300) was performed by PCR-ASP. Assuming that all samples with 130A have the *JK*01M* allele the results revealed an allele frequency in the control group of 4.2% with a genotype frequency of 92% 130G/G, 7.7% 130G/A and 0.3% 130A/A. The same PCR-ASP screening approach was applied to a Jordanian blood donor cohort and 130A was also detected in this population. These results prompted a search in the HapMap database (www.hapmap.org) for 130A in other well-defined populations. This revealed substantial variation between different populations in the distribution of 130G/A (Table 3 in paper V), with a peak prevalence of 130A at 45.7% in a cohort of Chinese origin. Despite how common this allele appears to be, it is striking that reports linking this allele to the Jk(a+^w) phenotype have not surfaced until now.

The weak or varying Jk^a antigen expression was further studied. By Western blotting, using the same anti-rat UT-B previously utilised in paper II, less Kidd gp was detected on RBCs from these samples than on control RBCs with normal JK phenotypes. The same pattern was shown by titration studies with anti-Jk^a and

anti-Jk3. The standard urea hemolysis test designed to screen for Jk(a-b-) samples was unable to identify Jk(a+^wb-) samples although two of the index samples indicated a decreased urea-to-water ratio at 2 minutes incubation. Real-time PCR analysis showed that the mean C_t values of *JK*-mRNA (cDNA) from *JK*02/02* samples demonstrated higher transcript levels than those of *JK*01/01* samples, although this did not reach significance.

The most interesting finding was by flow cytometry, where control RBCs from the 130A screening assay were included. This part of the study clearly showed that individuals hetero- or homozygous for the *JK*01M* allele expressed less Jk^a than corresponding individuals with the *JK*01* allele.

Continuing investigation within the JK blood group system

1. Development of an *in vitro* expression system for UT-B.

The need for an *in vitro* expression system was recognized as the next step forward in this interesting project to prove that *JK*01M* is responsible for the reduced expression of Jk^a antigen at the cell surface. With the help of a Masters of Science student, attempts to express *JK*01*, *JK*02*, and *JK*01M* cDNA in a cell culture system were performed. *JK*02* cDNA was successfully amplified, cloned and transfected into HeLa cells however, detection proved to be a limitation: since even mock-transfected HeLa reacted with the anti-Jk^a and anti-Jk^b.

*JK*01* and *JK*01M* cDNA could not be amplified despite several attempts. This might be explained by the lower amount of mRNA transcripts detected in *JK*01* individuals but has to be further investigated. However, mutagenesis of *JK*02* cDNA was also attempted with mixed success. This was due to the requirement of the experimental design to alter even the silent changes identified in the *JK*01M* allele.

2. Investigation of a family carrying the 130A mutation

Further to our studies described in paper V, we investigated samples from a Hispanic-American family of parents and six children. The index sample in this family was a daughter (C in Figure 9) with a positive direct antiglobulin test. Her RBCs had typed Jk(a+b-) at the referring center however the results of JK phenotype analysis on samples from family members were discrepant and the samples were referred to our laboratory. With the aim to ascertain antigen status of the patient and to rule out a suspected auto-anti-JK3, family studies were performed, which included serological analysis direct sequencing as described in paper I and flow cytometric analysis (Figure 10) with anti-Jk^a and anti-Jk^b as described in paper V. In this family three *JK* alleles were identified; *JK*01*, *JK*02* and *JK*01M*. However the presence of these three alleles could not explain the phenotype pattern observed, i.e. in some individuals carrying the *JK*01M* allele,

no antigen was detected whereas in others, Jk^a was weakly detected. No other changes were detected in the sequence analysis but we postulate that perhaps a completely silent variant of *JK*01M* exists which need to be further explored.

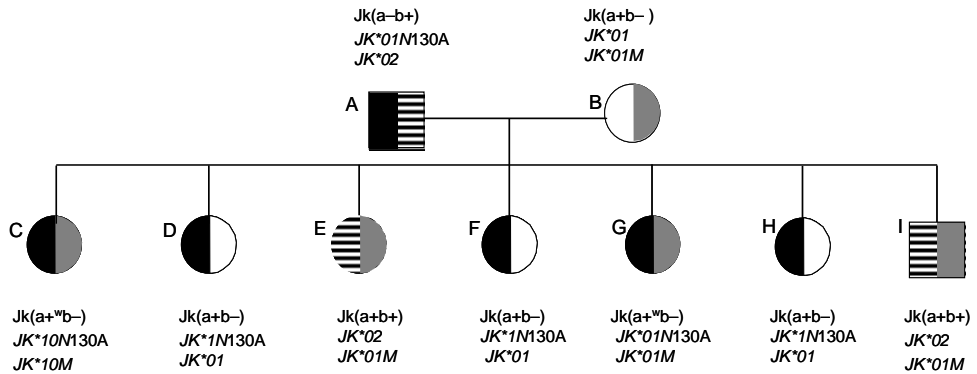


Figure 9. Pedigree of a family carrying *JK*01M* in combination with a possible silent variant of the same allele. The JK alleles are described as follows:

■ a possible *JK*01N* with 130A, ▨ normal *JK*02*, □ normal *JK*01* and ▩ the novel *JK*01M*.

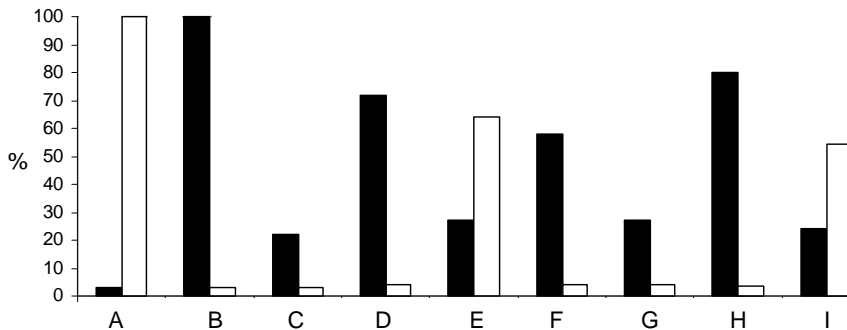


Figure 10. Flow cytometric analysis with anti-Jka (black bars), anti-Jkb (white bars) on RBCs from the investigated family. Phenotypes of the family members are described in Figure 9.

Mean fluorescence intensity (MFI) values are given as the percentage of the maximum MFI detected in this cohort.

Hypothetical membrane topology of UT-B

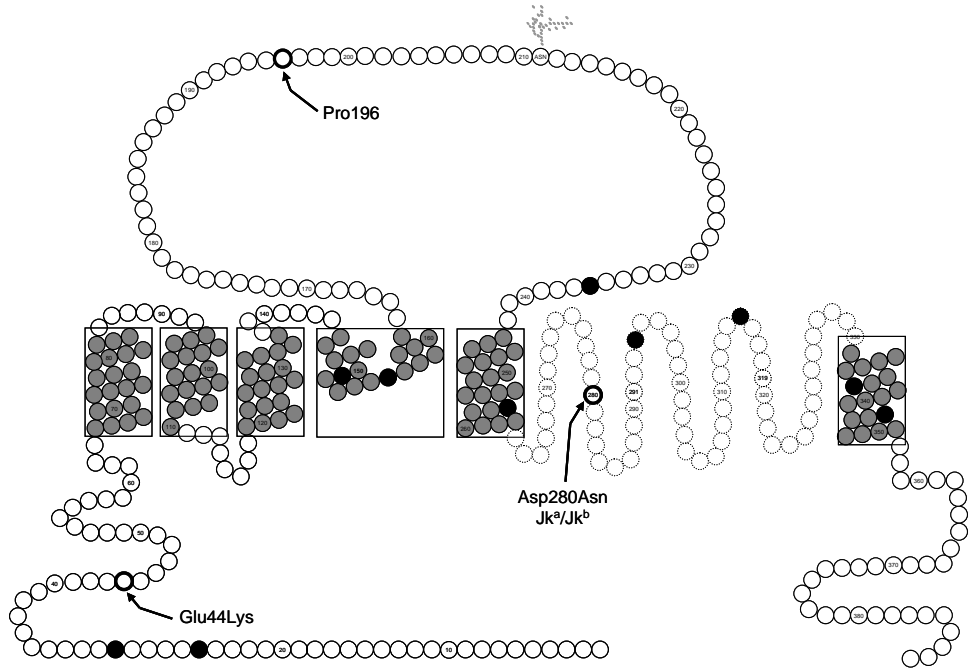


Figure 11. Hypothetical membrane topology of UT-B. This schematic representation is based on hydropathy analysis and comparison with representations of its homolog, UT-A. Amino acids are indicated by circles and every tenth residue is numbered. Transmembrane domains are shown as boxes and amino acids within them shaded in gray. Cysteine residues are represented by filled circles. Three important amino acids are indicated by thick circles and arrows: The Jka/Jkb-defining polymorphism Asp280Asn, the Glu44Lys resulting from the JK*01M allele associated with the Jk(a^w) phenotype, and finally Pro196 encoded by the 588G/A-containing triplet at nucleotide 194-196. Different hydropathy analysis software tools give conflicting resolutions concerning the degree of membrane insertion between the 5th transmembrane domain and the most C-terminally located one, and the implicated amino acids are therefore indicated with dotted lines. The N-glycan on Asn211 is indicated.

Conclusion

In this thesis, samples with apparent null or weak phenotypes within the KEL and JK blood group systems have been investigated with various methods to elucidate their serological and molecular genetic characteristics.

A total of ten novel alleles were identified. Of these, eight silence expression of the blood group glycoprotein whilst two are associated with reduced antigen strength. For each system, four null alleles and one weakening allele were found. The recognition and characterisation of a new phenotype in the JK system, Jk(a+weak), and its associated allele is perhaps the most interesting and surprising finding presented here.

The SNPs generating novel alleles described here follow the expected pattern, *i.e.* transitions are most common and found in six of ten variants. The remaining four alleles contained two transversions and two deletions.

In a few cases, samples with suspected rare phenotypes were investigated but no genetic alterations were identified, which may suggest that other mechanisms than silencing SNPs in the investigated regions are responsible for the observed findings.

This thesis contributes to and expands the overall understanding of allelic diversity within the KEL and JK blood group systems. It highlights the importance of characterising alleles in which alterations in the sequence may be distant from polymorphisms encoding blood group antigens.

The new gene variants provide more data for inclusion of allelic markers in DNA-based blood group testing tools to achieve safer phenotype prediction and eventually increased phenotype matching for patients in need of transfusions.

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I started with All you need is love and finish with A little help from my friends.

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Appendix: Papers I-V

Paper I

Genetic basis of the K₀ phenotype in the Swedish population

Elisabet S. Wester, Jill R. Storry, Karin Schneider, Birgitta Nilsson Sojka, Joyce Poole, and Martin L. Olsson

BACKGROUND: The absence of all Kell blood group antigens (K₀ phenotype) is very rare. K₀ persons, however, can produce clinically significant anti-Ku (K5) after transfusion and/or pregnancy and require K₀ blood for transfusion. Ten alleles giving rise to the K₀ phenotype have been reported: different populations were studied although none from Scandinavia.

STUDY DESIGN AND METHODS: Three K₀ samples were identified by blood banks in Sweden (Uppsala, Umeå, and Linköping) during a 20-year period. Kell antigen typing was performed with standard serologic techniques by the respective blood banks and K₀ status was confirmed by the International Blood Group Reference Laboratory in Bristol, England. Polymerase chain reaction and DNA sequencing of the *KEL* coding region (exons 1-19) was performed on genomic DNA.

RESULTS: The Uppsala K₀ was homozygous for a 1540C>T substitution in exon 13, leading to an immediate stop codon. The Umeå K₀ was homozygous for 1023delG in exon 8 that results in a frameshift and a premature stop codon in exon 9. In the Linköping K₀, a previously reported mutation g>a at +1 of intron 3 was found.

CONCLUSION: Two novel and one previously reported null alleles at the *KEL* locus are described. The identified nonsense mutations abolish expression of the Kell glycoprotein and are thus responsible for the K₀ phenotype in these Swedish families.

The *KEL* gene, cloned in 1991, is located on the long arm of chromosome 7 (7q33) with its 19 exons spanning 21.5 kb.^{1,2} It codes for the Kell blood group molecule (CD238), a Type II glycoprotein, with a single membrane-spanning region encoded by exon 3. The Kell glycoprotein of 93 kDa comprises 732 amino acids with an intracellular N-terminus.³ It has at least five N-glycosylation sites, although one site is lost in the presence of Met193, the K1 polymorphism.

A single disulfide bond links the Kell glycoprotein to XK, a multipass membrane protein that carries the Kx antigen.⁴ Some recent preliminary findings suggest that Kell is also attached to the red blood cell (RBC) cytoskeleton through an association with protein 4.1 and glycophorin C.⁵ Although it was originally thought to be erythroid-specific, Kell is present on RBCs and testes and in lesser amounts on brain tissue, lymphoid organs, heart, and skeletal muscles.^{6,7} Kell is a member of the neprilysin (M13) subfamily of zinc endopeptidases, and in vitro, Kell has been shown to cleave big endothelin-3 specifically, although its function on RBCs is unknown.⁸

ABBREVIATION: NMD = nonsense-mediated mRNA decay.

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The *KEL* alleles are inherited in a codominant fashion: the common allele, referred to here as *KEL**2, encodes all high-incidence antigens of the Kell blood group system.⁹ Apart from the K1 (K) and K2 (k) antigens, the Kell blood group system contains 22 other identified antigens. With the exception of Ku (K5) and Km (K20), these are all due to single-nucleotide changes and are divided into high- and low-incidence antigens.³ Antibodies against Kell antigens are clinically significant because they may cause hemolytic transfusion reactions and fetomaternal incompatibility with severe hemolytic disease of the newborn.¹⁰

The rare K_0 phenotype is defined by total absence of Kell antigens on RBCs and occurs by homozygosity or compound heterozygosity for silent alleles at the *KEL* locus. The absence of Kell glycoprotein has no known disease association: K_0 RBCs have normal discoid shape but a decreased amount of XK protein is expressed even though Kx antigen expression appears elevated. In contrast, the absence of the XK protein leads to the McLeod phenotype, which is associated with RBC acanthocytosis and late-onset neurologic defects and with the depressed expression of Kell protein.³

So far, three reports have been published, in which a total of 10 alleles with different point mutations giving rise to the K_0 phenotype have been described, in several populations.¹¹⁻¹³ We have investigated three K_0 samples of Swedish origin, referred to the Nordic Reference Laboratory for Blood Group Genomic Typing in Lund, to determine the molecular basis of the null phenotype. To our knowledge, these are the only K_0 samples found in Sweden to date.

MATERIALS AND METHODS

Samples

The Uppsala K_0 is a blood donor whose null status was identified after routine antigen typing. Blood samples in EDTA from the proband, his parents, a brother, and a sister were also tested.

The Umeå K_0 was the K_0 brother of a deceased patient with an antibody to a high-incidence antigen, presumed to be anti-Ku. RBCs in a segment from an autologous blood bag frozen in nitrogen were thawed for analysis by molecular biology. This sample was first detected and analyzed by phenotyping and antibody investigation in the early 1980s.

The Linköping K_0 was an elderly female patient with anti-Ku. This sample was also detected and analyzed in the 1980s, and RBCs were droplet-frozen as rare test RBCs.¹⁴ These were recovered more than 20 years after freezing, and DNA was isolated.

Serology

K_0 status was identified by standard serologic techniques that included adsorption-elution at the blood centers that

referred these samples. Confirmation and further serology were performed at the International Blood Group Reference Laboratory in Bristol, England.

Molecular biology

Genomic DNA was prepared by a salting-out method modified from Miller and coworkers¹⁵ and diluted in H_2O to 100 ng per μL . *KEL**1 and *KEL**2 polymerase chain reaction (PCR) were performed according to our routine in-house procedures.^{16,17}

The coding region of *KEL* was amplified by PCR in 13 segments. This was performed in a PCR system (GeneAmp Models 2400, 2700, or 9700, Perkin Elmer Cetus, Norwalk, CT) with primers and conditions previously described by Lee and colleagues.^{11,18} Fragments were separated electrophoretically for 25 minutes at 150 V with 3 percent agarose gel (SeaKem, FMC BioProducts, Rockland, ME) containing ethidium bromide (0.56 $\mu g/mL$). The $\Phi X174$ *Hae*III DNA ladder (Advanced Biotechnologies Ltd, UK) was used as a size marker. Sequencing was performed on a sequence detection system (ABI Prism 310, Applied Biosystems, Foster City, CA) and then analyzed with computer software (SeqScape, v2.0, Applied Biosystems). GenBank Accession number M64934 was used as a reference sequence. Numbering of nucleotides and amino acids is according to Lee and colleagues;² that is, the ATG start codon is located at nucleotides 121 to 123 in exon 1.²

RESULTS

Uppsala K_0

The proband's RBCs lacked all Kell antigens tested for and thus typed KEL:-1,-2,-3,-4,-5,-7,-11,-14,-18,-23. The Kx antigen expression was marginally stronger than control cells of normal phenotype. His sister's RBCs were investigated separately and were also confirmed to have the K_0 phenotype. Adsorption with anti-K2 and subsequent elution confirmed the absence of antigens on the cell surface. RBCs from the parents and one brother were KEL:-1,2. No antibodies against Kell were detected in any of the family members. The pedigree of this family is shown in Fig. 1.

The blood donor and his sister were homozygous for a single-nucleotide mutation, 1540C>T in exon 13 that changes Gln474Stop. The parents were both heterozygous for the same mutation (Fig. 2). A DNA sample from the KEL:-1,2 brother was not available for molecular analysis.

Umeå K_0

The RBCs from the Umeå K_0 typed KEL:-1,-2,-3,-4,-5. Anti-Ku was identified in the serum.

Homozygosity for a nucleotide deletion, 1023delG, was found in exon 8 (Fig. 2). This mutation leads to a -1 frameshift and creates a premature stop codon in exon 9, 28 codons downstream from the deletion.

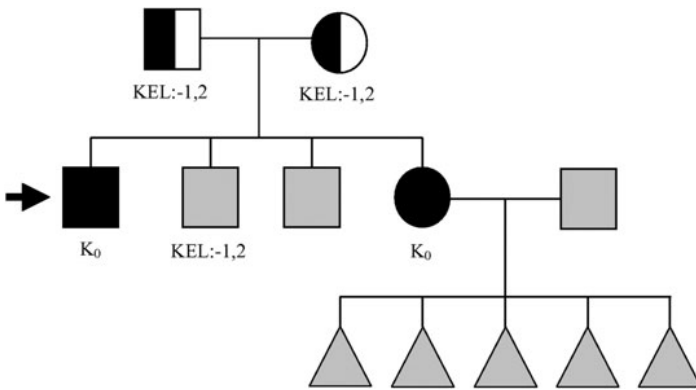


Fig. 1. Pedigree of Uppsala *K₀*. Pedigree of three generations in the Uppsala family including K1 and K2 phenotypes (where tested) of the donor, his parents, siblings, and the children of one sister. The proband is indicated by an arrow. The color coding indicates zygosity for the found *KEL* alleles: Black = *KEL*0*; white = *KEL*2*; gray = not tested.

mutation was previously reported by Lee and associates.¹¹ The mutation changes the conserved gt sequence at the donor splice site to at. Because the gt doublet is an important recognition signal for splicing, this mutation would result in skipping of exon 3, a prediction already verified by the transcript analysis of Lee and associates.¹¹

All mutations identified were on a *KEL*2* backbone allele that if functional would result in the following phenotype: *KEL*:-1,2,-3,4,5,-6,7,-10,11,12,13,14,16,-17,18, 19, 20,-21, 22,-23,-24,-25,26,27. This information was utilized for selection of the appropriate antiserum for adsorption-elution studies in the Uppsala *K₀*.

DISCUSSION

Kell is one of the most polymorphic blood group systems with at least 24 different antigens. With the exception of Ku (which is likely to be one or more broadly reactive epitopes on the Kell glycoprotein) and Km, all antigens result from missense single-nucleotide polymorphisms and subsequent single-amino-acid substitutions. The *K₀* phenotype is defined by the total lack of Kell antigens and is extremely rare: So far, 10 different alleles in a scarce number of individuals have been reported. These are listed at the following Web site: <http://www.bioc.aecom.yu.edu/bgmut/index.htm>. Although homozygosity for an inactivating mutation is more common, there are individuals who are heterozygous for different *KEL*0* alleles among the *K₀* cases reported.^{11,13} Table 1 lists the known and new *KEL*0* alleles.

We have investigated *K₀* samples from three different families to identify which *KEL*0* alleles are found in the Swedish population. We identified two new alleles in which premature stop codons are generated. If translated, these would encode truncated proteins of 473 or 328 amino acids, but by standard serology and immunoblotting it has been shown that no Kell protein is detected on the cell surface in *K₀* RBCs.¹¹ Where premature stop codons are generated, these observations are in accordance with our understanding of the

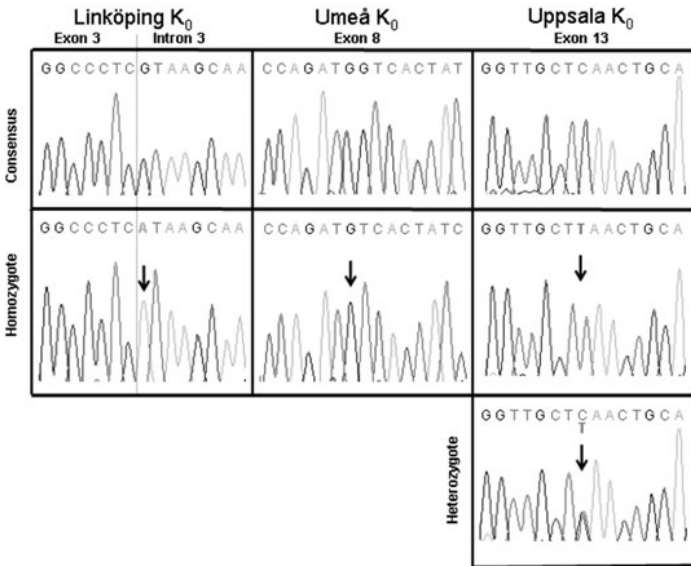


Fig. 2. Chromatograms of the three *K₀* samples studied. Results from sequencing the wild-type *KEL* gene as well as the *K₀* samples from Linköping, Umeå, and Uppsala. The positions of mutations are indicated with arrows.

Linköping *K₀*

These RBCs typed *KEL*:-1,-2,-3,-4,-5,-6,-7,-17. A strongly reactive anti-Ku was identified in the serum.

Homozygosity for the mutation g>a at +1 of intron 3 was observed in this sample (Fig. 2). The same splice site

TABLE 1. *KEL*0* alleles in different populations

Location*	Mutation	Consequence	Number of cases	Origin
i3 5'	+1g>c	Alternative splicing → Stop	1	Taiwan ¹²
i3 5'	+1g>a	Alternative splicing → Stop	>5; 1†	Reunion Island, USA, ¹¹ Sweden
e4	366T>A	C83Stop	1	Yugoslavia ¹¹
e4	502C>T	R128Stop	2	African American, USA ¹¹
i5 3'	-2a>g	Alternative splicing → Stop	1‡	Japan ¹³
e6	694C>T	R192Stop	1†	USA ¹¹
e8	1023delG	-1 frameshift → Stop	1	Sweden
e9	1162C>T	Q328Stop	1	Portugal ¹¹
e10	1208G>A	S363N	1	USA ¹¹
e12	1497G>A	W459Stop	1	Japan ¹³
e13	1540C>T	Q474Stop	1	Sweden
e18	2147G>A	S676N	1	Israeli Arab, USA ¹¹

* e = exon; i = intron.
† Heterozygous with e10 1208G>A.
‡ Heterozygous with e12 1497G>A.

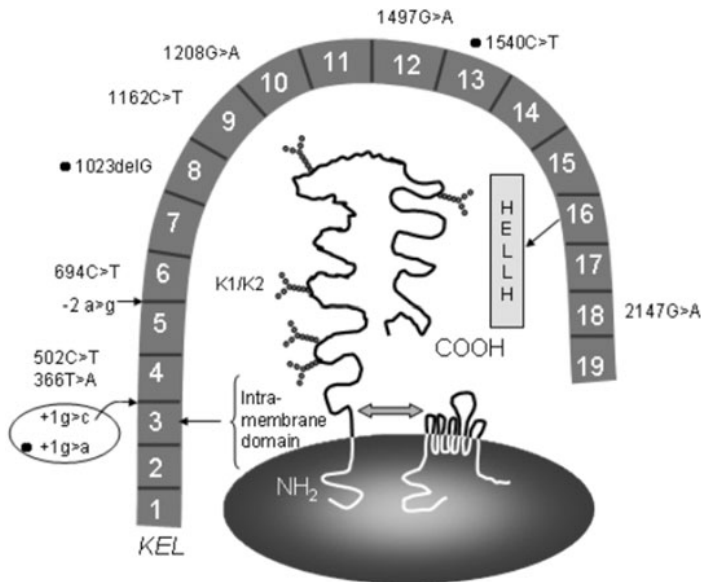


Fig. 3. Schematic representation of the Kell glycoprotein, the exons of the *KEL* gene, and the distribution of the *KEL*0* point mutations. A schematic representation of the Kell glycoprotein and the adjacent XK protein. Polymorphisms resulting in *KEL*0* alleles and the 19 exons of *KEL* are shown. Black dots indicates the mutations found in this study. Encircled—the most common mutation found in different populations. HELH refers to the zinc-binding catalytic site encoded by exon 16.

posttranscriptional quality control system, nonsense-mediated mRNA decay (NMD), which clears eukaryotic cells of premature termination codon-containing mRNAs. A premature stop codon that initiates NMD is defined by occurring at least 50 nucleotides upstream from the last exon-exon junction in a spliced RNA molecule. After splic-

ing, 20 to 24 nucleotides upstream of the exon-exon junctions will be marked by a protein complex. If such a complex is found after the stop codon, this will initiate RNA degradation, and as a result truncated and potentially deleterious proteins are not synthesized.^{19,20} The two novel alleles identified in this study that generate stop codons would comply with the current concepts of NMD. Indeed, it is likely that all *KEL*0* alleles described to date, which contain premature stop codons, would suffer the same fate (Fig. 3). In a previous report, Koda and coworkers¹³ suggested that NMD does not play a role in transcript degradation because mRNA transcripts were detected in the K_0 sample investigated; however, we think that the presence of transcripts and their degradation by NMD are not mutually exclusive and that it is likely that transcripts are detectable prior to the degradation process. Figure 3 also shows the missense mutations in exons 10 and 18 that result in the K_0 phenotype. It has been shown that these amino acid substitutions result in the intracellular retention and degradation of the mutant Kell proteins such that no protein is expressed on the cell surface.¹¹

The third allele identified in this study is identical to one previously reported, with a splice site mutation (g>a) in the exon 3-intron 3 junction (Fig. 2).¹¹ Transversion of +1g (g>c) was the first K_0 mutation reported in an individual from Taiwan.¹² Both mutations cause skipping of

exon 3 and introduce a downstream premature stop codon.^{11,12} Should any protein be transcribed, it would not be assembled in the RBC membrane because exon 3 encodes for the single-transmembrane region.² The invariant g in the intron 3 donor splice site appears to be prone to mutation because g>a and g>c have been described in different populations. This is the most common mechanism giving rise to the *K₀* phenotype.

It is interesting that we have identified two mutations unique to our small Swedish population and one mutation that has been found in rare individuals worldwide. The frequency of *KEL*0* alleles is not known but Lee and colleagues¹¹ predict that the individual nonsense mutations may be more frequent than previously thought, because any inactivating mutation present in a heterozygous state together with a wild-type allele will probably remain undetected.¹¹

Although the *K₀* phenotype is rare, characterization of null phenotypes is clinically relevant. Knowledge of mutations leading to null phenotypes should be considered when performing molecular analysis for common alleles in certain situations, for example, prediction of fetal phenotype and forensic investigations. False-positive predictions could be potentially harmful in these situations because current protocols depend on detecting the presence or absence of known single-nucleotide polymorphisms.

Antigens in the Kell blood group system are considered to be highly immunogenic. Therefore, it is somewhat surprising that the Uppsala *K₀* sister did not produce anti-Ku in spite of five pregnancies, although both brothers from Umeå and the Linköping patient did, following transfusion. Consistent with other reported cases, lack of Kell glycoproteins does not seem to cause negative effects because the individuals in our study, as well as in the other reports, have been healthy.

In summary, we have identified two new alleles in the Swedish population, bringing the total to 12 *KEL*0* alleles described. We have identified another person with the intron 3 +1g>a mutation confirming this mutation as the most frequent genetic basis for the *K₀* phenotype.

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Paper II

Erythroid urea transporter deficiency due to novel *JK^{null}* alleles

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BACKGROUND: The Kidd blood group antigens Jk^a and Jk^b are encoded by the red blood cell (RBC) urea transporter gene. Homozygosity for silent *JK* alleles results in the rare Jk(a-b-) phenotype. To date, seven *JK^{null}* alleles have been identified, and of these, two are more frequent in the Polynesians and Finns. This study reports the identification of other *JK^{null}* alleles in Jk(a-b-) individuals of different ethnic or geographic origins.

STUDY DESIGN AND METHODS: Nine Jk(a-b-) samples and a sample from a Jk(a-b+) mother of a Jk(a+b-) baby were investigated. Polymerase chain reaction amplification and sequence analysis of the *JK* gene was performed. Western blotting and urea lysis were used to confirm Jk(a-b-) RBCs.

RESULTS: Four novel alleles were identified: two different nonsense mutations, 202C>T (Gln68Stop) and 723delA (Ile262Stop) were identified on otherwise consensus *JK*1* and *JK*2* alleles, respectively. A missense mutation, 956C>T (Thr319Met), was identified in a *JK*1* allele from an African-American and a *JK*2* allele in two people of subcontinental Indian descent. Immunoblotting and urea lysis confirmed absence of JK glycoprotein in RBC membranes from a sample carrying the 956C>T mutation. Other previously described *JK^{null}* mutations were found in samples of origins other than in which they were first identified.

CONCLUSION: The molecular bases of the Jk(a-b-) phenotype are diverse and this is the first report of *JK^{null}* alleles in individuals of African and subcontinental Indian descent. Although rare, these alleles should be taken into consideration when planning genotyping strategies for blood donors and patients.

The Kidd blood group system (ISBT 009) was first described in 1951 when Mrs Kidd produced an antibody to an unknown blood group antigen during pregnancy.² This first antigen was named Jk^a and since then the antithetical antigen, Jk^b, as well as the null phenotype, have been identified (reviewed in Daniels³). A third antigen, Jk3, is common to both Jk^a and Jk^b carriers and is absent only on Jk(a-b-) red blood cells (RBCs). The Jk(a-b-) phenotype may be identified by the absence of Jk^a and Jk^b antigens in routine phenotyping and/or by the presence of anti-Jk3 in the plasma of immunized individuals. In addition, Heaton and McLoughlin showed that Jk(a-b-) RBCs were resistant to lysis by 2 M urea, a common diluent in automated blood analyzers and this could be used as a screening test.⁴ They suggested that perhaps the JK glycoprotein was involved in urea transport. Cloning and sequencing of the human RBC urea transporter gene (*SLC14A1*; synonyms: *UT-B1*, *HUT11*, *RACH1*) confirmed its identity as the *JK* gene and further, identified the molecular basis associated with the Jk^a/Jk^b polymorphism.⁵⁻⁷ The mature JK glycoprotein

ABBREVIATION: SNP = single nucleotide polymorphism.

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Part of this study has previously been reported in abstract form.¹

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(synonyms: Kidd, RBC urea transporter, UT-B1) of 389 amino acids is encoded by exons 4 through 11 with the translation start point in exon 4.⁸ The JK glycoprotein can be found on fetal cells at 11 weeks of gestation and is expressed not only on RBCs but also in the vasa recta and outer medulla of the kidney, and colon.^{9,10} Studies on rats show that glycosylated and nonglycosylated isoforms are also found in brain and bladder.^{9,11} Consistent with other transporter and channel proteins, JK glycoprotein is a type III transmembrane protein. One single *N*-glycan at Asn221 is predicted to carry ABH antigens but no other glycosylation is described.¹² The two antigens Jk^a and Jk^b result from a single amino acid difference Asp280Asn, encoded by a 838G/A substitution in the codominant alleles, *JK*1* and *JK*2*, and are predicted to be situated on the fourth extracellular loop.⁷ Irshaid and colleagues¹³ demonstrated that in addition to the missense single nucleotide polymorphism (SNP) that defines Jk^a and Jk^b, there were two other silent SNPs that were associated with each allele: 588 A/G; and intron 9-46a/g. In six of 10 *JK*1* alleles investigated, the *JK*1* allele was defined by 588 A and associated with intron 9-46a while all 16 *JK*2* alleles investigated were associated with 588G and intron 9-46 g.¹³

Seven *JK^{null}* alleles have been reported to date.^{8,13-18} Two give rise to the Jk(a-b-) phenotype in the Polynesian and Finnish populations in which the frequency of this phenotype is elevated. The frequency of the other alleles is unknown but extremely low. Although Jk(a-b-) people lack the RBC urea transporter there is no apparent dysfunction of the RBCs under normal conditions. Additionally, there is redundancy within the urea transporters in the kidney and there is only minor impairment of function in that these individuals are unable to fully concentrate urine.¹⁹

In the Japanese population, the presence of a dominant inhibitor gene *In(Jk)* has been demonstrated as another origin for the null phenotype. In these cases, weak antigen expression may be found by adsorption/elution techniques. Anti-Jk3 has not been found in this group.²⁰ The genetic background for *In(Jk)* has not been identified, however, the *JK* gene appears to be unaffected.

We report here the investigation of nine Jk(a-b-) samples of different ethnic and geographic background. Also included is a case report of a family in which there was an apparent maternal exclusion based on the Jk phenotyping. We identified three new mutations carried on four alleles, as well as five samples that carried *JK^{null}* alleles reported previously.

MATERIALS AND METHODS

The numbering of nucleotides in this paper starts with the A of the initiation codon in exon 4 as nucleotide number 1. The same codon encodes amino acid number 1.

Samples

Samples from nine Jk(a-b-) individuals were investigated. All these had been identified by standard serological techniques, including routine phenotyping, as well as adsorption and elution with polyclonal Jk phenotyping reagents in most cases. Screening and identification of antibodies was performed according to the routine procedures of the referring laboratories. A *JK^{null}* allele was suspected in a Jk(a-b+) mother whose child's RBCs typed Jk(a+b-). The father's RBCs also typed Jk(a+b-).

Molecular biology

Genomic DNA was prepared by a salting-out method modified from Miller and coworkers²¹ and diluted in sterile water to 100 ng/ μ L. Allele-specific polymerase chain reaction (PCR) to discriminate between *JK*1* and *JK*2* followed by screening for known *JK^{null}* mutations was performed according to routine in-house procedures.^{13,22} If conclusive results were obtained, no further investigation was performed. Samples in which known mutations had been excluded were sequenced as follows: the coding region, as well as flanking intron sequences (\pm 30 nt), of *JK* were amplified by PCR in six segments. In two samples in which a missense mutation only was identified, exons 1 to 3 and the promoter region⁸ were sequenced as well. All primers (Table 1) were synthesized by DNA Technologies A/S (Aarhus, Denmark) according to the *HUT11A* sequence (GenBank Accession No. NM_015865). For the PCR procedure, 100 ng of DNA in a total volume of 10 μ L, with 2 nmol of each dNTP, 5 pmol of each primer, 0.5 U TaqGold (Perkin Elmer/Roche Molecular Systems, Branchburg, NJ) in the buffer supplied, was used. Amplification was performed with a commercially available PCR system (GeneAmp PCR System 2400, 2700, or 9700, Perkin Elmer Cetus, Norwalk, CT) under the following conditions: 7 minutes at 96°C followed by 35 cycles of 30 seconds at 96°C, 30 seconds at 58°C, 60 seconds at 72°C, and then a final elongation of 7 minutes at 72°C. PCR products were separated by electrophoresis for 25 minutes at 150 V with 3 percent agarose gel (SeaKem, FMC BioProducts, Rockland, ME) containing ethidium bromide (0.56 μ g/mL). The Φ X174HaeIII DNA ladder (Advanced Biotechnologies Ltd, London, UK) was used as a size marker. The PCR product was eluted from the gel with a gel purification kit (QIAquick, Qiagen Nordic, Crawley, UK). Sequencing of the PCR products was performed with primers according to Table 1, on a sequence detection system (ABI Prism 3130, Applied Biosystems, Foster City, CA) with reagents (BigDye, Applied Biosystems) and then analyzed with software (SeqScape, v2.5, Applied Biosystems).

Urea lysis test

Because Jk(a-b-) RBCs resist lysis by 2 mol per L urea, a lysis test²³ was performed on one Jk(a-b-) sample in which

TABLE 1. Oligonucleotide primers used in this study

Amplified fragment	Primer name*	Nucleotide sequence (5'→3')	PCR product
Promoter	JkPF JKPR	GCTGGCCTTCAGAAAACAAC GGATGAGTACTTACACCAC	610
Exon 1	Jk5'Fesw Jkin1Resw	GATGCCTGAAGTGGGAGGAG CTTAGAACTGTGAAGACAAGCC	256
Exon 2	Jkin1Fesw Jkin2Resw	ATCAAGACTTCTGGGAGTAGTG CATTAAACACTCATGGCTAGC	183
Exon 3	Jkin2Fesw Jkin3Resw	GTGGTCATTAGAGCTAGGGCA CCCACCTCCCAGGCTCTGC	318
Exons 4-5	Jkin3F1 Jkin5R Jkin4R†	GGCCCCAAAGCTGGCTAATC CCACAGTAACTGGTCAGCC CCAAAGCTACTTCTCCTGTC	1039
Exon 6	Jkin5F3 Jkin6R	CAAGTGCAACCAAGCTCAC CTGCCATATAACAACCTCCATTC	216
Exon 7	Jkin6F Jkin7Rll	TGCTTTGTACATGCACATTC TTAGACCCTAATGCTCTGGAG	402
Exons 8-9	Jkin7F Jkin9R2 Jkin8Resw†	AGAGGTAAGGTATGCCAATC ACTGCTTATCCTTGATTGAG TCCCTAGGAAGCCAAGATCTC	677
Exon 10	Jkin9Fesw Jkin10Resw	ACAGAGCCCATGGAGCTCC TGTAAGCTGGTCAATTACGCT	282
Exon 11	Jkin10F Jk3'UTRII	TGCACAGAATCCAGAGCAATG CGTCTGTAATCTGTTCTTATGC	443

* Primer that is used for sequencing only.

F and R = forward and reverse primer, respectively. in = intron.

an amino acid substitution was detected. Briefly, test tubes containing 0.25 mL of a 2 percent suspension of RBCs were centrifuged. The supernatant was removed and the RBCs were mixed with 1 mL of 2 mol per L urea or water. After 2 minutes of incubation at room temperature, the tubes were centrifuged, and the supernatant was tested in a spectrophotometer (plasma/low Hb, HemoCue, Ängelholm, Sweden) to assess free hemoglobin. The tubes were remixed and allowed to stand for 15 minutes at room temperature and reevaluated. Jk(a+b+) RBCs and Jk(a-b-) RBCs of known molecular background were tested in parallel.

Western blot analysis

To confirm absence of JK glycoprotein in the RBC membranes of the same Jk(a-b-) sample described above, Western blot analysis was performed with an anti-rat UT-B1 that had been shown previously to cross-react with the JK glycoprotein.¹¹ RBC ghosts were prepared according to the method of Dodge and colleagues.²⁴ Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting was performed as described.¹¹ Briefly, proteins were separated by electrophoresis on a denaturing 10 percent SDS polyacrylamide gel with a 4 percent stacking gel and then transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon, Millipore AB, Solna, Sweden). After transfer, the PVDF membrane was blocked in 5 percent nonfat milk/phosphate-buffered saline/0.01 percent Tween (5 percent milk/PBS-T) and then incubated with anti-rat UT-B1

diluted 1:5,000. After stringent washing with PBS-T, the membrane was incubated with the secondary antibody: a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Invitrogen AB, Lidingö, Sweden), diluted 1:20,000. Bands were developed with west pico chemiluminescent substrate (SuperSignal, Pierce, Rockford, IL) according to the manufacturer's protocol. A prestained broad-range SDS-PAGE standard (Bio-Rad, Sundbyberg, Sweden) was used as a size marker.

To control for protein loading, the membrane was stripped by submerging the membrane in stripping buffer (100 mmol/L 2-mercaptoethanol, 2% SDS, 62.5 mmol/L Tris-HCl, pH 6.7) and incubated at 50°C for 30 minutes. After two washes in PBS-T, the membrane was blocked as above and then incubated with anti-glycophorin A,B (clone E3, Sigma-Aldrich Sweden AB, Stockholm, Sweden), diluted 1:7500 in PBS-T

for 1 hour at room temperature. After stringent washing, the membrane was further incubated with HRP-conjugated anti-mouse globulin (Bio-Rad), diluted 1:7500 in 5 percent milk/PBS-T for 1 hour at room temperature, washed, and exposed to Pierce SuperSignal detection reagent, as above.

RESULTS

Four new *JK^{null}* alleles were identified among the samples investigated. A summary of the results is presented in Table 2, and the mutations are described as follows:

Genomic deletion from intron 3 to intron 5

The genomic lesion that spans exon 4 and exon 5 of the *JK* gene was observed in a woman of Bosnian origin, whose plasma contained anti-Jk3. This deletion has been described previously in two other individuals, of Tunisian¹⁵ and English¹⁶ descents. The breakpoint sequence found was identical to the earlier reports.

202C>T

Two American siblings of Caucasian origin were identified by different laboratories. The sister was a Jk(a-b-) patient with anti-Jk3 in her plasma. Her transfusion history was unknown but she had three children (not included in this study), all typed as Jk(a+b-). The Jk(a-b-) phenotype of the brother was found by the identification of an anti-Jk3 in routine pretransfusion testing. Both siblings were

TABLE 2. Previously published and the four novel *JK^{mut}* alleles (*) in this report*

Mutation affected exon (e) and intron (i)	e5 202C>T	i5-1 g>a	i5-1 g>c	e6 582C>G	i7+1 g>t	e8 723delA	e9 871T>C	e10 956C>T
Consequence	Deletion of 3-15	e6 skipped	e6 skipped	Tyr194Stop	e7 skipped	Ile262Stop	Ser291Pro	Thr319Met
Allelic backbone	<i>JK*1</i>	<i>JK*2</i> or <i>JK*1</i> ¹⁶	<i>JK*2</i>	<i>JK*1</i>	<i>JK*2</i>	<i>JK*2</i>	<i>JK*2</i>	<i>JK*1</i> or <i>JK*2</i>
Ethnic/geographic origin	English ¹⁶ Tunisian ¹⁵ Bosnian	Polynesian ^{6,18} Chinese ⁹	Chinese ¹⁷	Swiss ¹⁶	French ⁸	Hispanic-American ●	Finnish ^{3,14}	African-American ● Indian ●
Probands (families reported)	2 (1)	Many	1 (1)	4 (2)	1 (1)	1 (1)	Many	3 (3)

* Nucleic and amino acid changes are in comparison to the coding region, that is, exons 4-11 of the consensus *HUT11A* (GenBank NM_015665).

confirmed to be Jk(a-b-) by adsorption and elution tests, and sequence analysis revealed that the siblings were homozygous for *JK*1* alleles carrying a novel nonsense mutation, 202C>T in exon 5 (Fig. 1), that is predicted to change Gln68Stop.

Intron 5 +1g>a

A woman with anti-Jk3, probably of Vietnamese origin but living in the south of Sweden, was homozygous for the common Polynesian mutation, intron 5 +1g>a,^{8,13} that is carried on a *JK*2* allele. A sample from her sister, whose RBCs typed Jk(a+b-), genotyped as *JK*1/2* and was heterozygous for the same mutation.

582C>G

Homozygosity for a mutation previously reported in a Swiss family (582C>G)¹⁶ was found in a sample from another Swiss proband with anti-Jk3. No apparent connection between this and the originally reported proband was established. Similar to the original Swiss family, 582G was associated with the *JK*1* allele.

723delA

A female blood donor of Hispanic American descent, with no anti-Jk3 in her plasma, was found through mass screening. She was a compound heterozygote for the previously described Polynesian mutation, intron 5 +1 g>a,^{8,13} as well as a novel mutation, a nucleotide deletion, 723delA in exon 8 (Fig. 1). This mutation causes a frameshift at codon 241 and creates a premature stop signal in the same exon at codon 262. Both mutations were carried on *JK*2* alleles.

871T>C

A Swedish male patient of Scandinavian origin, with anti-Jk3 in his plasma, was homozygous for the Finnish mutation (871T>C) carried on a *JK*2* allele.¹³

956C>T

Sample 1

Sample 1 was from an African-American male blood donor without anti-Jk3 in his plasma but Jk(a-b-) by adsorption and elution tests. Homozygosity for a novel mutation, 956C>T in exon 10 (Fig. 1) was detected that encodes an amino acid change of Thr319Met. Furthermore, although the sample was also homozygous for 838G, which encodes Asp280 (the amino acid defining the Jk^a antigen) the silent nucleotide polymorphisms at 588

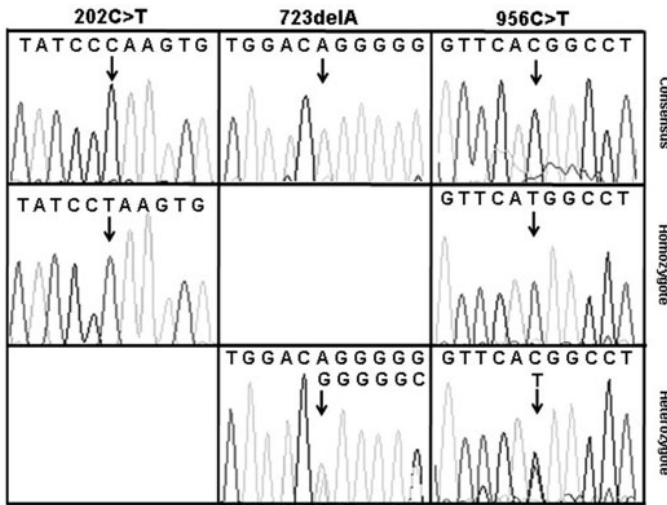


Fig. 1. Chromatograms of the four JK^{null} samples with novel mutations. Results from sequencing the consensus JK gene as well as JK alleles carrying the following mutations: 202C>T, 723delA, and 956C>T. The positions of mutations are indicated with arrows.

TABLE 3. Comparison of the allele-associated nucleotides in the JK^*1 and JK^*2 alleles with alleles containing the novel 956C>T mutation

Allele identity	Discriminating nucleotide position			
	588	838	i9-46	956
JK^*1	A	G	a or g	C
JK^*2	G	A	g	C
African-American Jk(a-b-)	G	G	g	T
Indian Jk(a-b+)	G	A	a/g	C/T
Indian-Pakistani Jk(a-b-)	G	A	a	T

and intron 9 to 46 shown previously to be allele-related,¹³ were consistent with a JK^*2 allele (Table 3).

Because the novel mutation is a missense mutation only, additional studies were performed: testing for function of the RBC urea transporter by urea lysis and testing for presence of JK glycoprotein by Western blotting. The urea lysis test showed that RBCs from the proband followed the same pattern as Jk(a-b-) control RBCs of Finnish and Polynesian origin. That is, no hemolysis was detected after 15 minutes while normal Jk(a+b+) RBCs were lysed completely within 2 minutes. All RBC samples were completely hemolyzed by the addition of water at 2 minutes. Western blot analysis (Fig. 2) with a cross-reactive anti-rat UT-B1 demonstrated staining of a broadly migrating band in normal Jk(a+b+) RBC membranes. Weak staining was observed in RBC membranes from this sample, consistent with that observed in the Jk(a-b-) controls.

Sample 2

A case of "maternal exclusion" in a family of Indian origin led to the identification of a second example of the new mutation 956C>T, this time on a JK^*2 allele. The mother typed Jk(a-b+), the father Jk(a+b-), and the baby Jk(a+b-). Anti- Jk^a was identified in the mother's plasma. Routine allele-specific PCR confirmed the phenotypes but sequencing revealed heterozygosity for 956C/T carried by a JK^*2 allele with unexpected nucleotides at positions 838 and intron 9 to 46¹³ in combination with a normal JK^*2 allele (Table 3).

Sample 3

The third sample with this mutation was from a Pakistani woman in her third pregnancy, whose plasma contained anti- Jk^a , - $Jk3$, and -K. She was homozygous for the 956C>T mutation carried on JK^*2 alleles, together with the same discrepant allelic markers observed in the JK^{null} allele in sample 2 (Table 3). Cord RBCs tested at delivery typed Jk(a+b-) and an eluate contained anti- $Jk3$. The baby underwent two exchange transfusions based on the elevated bilirubin level. No family history of consanguinity was available, and the RBCs of two sisters typed Jk(a+b+).

Sequences carrying novel mutations were deposited at NCBI GenBank (<http://www.ncbi.nlm.nih.gov>) with the accession numbers EF571316 (202C>T), EF571317 (723delA), and EF571318 (956C>T).

DISCUSSION

In this article we report three new JK^{null} mutations carried on four alleles. Mutations that silence alleles within the JK system follow the same pattern as in other genes, that is, nonsense mutations leading to premature stop codons, incorrect outsplicing during transcript processing, or missense mutations resulting in amino acid substitutions. Two novel nonsense mutations were found, which are expected to inactivate the transcript with rapid degradation by the nonsense-mediated mRNA decay pathway.²⁵ Interestingly, the same novel missense mutation was identified in three unrelated samples. Though no antigen was detected by adsorption-elution tests, absence of antibody in one proband prompted further investigation by urea lysis and Western blotting. Resistance to urea lysis was

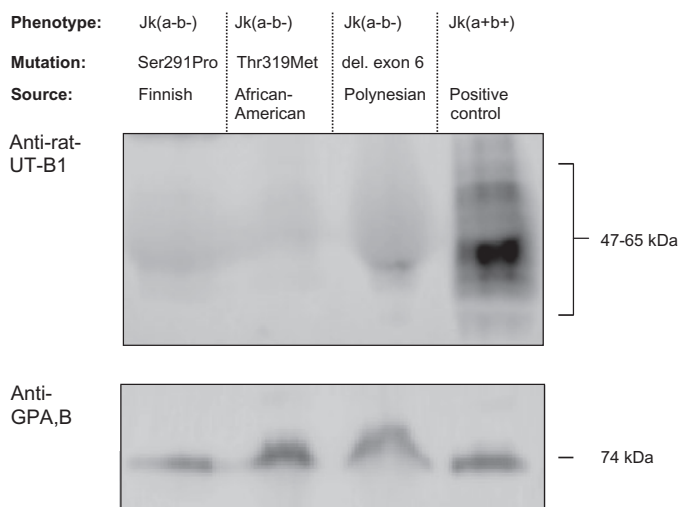


Fig. 2. Detection of JK glycoprotein in RBC membranes. (Top panel) Blotting of Jk(a+b+) RBC membranes with anti-rat UT-B1 demonstrated a broadly staining band synonymous with the expected band of approximately 47 to 65 kDa, consistent with N-glycosylated UT-B1.¹¹ Control Jk(a-b-) RBC membranes (of Finnish and Polynesian origin) demonstrated faint staining; however, an even weaker staining was detected in Jk(a-b-) RBC membranes from the African-American donor with the novel 956C>T mutation (encoding T319M). (Bottom panel) Blotting the same membrane with anti-GPA,B demonstrates the GPA 74-kDa dimer and verifies that proteins are loaded in all lanes.

demonstrated and although a very faint band was observed by Western blotting this was comparable to results in the original report in which the antibody was used. A single amino acid change of threonine to methionine might not be expected to affect protein expression; however, there are precedents in other multipass membrane proteins such as Band 3 and RhAG, where single amino acid changes close to or within the transmembrane domains can ablate the expression of the protein.^{26,27} Furthermore, the Jk(a-b-) phenotype among Finns is due to another single amino acid substitution (Ser291Pro).^{13,14} Both these amino acid changes lie within predicted transmembrane-spanning domains of the JK glycoprotein.⁵

We first identified the 956C>T mutation in an African-American blood donor, and to our knowledge this is the first description of a *JK^{null}* allele of African origin although the Jk(a-b-) phenotype in this population has been reported briefly in an abstract.²⁸ The mutation was carried on a *JK²* allele (albeit altered), which is more frequent in this population.²⁹ We later identified the same mutation in samples from two unrelated women, both from the Indian subcontinent. In both samples the mutation was carried on *JK²* alleles, which suggests but does not prove that the

mutation arose independently on the African and Indian continents. This is the first report of a *JK^{null}* allele in India, and there are only few publications describing frequency of blood groups other than ABO and Rh in Indian populations. One report published in 1980 describes studies on many genetic markers in different ethnic groups in Southern India and the authors found that 13 of 211 Hindus and one of 87 Muslims phenotyped as Jk(a-b-).³⁰ In another report from 1997 the Jk(a-b-) frequency was 0.54 percent in North Indian blood donors.³¹ It is somewhat surprising that there has been no follow-up on these findings and in general there is a paucity of literature on the molecular basis of blood groups in India. The 956C>T mutation was not found when performing database SNP searches in GenBank, Ensembl and HapMap (<http://www.ncbi.nlm.nih.gov/>, <http://www.ensembl.org/index.html>, and <http://www.hapmap.org/>), which adds substance to the fact that this is a mutation and not a common SNP. The silencing mutations we describe here are spread throughout the gene, which suggests random spontaneous mutations. No hot-spot areas are apparent, with the possible exception of the invariant acceptor splice site of the 3' end of intron 5, which is mutated in Polynesian Jk(a-b-) individuals + 1g>a^{8,13} but which in a single report from China is mutated + 1g>c.¹⁷ The Jk(a-b-) phenotype, and therefore the absence of the RBC urea transporter, is not associated with any pathology and the role of the JK glycoprotein in the kidney is apparently compensated for by other urea transporters. Jk(a-b-) individuals, however, present a clinical management challenge because anti-Jk3 is produced readily in response to transfusion and pregnancy and Jk(a-b-) blood is not routinely available.

In conclusion, we demonstrate here that although the Jk(a-b-) phenotype is rare, the underlying molecular bases are varied among the different populations. Aside from the *JK^{null}* alleles previously identified in European and Asian populations, our study also elucidates molecular bases of *JK^{null}* alleles in people of African and Indian descent.

Our results highlight the need to consider the population under investigation when designing molecular assays for phenotype prediction. Although it is unreasonable and unnecessary to accommodate all known mutations in manual testing, as high-throughput genotyping


platforms such as DNA microarray become more widely available, these alleles may readily be included.

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Paper III

A simple screening assay for the most common *JK*0* alleles revealed compound heterozygosity in Jk(a–b–) probands from Guam

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Running title: PCR method for *JK*0* detection

Key words: JK blood group system, null phenotypes, molecular basis, PCR-ASP.

Abstract

The Jk(a-b-) phenotype results from alterations in the *JK* gene and is characterised by absence of the RBC urea transporter in the cell membrane. The frequency of Jk(a-b-) varies among populations, but is most commonly found in people of Polynesian and Finnish descent. Although rare, Jk(a-b-) individuals present a clinical challenge since anti-Jk3 is produced readily in response to transfusion and pregnancy, and Jk(a-b-) blood is not routinely available. Identification of Jk(a-b-) patients and donors is most often performed serologically. However, 10 *JK*O* alleles have been identified and this information can be adopted for use in DNA-based typing. We selected five *JK*O* alleles that had been encountered by our reference laboratory in two or more samples from unrelated individuals, and designed an allele-specific primer PCR assay for use as an initial screening tool. Following in-house validation, we tested genomic DNA from a family; a mother and her two sons referred to us for genetic investigation of their Jk(a-b-) phenotypes. Two different nucleotide substitutions, -1g>a in intron 5 and 956C>T in exon 10, originally associated with Polynesian and Indian/African populations respectively, were identified in the family. The mother and one son were compound heterozygotes, while the second son was homozygous for intron 5 -1g>a. We conclude that the effort to design and validate such a screening assay was cost-efficient when compared to DNA sequencing costs. Furthermore, selection of the more common *JK*O* mutations was a practical approach which resulted in rapid identification of the genetic bases behind the Jk(a-b-) phenotypes in this unusual family. Although an obvious target for eventual inclusion into high-throughput genotyping platforms for clinical diagnostic services, current systems are very limited. Our approach provides a simple and inexpensive method for the identification of these rare alleles.

Introduction

The antigens of the Kidd (JK; ISBT009) blood group system are carried on the RBC urea transporter (Kidd glycoprotein, SLC14A1),¹ a multipass membrane-spanning glycoprotein. Individuals who lack the Kidd glycoprotein on their RBCs express the Jk(a–b–) phenotype, also known as Jk^{null}. This phenotype is associated with a mild insufficiency in urine concentration (which goes largely undetected in normal conditions) but may often be identified by the presence of anti-Jk3 in the plasma of immunized individuals. The absence of the RBC urea transporter renders Jk(a–b–) RBCs resistant to lysis by 2 M urea,^{2,3} which is used as an efficient screening test in populations where the Jk(a–b–) phenotype is more prevalent.⁴⁻⁸

The Jk^a and Jk^b antigens depend on the amino acid at position 280, aspartic acid for Jk^a and -asparagine for Jk^b.⁹ The Jk(a–b–) phenotype derives from homozygosity or compound heterozygosity for mutations in the *JK* gene (*SLC14A1*; synonyms: *UT-B1*, *HUT11*, *RACH1*), so-called *JK*0* or *JK*0* alleles. A rare dominant Jk(a–b–) phenotype named In(Jk) was reported in two Japanese individuals identified by 2 M urea lysis screening,¹⁰ although the molecular basis has not been identified.

The *JK* locus comprises 11 exons carried on chromosome 18, and several different genetic alterations have been described to cause the Jk(a–b–) phenotype. The two most frequent *JK*0* alleles are found in people of Polynesian (intron 5 –1g>a) and Finnish (871T>C) descent¹¹⁻¹³ where the incidence of the Jk(a–b–) phenotype was shown to be 0.27% and 0.03% respectively.^{5,7} Other *JK*0* alleles have been only sporadically detected in other populations.¹⁴⁻¹⁹ In this report, we have named silencing *JK* alleles generally as *JK*0* however; where the *JK*01* or *JK*02* backbone is known, this is indicated as *JK*01N* or *JK*02N* in line with current proposals under consideration by the ISBT committee on terminology for red cell surface antigens (<http://ibgrl.blood.co.uk/>). Although rare in the general population, Jk(a–b–) individuals present a clinical challenge since anti-Jk3 may be produced in response to transfusion and pregnancy, and Jk(a–b–) blood is not routinely available. Limited resources of anti-Jk3 make large-scale phenotype screening programs for Jk(a–b–) blood impractical.

Screening methods for blood group polymorphisms using genomic DNA are becoming widespread in many laboratories. In instances where a rare null allele is found more commonly in one population than in others, it is practical and cost efficient to test only for that mutation and large numbers of samples can be tested at a time. We sought to investigate the usefulness of developing an allele-specific primer PCR (PCR-ASP) screen for those *JK*0* alleles that had been found in two or more unrelated individuals as a primary analysis for samples referred to the Nordic Reference Laboratory for Blood Group Genomic typing for identification and/or confirmation of their Jk(a–b–) phenotype. Our algorithm was such that samples in which a causative polymorphism was identified could be reported directly. Exclusion by the assay of these five mutations would prompt DNA sequence analysis of exons 1 to 11 of the *JK* gene, a much more time-consuming and labor-intensive undertaking.

Materials and Methods

*JK*0* mutations that fulfilled our criteria for inclusion are listed as follows (shown in Figure 1): deletion of exon 4 and 5 (Δ exons 4+5),^{14,15} intron 5 –1g>a,^{11,12,17} 582C>G,²⁰ 871T>C,^{12,13} and 956C>T.¹⁸ Allele-specific primers (Table 1) were designed to detect both the normal and mutant alleles according to the *HUT11A* sequence (GenBank accession no. NM_015865) and were synthesized by DNA Technologies A/S (Aarhus, Denmark). A mismatch was introduced in two primers to enhance allele specificity (underlined in Table 1). We performed the new assay in parallel with our routine in-house PCR-ASP for *JK*01/JK*02*.²¹

For the PCR reaction 100 ng of DNA was used in a total volume of 10 μ L, using 0.2 mM dNTPs, 0.25 pmol of each primer, 0.25 pmol of control HGH primers, 0.5 U TaqGold™ (Perkin Elmer/Roche Molecular systems, Branchburg, NJ, USA) in the buffer supplied. Amplification was performed in the GeneAmp PCR system 2700 or 2720 (Perkin Elmer Cetus, Norwalk, CT, USA) as follows; 7 minutes at 97°C, then 35 cycles of 30 seconds at 94°C, 30 seconds at 62°C, 40 seconds at 72°C and a final elongation of 2 minutes at 72°C. PCR products were separated by electrophoresis for 25 minutes at 150

V using 3% agarose gel (SeaKem. FMC BioProducts, Rockland, MA, USA) containing ethidium bromide (0.56 µg/ml).

Once optimised, PCR reagent mixes for each mutation and for the corresponding consensus nucleotide were prepared to a final volume of 500 µL, and frozen at -20°C after quality control. Samples carrying the *JK*0* mutations, and samples of normal Jk type were from our in-house DNA collection.

Genomic DNA was prepared either by a salting-out method modified from Miller *et al.* and diluted in sterile water to 100 ng/µl,²² or by the Qiagen Blood and Body Fluid protocol (Qiagen Inc, Valencia, CA; USA).

The numbering of nucleotides in this paper starts with the A of the initiation codon in exon 4 as nucleotide number 1. The same codon encodes methionine at position number 1.

Results

Following optimisation of each PCR-ASP to the conditions listed above, the new screening tool readily detected both mutation and consensus SNPs for each tested allele (Figure 2), and homozygote and heterozygote controls gave the expected results. Once the assay had been established and the expected bands shown to be robust and specific, DNA analysis of the individuals included in the case report was performed.

Case Report

Samples from a woman, originating from Guam, and her two sons were received for serological investigation. An earlier investigation of the woman's plasma performed by the referring hospital demonstrated an anti-Jk^b although she had not been transfused. The sample at the time of the current investigation demonstrated anti-Jk3. Her sons were tested together with other family members to locate potential compatible donors.

The RBCs from all three samples typed Jk(a-b-) by serology and showed no hemolysis by the urea hemolysis test. Genomic DNA from these samples was tested by the HEA BeadChip™ Kit (Immucor Inc, Norcross, GA) and all three were identified as *JK*02* homozygotes.

However, the screening assay results showed that the mother and one of her sons carried two different mutations and thus were compound heterozygotes for the intron 5 -1g>a mutation and 956C>T in exon 10. The other son was homozygous for intron 5 -1g>a (Figure 3). The three individuals typed *JK*02* by the in-house PCR-ASP.²¹ The pedigree of this unusual family could be constructed to explain the phenotypic and genetic results (Figure 3).

Discussion

With the more widespread use of genotyping for predicting blood group phenotypes, null alleles must be taken in to consideration even if they are uncommon. Different approaches for DNA-based typing permit different levels of inclusiveness. In a more high-throughput automation setting, where space on a chip or bead is not overly restricted then all known null mutations can be included. On the other hand, in a manual setting, cost effectiveness of any assay lies in the relative frequency of a null mutation in a given population or in the expected cohort of referred samples. Based on this, the PCR-ASP described was designed to include *JK*0* mutations that had occurred in two or more unrelated individuals and to use it as a primary screening tool in the investigation of cases with a suspected Jk(a-b-) phenotype. Thus, only samples that are unresolved by the screening assay will be subjected to costly and labor-intensive DNA sequencing analysis of all eleven exons in the *JK* gene.

Since these samples are uncommon even in a major reference laboratory, a simple one-mutation-in-one-PCR-tube approach was chosen and no attempt to multiplex the assay was performed. In multiplex assays, competition for DNA (often of varying or unknown quality when referred from another laboratory) can lessen the robustness of the individual allele-specific PCR reactions. We have tried this approach previously but found that the reproducibility of the assay was poor when performed infrequently.²¹ However, our current approach is balanced by the advantage that novel SNPs of sufficient frequency are easily incorporated in to the assay. As a matter of fact, the two CE-marked

products approved for blood group diagnostic use in Europe include either no *JK*0* markers (BAGene, BAG Health Care GmbH) or detection only of the Finnish and Polynesian variants (BLOODChip⁺, Progenika Biopharma SA). A third commercial blood group genotyping product (BeadChipTM, Immucor, Inc.) not yet approved for diagnostic use also includes detection of *JK*01/JK*02* only.

We were fortunate enough to be able to test the PCR-ASP assay by analysing samples from an especially interesting family: a mother and two sons all of whom carried the Jk(a-b-) phenotype. Somewhat expected from the ethnic background, we identified the Polynesian SNP (intron 5 -1g>a) on a *JK*02*-allele (*JK*02N*) in all three individuals. One of the sons was homozygous for this SNP, thus the father (not included in this investigation) must at least be heterozygous. The intron 5 -1g>a mutation has the highest population frequency and has previously been described in the overall Asian area in which Guam is situated.^{19,23} The other allele, 956C>T, detected in samples from the mother and the other son was more surprising since this SNP has been described previously in combination with a *JK*02* allele in two samples only which originated from the Indian subcontinent.¹⁸ However, null genes are under-diagnosed in general and especially so in populations in which more sophisticated DNA techniques are not in common practice. We anticipate from our studies and from screening studies being performed in other laboratories (Connie Westhoff, personal communication) that the latter mutation is more common than we first thought. This highlights the importance of understanding the genetic variation within a regional population under study, and shows that it can be worthwhile to incorporate null mutations into any strategy for genotype/phenotype testing.

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Figure legends

Figure 1. Cartoon of an RBC bearing the erythrocyte urea transporter framed by the *JK* gene of 11 exons. The coding exons 4-11 are shown in dark grey (white text) and the non coding part, exons 1-3 are light grey (black text). *JK*O* mutations, included in the assay, are indicated on the gene as well as the polymorphism encoding Jk^a and Jk^b.

Figure 2. Representative PCRs showing the A) specific *JK*O* and B) consensus bands for the five selected mutations. The Φ X174 *Hae*III DNA ladder (Advanced Biotechnologies Ltd, UK) was used as a size marker. P indicates a positive reaction and N a negative. In every set a PCR-reaction with H₂O instead of DNA was run in parallel to ensure that the PCR reagents were not contaminated.

Figure 3. The family pedigree together with the results of the PCR-ASP performed on samples from a Jk(a-b-) mother and her two Jk(a-b-) sons. The ASP-PCR kit is arranged as follows: lane 1: exon 4/ Δ exon 4+5; lane 2: intron 5 -1g/a; lane 3: 582C/G; lane 4: 871T/C; lane 5: 956C/T. Bands amplified by allele-specific primers corresponding to intron 5 -1g/a and 956C/ T are indicated by the arrows. An internal control is run in parallel in all PCR reactions and the Φ X174 *Hae*III DNA ladder (Advanced Biotechnologies Ltd, UK) was used as a size marker.

The pedigree shows the intron 5 -1a allele in black and the 956T allele in grey. White indicates that the allele is unknown. Samples from the boys' father were not tested (n.t.) but one allele is presumed to carry the intron 5 -1a mutation.

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Table 1. Primers used in the *JK*0* assay

A. Primers for detection of the mutations

PCR target	Primer name	Nucleotide sequence (5' → 3')	Amplicon size (bp)
Δexons 4+5	JK-i3-F2	AGTCTTCTCAACTAGACTGAAC	252
	JK-i5-R10	CCCTCCCTAAAGGAACTGGC	
Intron 5 -1a	JK-i5-F2b	CTGTGTCTCTTGCCCCACAA	149
	JK-470-R13	CAAGTCATGGACATAGCACA	
582G	JK-582G-F	CTTTCAGCCACAGGACATTAGA	274
	JK-i7-RI	GACCCTAATGCTCTGGAGTAG	
871C	JK-780-F3	CATGCTGCCATAGGATCATTGC	332
	JK-871C-R	TTGCAATGCAGGCCAGAGG	
956T	JK-in9-F	ACAGAGCCCATGGAGCTCC	211
	JK-956T-R2	CCGACTCCAAGATAGGCCA	

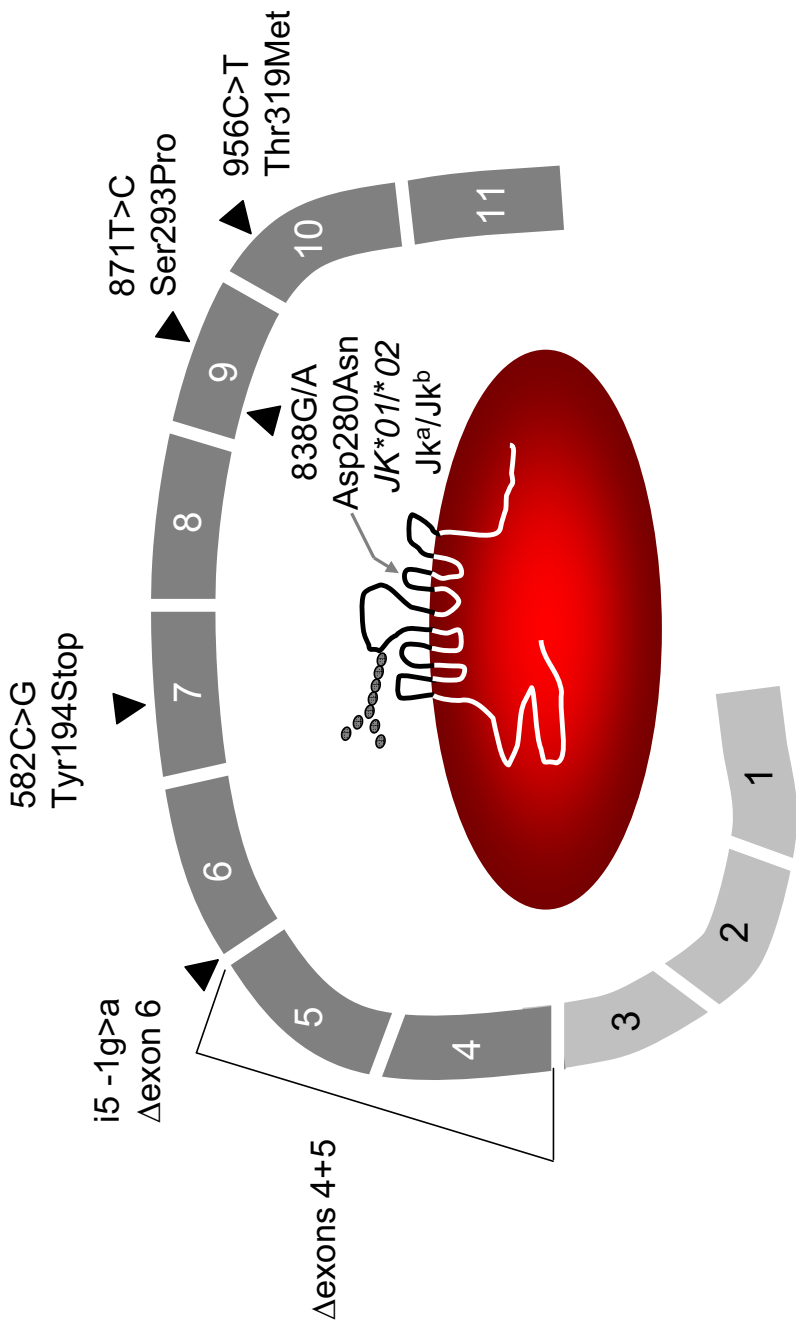
B. Primers for the detection of consensus nucleotides

PCR target	Primer name	Nucleotide sequence (5' → 3')	Amplicon size (bp)
Exon 4	JK-i3-F2	AGTCTTCTCAACTAGACTGAAC	534
	JK-i4R	CCAAAGCTACTTCTCCTGTC	
Intron 5 -1g	JK-i5-F1	TGTGTCTCTTGCCCCACAG	148
	JK-470-R13	CAAGTCATGGACATAGCACA	
582C	Jk-582C-F	CTTTCAGCCACAGGACATTAC	271
	JK-i7-RII	TTAGACCCTAATGCTCTGGAG	
871T	JK-780-F3	CATGCTGCCATAGGATCATTGC	333
	JK-871T-Rmis2	ATTGCAATGCAGGCCA <u>T</u> AGA	
956C	JK-in9-F	ACAGAGCCCATGGAGCTCC	211
	JK-956C-Rmis	CCGACTCCAAGATAGG <u>A</u> CG	

Mismatched nucleotides are underlined.

C. Control primers

	Primer name	Nucleotide sequence (5' → 3')	Amplicon size (bp)
	HGH-F	TGCCTTCCCAACCATTCCCTTA	434
	HGH-R	CCACTCACGGATTTCTGTTGTGTTTC	



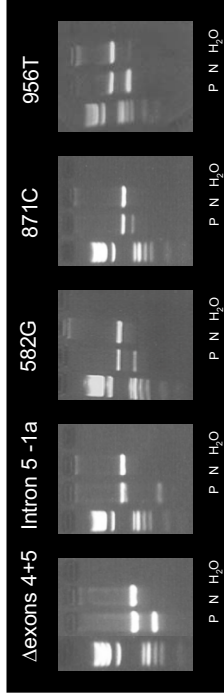
Mw
(bp)

1353
1078
872
603

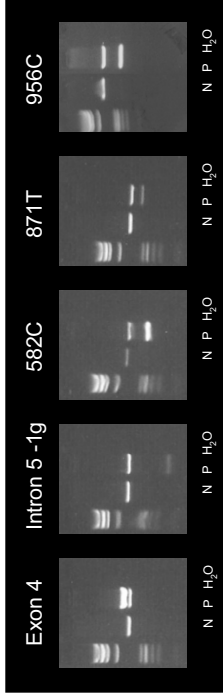
310
271/281
234
194

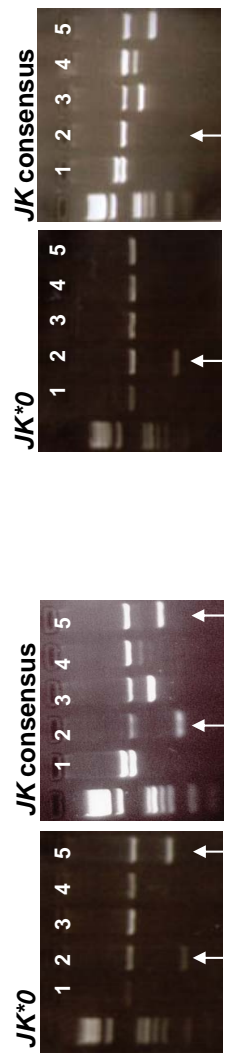
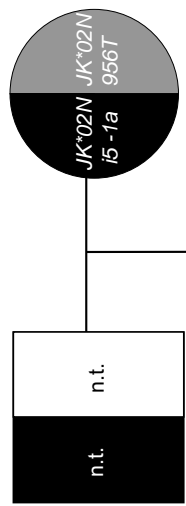
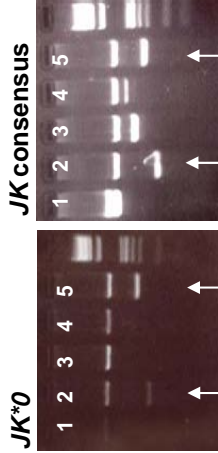
118
72

A: JK*0 PCR



B: Consensus PCR





Paper IV

***KEL*02* alleles with alterations in and around exon 8 in individuals with apparent *KEL:1,-2* phenotypes**

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Part of this study has previously been reported in abstract form [1,2].

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Running head: Novel *KEL*02* alleles cause mistyping

Key Words: Blood group antigen, erythrocyte, Kell, K₀, weak expression, genotype.

Abstract

Background and Objectives: Antibodies to antigens in the Kell blood group system, especially anti-KEL1, are involved in both haemolytic disease of the newborn and foetus and haemolytic transfusion reactions. Correct typing results are important and discrepancies between serologic and genetic typing must be resolved. Here, we describe the investigation of three healthy individuals who were initially phenotyped as KEL:1,-2.

Materials and Methods: Antigen typing was performed by standard serological techniques and by flow cytometric analysis. The *KEL*01/02* polymorphism was tested by an allele-discrimination TaqMan assay as well as by PCR-ASP and PCR-RFLP. DNA sequencing of the *KEL* coding region was also performed.

Results: Two *KEL*02N* alleles with mutated splice sites around exon 8 were identified: intron 7 – 1g>c (novel) and intron 8 +1g>t (previously reported in one case of K_n). In the third sample, a missense mutation in exon 8, 787G>A (novel) predicting Gly263Arg, was detected on a *KEL*02* allele, and associated with dramatically weakened KEL2 antigen expression.

Conclusion: Resolution of discrepant phenotype/genotype results identified silencing mutations in or around exon 8. A combination of molecular and serologic methods has the potential to improve the quality of test results and was required to ensure both the accurate KEL2 antigen status and *KEL*01* zygosity of these individuals.

Introduction

The *KEL* gene, situated on chromosome 7q33, encompasses 19 exons distributed over ~21.5 kb of genomic DNA [3]. It encodes Kell (CD238), a 93 kDa type II transmembrane glycoprotein of 732 amino acids that carries the currently recognised 31 KEL blood group antigens, including KEL1 (K) and KEL2 (k) within the Kell system (ISBT 006; symbol KEL) [4]. In addition to RBCs, the Kell glycoprotein is also expressed in testis and skeletal muscles [5]. All KEL antigens are encoded by missense single nucleotide polymorphisms (SNPs) that lead to amino acid substitutions. The majority of KEL antigens are either of very high or low prevalence, and only infrequently cause major clinical problems. However, KEL1 antigen is highly immunogenic and the resulting anti-KEL1 is clinically significant since it may cause haemolytic transfusion reactions (HTR) as well as haemolytic disease of the foetus and newborn (HDFN). In Caucasians the KEL1 antigen has a prevalence of approximately 9% and the KEL:1,-2 (K+k-) phenotype is rare, with a prevalence of about 0.2% [6,7]. The 578C/T polymorphism in exon 6 results in either Thr193 (KEL2) or Met193 (KEL1) in the Kell glycoprotein [8].

The Kell glycoprotein is part of the M13 zinc endopeptidase family. It cleaves the inactive form of endothelin-3 (b-ET3) to active endothelin-3, a potent blood vessel constrictor [9]. While function has been demonstrated readily *in vitro* both by recombinant Kell glycoprotein and by red blood cells (RBCs) of known KEL phenotypes [10,11], the *in vivo* role of the enzyme on the RBC surface and possible differences between phenotypes remain unclear. Antibodies to KEL blood group antigens mostly consist of IgG and thus readily pass the placental barrier, and are the third most common cause of HDFN after RhD and ABO antibodies. During pregnancy these antibodies, especially anti-KEL1, may cause suppression of foetal erythropoiesis as well as hemolysis [12,13]. Kell glycoprotein was shown by *in vitro* models to be the earliest blood group molecule expressed during erythropoiesis [14-16]. This may explain why affected KEL1-alloimmunized foetuses have lower numbers of circulating pre-mature RBCs compared to foetuses with haemolytic disease due to other antibodies such as anti-D. The KEL1 antigen is also readily immunogenic in a transfusion setting, thus all blood donors in our region are phenotyped for KEL1, and KEL:-1 blood is selected for all female transfusion recipients under 50 years old. Based on this practice, it is important that blood donors are typed correctly and that reagent RBCs for antibody identification are well characterised, also regarding dosage of KEL1 and KEL2, i.e. zygosity for *KEL*01* and *KEL*02*. It is also important to accurately type individuals to determine if a medical intervention should be undertaken or not. One such scenario is the phenotyping of a prospective father to predict the risk of KEL1-related HDFN of a current or future foetus to an immunized mother since homozygosity in the father for an antigen to which a pregnant woman is immunized would prompt careful monitoring of the obligate heterozygous at-risk foetus.

The rare K_0 and K_{mod} phenotypes are generally found by serological testing, either as the absence or very weak expression of common KEL antigens, and/or by the identification of anti-KEL5 (anti-Ku) in the tested person's plasma that reacts with all RBCs except K_0 cells. To date, there are multiple different *KEL*0* alleles described, all of which are carried on a *KEL*02* allelic backbone [17-22] summarized in Blood Group Antigen Gene Mutation Database (dbRBC) [23]. The K_{mod} phenotype has been attributed to many different altered alleles of which the majority are modified *KEL*02* alleles [21,24]. *KEL*01* alleles that give rise to altered KEL1 expression have been reported with SNPs 577T>A (Thr193Ser), 578C>G (Thr193Arg) respectively which both affect the N-glycosylation site Asn191 [25,26]. Thus, it is important to consider these alleles when predicting the phenotype from *KEL*01/02* genotyping results.

The purpose of this study was to investigate the genetic background in three samples from healthy individuals with discrepant pheno- and genotyping results generated from standard serology and initial SNP-derived DNA testing.

Material and Methods

Blood samples

After informed consent, blood was collected by venipuncture in EDTA, acid citrate dextrose (ACD) or citrate dextrose phosphate-adenine (CPD-A1) anticoagulants and separated into RBCs for serologic analysis and leucocytes for nucleic acid extraction. The samples were divided into three groups:

i) Eleven Danish blood donors who were included in a study to confirm apparently KEL:1,-2 blood donors by *KEL*01/02* genotyping. The index case from this cohort is referred to as sample 1. ii) A Danish KEL:1,-2 test reagent donor (sample 2), whose *KEL*01* homozygosity was to be confirmed. Samples from the donor's family (her parents and sister) were also available for testing. iii) A case of allo-immunization during pregnancy originating from the Netherlands where the KEL:1,-2 prospective father (sample 3), and partner to a woman with anti-KEL1, was typed as part of the diagnostic work-up.

RBCs and DNA from our in-house test reagent panel were included as controls.

KEL1 and KEL2 expression by standard serology

Standard serological techniques for phenotyping in tubes and gel cards were used. CE-marked mono- and polyclonal anti-KEL1 and anti-KEL2 reagents from a number of commercial manufacturers were used by the referring laboratories and testing was performed according to the manufacturers' instructions. Adsorption using polyclonal anti-KEL2 followed by acid elution was performed on RBCs from three index samples.

KEL1, KEL2 and Kell glycoprotein expression by flow cytometric analysis

To further evaluate antigen expression of KEL1, KEL2 and Kell glycoprotein, RBCs from the blood donor and test reagent donor were analysed by flow cytometry. Anti-KEL1 (human monoclonal IgM lot 407000 Diagast, Cedex, France) and anti-KEL2 (human IgG, lotnr.kC56A-2, Immucor Norcross, GA, USA) were used as primary antibodies with a phycoerythrin (PE)-conjugated rabbit anti-human immunoglobulin (Dako, Glostrup, Denmark) selected as the secondary antibody. Anti-Kell, a murine monoclonal (MIMA-24 New York Blood Center, USA) was used in combination with a phycoerythrin (PE)-conjugated rat-anti-mouse Ig kappa light chain reagent (Becton Dickinson, CA, USA). Approximately 500,000 washed RBCs were added to 50 µL of PBS in a microtitre plate. Cells were fixed for 10 minutes by the addition of 100 µL of 0.1% glutaraldehyde to reduce agglutination of antigen-positive cells. Incubation was performed at room temperature under constant mixing. The plate was then centrifuged at 300 x g for 1 minute and the supernatant was discarded. Fifty µL of PBS and 5 µL of primary antibody were added to each well, incubated for 10 minutes at room temperature under constant mixing, and then washed twice with 150 µL of PBS. Another 50 µL PBS and 5 µL of secondary antibody were added, incubated for 10 minutes in the dark at room temperature under constant mixing, and washed twice with 150 µL of PBS. Finally, the RBCs were resuspended in 300 µL of PBS and analysed on a flow cytometer (FACScan, Becton Dickinson) using the sheath fluid recommended by the manufacturer (FACSflow, BD Biosciences, Erembodegem-Aalst, Belgium). From a sample volume of 300 µL, 10,000 events were collected at a flow rate of 60 µL/minute. Control RBCs of known KEL phenotypes were included in each run.

Real-time PCR for testing the *KEL*01/02* polymorphism

For samples 1 and 2 genomic DNA was extracted from whole blood using the Maxwell[®] 16 System Blood DNA Purification Kit (Promega, Madison, WI, USA). Genotyping for the 578C>T substitution (SNP data base rs2293266), was performed by real-time polymerase chain reaction (rt-PCR) using TaqMan 2*PCR Master mix (Applied Biosystems, Foster City, CA, USA). DNA amplifications were carried out in 25 µL polymerase chain reactions (PCR) containing 20 ng DNA, 0,9 µM primers (Reverse primer 5'- GGAAATGGCCATACTGACTCATCA and forward primer 5'- GCATCTCTGGTAAATGGACTTCCTT) and 0,2 µM probes (the labeled oligonucleotides: VIC[®] 5'- CTCAGCGTTCGGTTAA and FAM[™] 5'-TCTCAGCATTCGGTTAA). Amplification was performed in 96-well plates, with the following protocol on a GeneAmp PCR 9700 (Applied

Biosystems): 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The genotype was determined by measuring the end-point fluorescence on a 7900 HT Sequence Detection System (Applied Biosystems) using the SDS version 2.3 software.

Sample 3 was recognized by a Custom TaqMan SNP Genotyping Assay for *KEL*01/02* allele discrimination (KELL-A article no. 4332072 from Applied Biosystems) performed with genomic DNA under the conditions specified by the manufacturer.

PCR-ASP and PCR-RFLP for confirmation of the *KEL*01/02* polymorphism and sequencing of the *KEL* gene

Genomic DNA was prepared as above and diluted in H₂O to 100 ng/μl. The *KEL*01/02* genotypes of all samples were confirmed by PCR with allele-specific primers (PCR-ASP) [27,28] and PCR with restriction fragment-length polymorphism (PCR-RFLP) [8].

Samples with discordant pheno- and genotyping results (sample 1, 2 and 3) were further investigated by sequencing of the *KEL* gene; exons 1 - 19 including the intron borders as previously described [18,20,29]. As a reference sequence, the *KEL*02* allele with GenBank accession no. M64934 was used. According to current practice, nucleotide 1 indicates the first nucleotide of the translation-initiating start codon [30]. In the case of the *KEL* gene this is located 120 bp downstream of the first nucleotide in exon 1. This is also the reason why the *KEL1* vs. *KEL2*-differentiating SNP (now 578T/C) was written as 698T/C in early reports.

Terminology

We have used the officially accepted blood group terminology with addition of the current proposals under consideration by the ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology (<http://ibgri.blood.co.uk/>). According to this proposal, silenced *KEL*01* and *KEL*02* alleles are named *KEL*01N* or *KEL*02N*, respectively, and *KEL*01M* or *KEL*02M* used for alleles causing an altered expression.

Results

***KEL*01/02* genotyping**

Using a real time allelic discrimination assay, ten of eleven Danish blood donors typed as *KEL*01* homozygotes as expected. Surprisingly, the eleventh blood donor (sample 1) was heterozygous for *KEL*01* and *KEL*02* as were the Danish reagent test RBC donor (sample 2) and the Dutch prospective father (sample 3). Based on the allelic discrimination results, these three samples were further tested by PCR-RFLP and PCR-ASP and heterozygosity for the *KEL*01/02* polymorphism, 578T/C, in exon 6 was confirmed. In parallel to our investigation of sample 3, the foetus was typed with DNA from amniotic fluid as homozygous for the *KEL*02* SNP.

Blood group serology

Samples 1, 2 and 3 typed KEL:1,-2 by standard serology. Following the results of the genomic analysis, adsorption/elution with anti-KEL2 was performed on these three discrepant samples. Weak expression of KEL2 antigen was demonstrated on the RBCs of sample 1 whilst those of the samples 2 and 3 indicated no KEL2 expression. RBCs from the sister and father of sample 2 typed KEL:-1,2 but her mother's RBCs typed KEL:1,2.

Flow cytometric analysis of epitopes on Kell glycoprotein

Expression of KEL1/KEL2 antigens and Kell glycoprotein was measured by flow cytometry. As expected, RBCs from KEL:1,2 samples expressed less KEL1 and KEL2 than homozygous control RBCs with the phenotypes KEL:1,-2 and KEL:-1,2, respectively. Conversely, RBCs from the two Danish apparently KEL:1,-2 samples (samples 1 and 2) had a level of KEL1 antigen expression closer to the *KEL*01*-homozygous KEL:1,-2 control RBC donor than *KEL*01*-heterozygous KEL:1,2 RBC donors (Figure 1A). However, analysis with a monoclonal antibody recognizing a KEL1/KEL2-

independent epitope demonstrated lower expression of the Kell glycoprotein in the index samples than five of six control RBCs of the common phenotypes (Figure 1B). Notably, one of the *KEL*01*-homozygous donors used as a control, had a total Kell glycoprotein expression similar to that found in the two index samples tested.

Sequencing of *KEL*

The discrepant genotyping results prompted further investigation of the apparently silenced allele(s) in the three index samples. The *KEL* coding region including the exon-intron borders were sequenced. Three different single nucleotide mutations in and around exon 8 were identified (Figure 2): In DNA from the Danish blood donor (sample 1), heterozygosity for a novel mutation, 787G>A was identified, giving rise to an amino acid substitution at position 263 (Gly263Arg) (GenBank accession no. EU362929) carried on an exon 8 otherwise consensus sequence. The two remaining samples were heterozygous for mutations of the invariant splice-site motifs in the introns flanking exon 8. In DNA from the Danish reagent donor (sample 2) we identified heterozygosity for intron 8 +1g>t, and reported it as a novel mutation in 2006 [1]. This mutation was also reported in 2007 to be present in compound heterozygous form in a K_0 sample originating from Germany [21]. Exon 8 including approximately 25 nucleotides of surrounding introns were sequenced in samples from the reagent donor's family as well, and by this we could follow the inheritance of this *KEL*02N* allele from father to daughter (Figure 3). Sequence analysis of DNA from sample 3 identified heterozygosity for intron 7 -1g>c, which is another novel mutation (GenBank accession no. bankit 1316809). Results from analysing the three index samples by blood group serology and sequencing of *KEL* are summarised in Table 1.

Discussion

We investigated discordant phenotype/genotype results on samples originating from three healthy individuals who typed *KEL*:1,-2 by routine serology. Our investigation revealed three different mutated *KEL*02* alleles, two of which were apparently *KEL*02N* alleles and the third was a *KEL*02M* that resulted in very weak expression of the *KEL2* antigen detected by adsorption/elution only. The monoclonal anti-*KEL2* used for flow cytometry has been optimized mainly for serological routine methods but it is possible that other reagents not tested here could have given a weakly positive signal. However, neither flow cytometry nor standard haemagglutination was able to detect the *KEL2* antigen demonstrated by adsorption/elution in this case. These types of samples, referred to as *KEL2el* by Körmöczy *et al.* [21] have historically been termed K_{mod} by most workers in the field. The novel missense mutation identified in this donor, 787G>A, is located in exon 8. Interestingly, the SNP responsible for the *KEL3* (Kp^a) antigen, 841C>A is also located in this exon and encodes a change Arg281Trp [31]. The effect of the *KEL3*-related mutation on decreasing the expression of *KEL2* (and other *KEL* antigens encoded on the same allele) is well known [6]. It has been demonstrated *in vitro* that the Kell glycoprotein carrying the *KEL3* antigen is less expressed at the cell surface due to retention of most of the glycoprotein in a pre-Golgi compartment, and this might well be the explanation in our case [32]. In this sample, the exon 8 sequence showed homozygosity for *KEL*04* so the down-regulation of *KEL2* expression is likely to depend on the substitution at position 263.

The two *KEL*02N* alleles had mutations in the invariant donor and recipient motifs of the splice site regions surrounding exon 8; intron 7 -1g>c and intron 8 +1g>t, respectively. Intron 7 -1c abolishes the 3'-splice site and there are several potential consequences of this mutation such as exon skipping or utilization of a cryptic splice site nearby [33]. Intron 8 +1t disrupts the 5'-splice site and based on evidence in other genes with 5'-splice site mutations at this position, it is likely that exon 8 is skipped in the transcript maturation process [33]. It is highly probable that these splice site mutations are responsible for the absence of the *KEL2* and other antigens normally encoded by this allele. However, skipping of exon 8 would not alter the reading frame or introduce a premature stop codon but result in a shortened sequence that may either be unfavourable at the transcriptional or translational level.

Interestingly, the three alleles described here are all situated in and adjacent to exon 8 which could lead us to speculate on a hot spot area for gene alterations since other nucleotide positions causing null phenotypes and polymorphisms encoding KEL blood group antigens, e.g. KEL3/KEL4/KEL21 (Kp^a/Kp^b/Kp^c); KEL11/KEL17; KEL25/KEL28, can be found in this region; however the list of reported alleles in the Blood Group Antigen Gene Mutation database describes mutations in almost all *KEL* exons and in introns as well [23].

In a blood bank setting investigation of the three types of samples described here is very important: Blood donors are typed to ensure that no antigen-positive blood is transfused to patients with the corresponding antibodies. In other cases, such as to multi-transfused patients and to females of gestational age, the aim may be to prevent antibody formation. In multi-transfused patients, there is good evidence to suggest that by preventing immunisation by RH and KEL antigens, widespread immunisation to other blood group antigens is prevented – a so-called threshold effect [34]. In women of gestational age, it is important to prevent the production of antibodies particularly anti-D and anti-KEL1, both of which may cause serious HDFN. The second sample type is reagent test RBCs which are used for detecting and identifying antibodies in patients' plasma. According to national recommendations, for instance from the Swedish Society of Transfusion Medicine and the Danish Society of Clinical Immunology, reagent RBCs used for the detection of antibodies should have a double dose of the appropriate antigen, i. e. the donors should be homozygous for the allele of interest, with the aim of detecting all antibodies including antibodies of low titre. In some blood groups homozygosity versus heterozygosity may readily be deduced by results from routine serology by a dose effect, but in others genotyping by SNP-testing or sequencing is the only way to obtain a correct answer. We have previously suggested to use blood group genotyping for further characterization of test RBC donors to avoid inclusion of so-called pseudo-homozygous donors, e.g. those typing as C+E-c-e+ or Fy(a+b-) and therefore assumed to be *R¹R¹* and *FY*1/1* while in reality having the genotypes *R¹r¹* and *FY*1/X* or *FY*1/0*, respectively [35]. Conversely, heterozygous donors are sought when using test RBCs to establish antibody titre in the plasma from a pregnant woman, thus reflecting the antigen profile of the foetus. However, all this is based on the idea that donors homozygous for a certain allele will indeed express more of the corresponding antigen. For the above-mentioned examples and also others including the clinically important Jk^a and Jk^b antigens, there are studies to support this, but notably, the flow cytometric data presented here suggest that pseudo-homozygosity for the *KEL*01* allele (e.g. heterozygosity for *KEL*01* and *KEL*02N*, as exemplified in this study) may actually lead to a KEL1 antigen site density on RBCs that is closer to that found in *KEL*01* homozygous samples than what is observed in *KEL*01/02* heterozygotes (Figure 1). Although this interesting finding needs confirmation by other investigators using different monoclonal anti-KEL1 reagents, similar indications were presented by Körmöczi *et al.* [21]. However, real *KEL*01* homozygotes may still be preferable as test RBC donors since their KEL1 antigen expression appears to be marginally higher and the issue of weak KEL2 expression will not have to be taken into account (e.g. by adsorption/elution studies).

It should be noted that the flow cytometric data with the monoclonal antibody recognizing an KEL1/KEL2-independent epitope on the Kell glycoprotein gave rise to an unexpectedly low antigen site density in one of the apparently *KEL*01*-homozygous control samples (Figure 1B). Although this sample typed as KEL:1,-2 and genotyped as *KEL*01/01* by real-time PCR for the polymorphism at nt. 578, we cannot completely exclude that one of its alleles is altered so that lower than normal Kell glycoprotein levels are expressed. Apart from this outlier, these data suggest that a double dose of unaltered Kell glycoprotein results in the highest expression levels, as expected.

The third sample type of interest involves parental and foetal testing in pregnancies at risk for HDFN. When using prenatal genotyping to predict the blood type of a foetus, samples are often obtained from both parents to compare the results. In alloimmunization during pregnancy it is of special interest to investigate if the foetus is at risk of being affected by the antibody. In the case described in this study, the foetus has only a 50% instead of 100% risk to be affected since the prospective father, as shown by our investigation, carries only one functional *KEL*01* allele instead of two as predicted by phenotype. When comparing phenotype with genotype, a real-time assay may be considered good laboratory economy, since it is relatively fast and enables a medium-throughput testing. Samples with discordant results can be tested by more laborious DNA testing such as PCR-ASP or sequencing.

Today, the considerable heterogeneity of genetic bases underlying K_0 and K_{mod} phenotypes [21,36] make it impossible to control for with methods other than DNA sequencing. Looking at a future perspective however, a large number of genetic alterations resulting in silenced or modified expression of the Kell glycoprotein may be detectable in microarray or other platforms for high-throughput screening purposes.

In summary, the three variant *KEL* alleles described in this paper features the *KEL**02-specific 598T polymorphism in combination with alterations in or around exon 8. They would not have been found without the use of both serology and genotyping and highlight the potential consequences of inaccurate prediction of phenotype by genotyping methods alone in different situations in clinical transfusion medicine.

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Tables**Table 1.** Summary of the apparent phenotypes and genotypes of samples 1 - 3.

Sample no	Gender	Ethnicity /geography	Phenotype	KEL*01/KEL*02 578T/578C	Genetic alteration	Genotype
1	Male	Danish	KEL:1,-2 [†]	T/C	787G>A	<i>KEL*01/2M</i>
2	Female	Danish	KEL:1,-2	T/C	intron 8 +1g>t	<i>KEL*01/02N</i>
3	Male	The Netherlands	KEL:1,-2	T/C	intron 7 -1g>c	<i>KEL*01/2N</i>

[†]KEL2 antigen subsequently detected by adsorption/elution only.

Figure legends

Figure 1.

Flow cytometric analysis of Danish samples

(A) Anti-KEL1 and anti-KEL2 were used for flow cytometric analysis of control samples, blood donor (sample 1) and the test reagent donor (sample 2) with family. KEL1 (grey bars) and KEL2 (white bars) represent antigen expression by mean fluorescent intensity (MFI) and the index samples are indicated by arrows. Phenotypes and genotypes are given under the graph.

(B) Flow cytometric analysis with anti-Kell. The Kell glycoprotein expression on RBCs from the two Danish donors (sample 1 and 2) and common and rare control RBCs of the phenotypes indicated is shown. The MFI obtained with K₀ RBCs was used as the background value and subtracted from all results. The highest MFI was set to 100% and the other expression values are shown as percentages of the maximum value.

Figure 2.

Chromatogram visualizing the three identified mutations in and adjacent to exon 8

The upper sequence is the consensus sequence and the lower describes the three identified point mutations: intron 7 -1c in sample 3 originating from Netherlands, 787A in a sample 1, a Danish blood donor and intron 8 +1t from sample 2, a Danish test reagent cell donor. The mutations are all carried on *KEL*02* alleles in combination with a *KEL*01* allele in *trans*.

Figure 3.

Pedigree showing the inheritance of a silent *KEL*02* allele in a Danish sample

The pedigree shows four members of sample 2, the test reagent donor, and inheritance of *KEL*02N* from father to one of the daughters. Black indicates a *KEL*02N* allele, grey a *KEL*01* allele and white a *KEL*02* allele.

Figure 1A

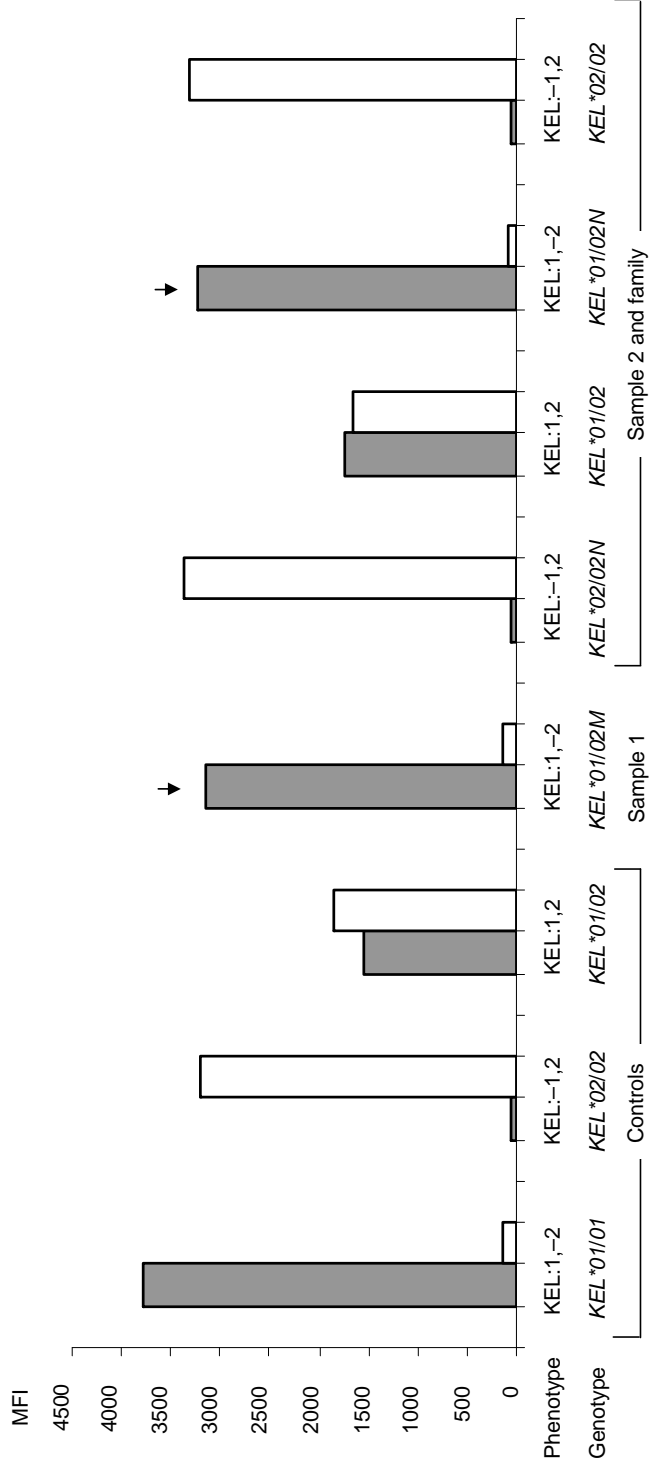


Figure 1B

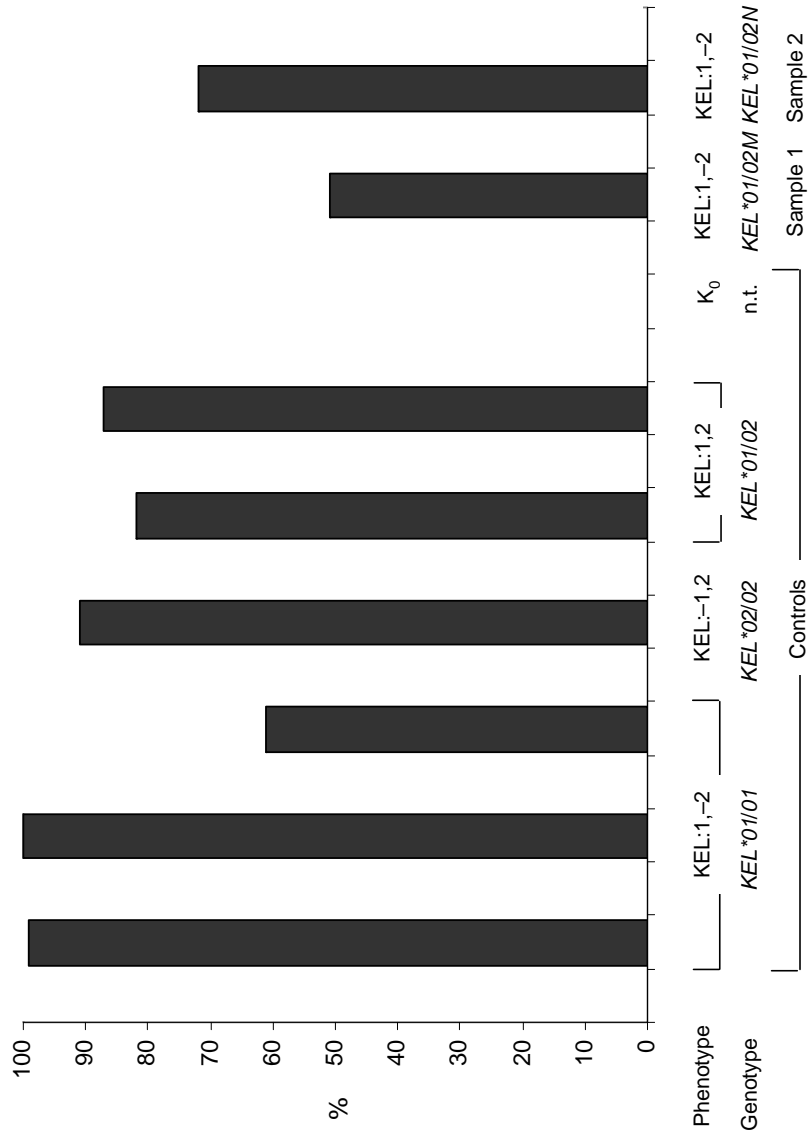


Figure 2

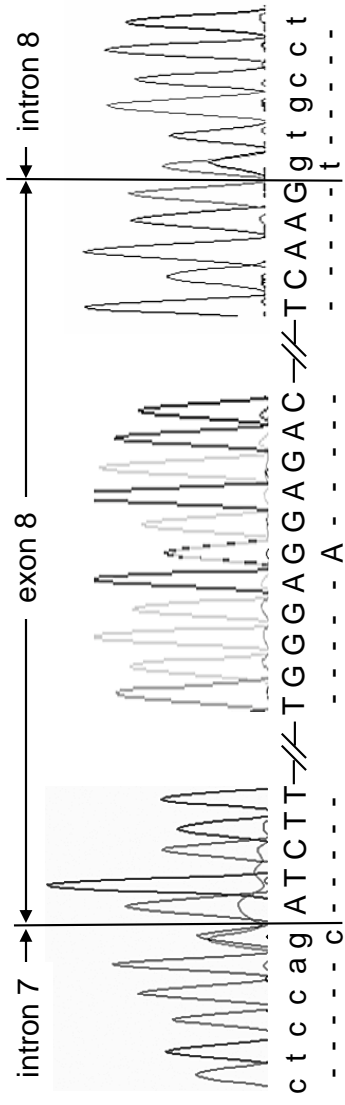
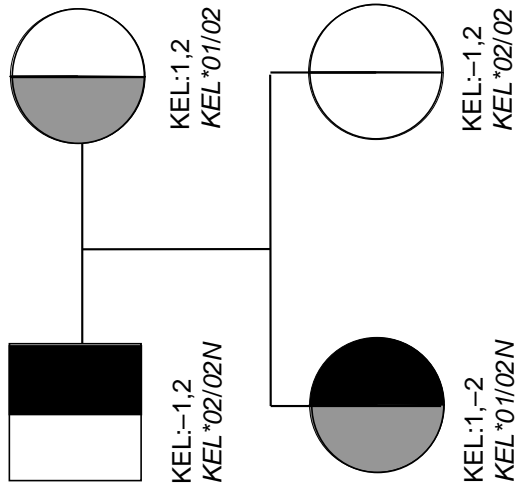


Figure 3



Paper V



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