Genetic diversity of KELnull and KELel: a nationwide Austrian survey

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BACKGROUND: Besides ABO and RH, the KEL blood group system, including the two antithetical antigens KEL1 and KEL2, is the most important owing to the frequent appearance of anti-KEL alloantibodies and their considerable clinical significance. So far, only limited information was available on KEL variant alleles determining the rare silent KELnull and KELel phenotypes with absent or diminished KEL antigen expression detected only by adsorption-elution techniques, respectively.

STUDY DESIGN AND METHODS: For a systematic investigation of the KELnull and KELel phenotypes, 401 KEL:1,-2 samples (representing 2.6% of all Austrian KEL:1,-2 samples) and 811 KEL:1,2 samples were genotyped for the *KEL**1/*KEL**2-specific single-nucleotide polymorphism. All heterozygous *KEL**1/*KEL**2 and 4 additional KELnull samples were subjected to detailed immunohematologic examination and allele-specific sequencing.

RESULTS: In 14 KEL:1,-2 samples, discrepant *KEL**1/*KEL**2 heterozygosity was observed, indicating the presence of silent or barely expressed *KEL**2 alleles, whereas all KEL:1,2 individuals were homozygous for *KEL**2. In the course of further molecular analysis, 8 novel *KEL**2null and 2 *KEL**2el alleles were discovered, representing 67 and 33 percent of previously known *KEL**2null- and *KEL**2el-encoding alleles, respectively. In addition, two different known *KEL**2null and *KEL**2el alleles each were confirmed. The immunohematologic properties of KEL variant red blood cells were defined by extended KEL phenotyping and flow cytometric KEL1, KEL2, KEL4, and KEL7 antigen as well as total Kell protein quantification.

CONCLUSION: For the first time, exact KELnull and KELel population frequencies could be established in this population.

The human red cell (RBC) membrane Kell glycoprotein (CD238 [MIM110900]) is a proteolytic enzyme encoded by the 19-exon *KEL* gene on the long arm of chromosome 7 (7q33).[1] It carries the KEL blood group system antigens and consists of 732 amino acids with a 47-amino-acid intracellular N-terminus, a single 20-amino-acid transmembraneous domain, and a 665-amino-acid extracellular C-terminus. The extracellular domain has six putative N-glycosylation sites and 15 extracellular cysteine residues, suggesting extensive protein folding by intramolecular disulfide bonds.[2,3]

KEL is the most important blood group system after ABO and RH due to the fact that all of the frequently occurring KEL-specific antibodies must be considered

ABBREVIATION: PCR-SSP = polymerase chain reaction with sequence-specific priming.

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clinically significant. The substantial immunogenicity of the low-prevalence KEL1 antigen (K, "Kell") and its antithetical KEL2 antigen (k, "Cellano") determine their particular clinical importance. Confrontation with foreign KEL antigens by blood transfusion or pregnancy may induce alloimmunization, with the potential to trigger hemolytic transfusion reactions and severe hemolytic disease of the fetus and newborn.[4-6]

The *KEL**1 and *KEL**2 alleles coding for KEL1 and KEL2 antigens, respectively, differ by only one T-to-C base-pair substitution at coding nucleotide 578. This leads to an amino acid exchange in the Kell glycoprotein, with Met193 or Thr193 determining the KEL1 or KEL2 antigen, respectively. Notably, Thr193 is part of a putative N-glycosylation consensus sequence.[7] Approximately 9 percent of investigated English blood donors are positive for KEL1 and the resulting allele frequencies of *KEL**1 and *KEL**2 are 0.0462 and 0.9538, respectively.[8]

Individuals of KELnull ("K_0") phenotype with a reported frequency of approximately 1:15,000 to 1:25,000 individuals not only lack any Kell enzyme–related biochemical activity but are also devoid of any KEL antigen traceability.[3] To avoid anti-KEL formation, such individuals are preferably transfused with KELnull blood, which is a challenging situation with respect to the availability of matched blood units. So far a total of 12 different *KEL**2null alleles have been described, which were found in homozygous or compound heterozygous combinations in individuals typed KELnull by serologic methods. All of these alleles were caused by single-point mutations of the *KEL**2 allele, leading to the formation of translational stop signals in eight cases and in two cases each to altered splice sites and apparently destructive amino acid exchanges. The splice site mutant allele *KEL**2(IVS3+1G>A)null, the nonsense mutant allele *KEL**2(R128X)null only found among African American people, and the *KEL**2(S363N)null allele predicting an apparently destructive amino acid exchange were observed 17, 4, and 2 times, respectively, whereas all other *KEL**2null alleles were single observations. These data, however, are derived from known KELnull individuals, whose frequency in an average population was not satisfactorily established so far.[9-12]

More recently, the molecular basis for the KELmod ("K_{mod}") phenotype was described in which KEL antigens are expressed very weakly.[13] If adsorption-elution techniques are necessary for serologic KEL antigen detection, such phenotypes should preferably be referred to as "KELel" in analogy to RH nomenclature.[14] Therefore, the latter term is used throughout this article. Several different alleles contribute to this phenotype when inherited in a homozygous or compound heterozygous manner, generally caused by single *KEL**2 point mutations. Interestingly, three of the four known *KEL**2el alleles predict Kell protein amino acid exchanges, whereas one represents a nonsense point mutation.[13] The scarce frequency data for the KELel phenotype suggest that the causing alleles are not uncommon.[3]

In this study, the frequency of KELnull and KELel phenotypes in the Austrian population and their heterogenous genetic background were investigated. We studied 401 samples phenotyped KEL:1,-2 (K+k–) by serology from three representative Austrian areas. *KEL**1/*KEL**2 genotyping revealed a total of 14 seemingly *KEL**1/*KEL**2 heterozygous samples, which featured either absent or markedly depressed KEL2 antigen expression. KEL2 antigen status in these samples was determined by direct hemagglutination and, in case of negativity, confirmed by adsorption-elution procedures. Additionally, four samples of known KELnull phenotype were investigated. In this most comprehensive study on Kell variant genetics so far, eight novel *KEL**2null and two novel *KEL**2el alleles were discovered, expanding the total number of known *KEL**2null and *KEL**2el alleles by 56 percent. For the first time, statistically relevant KELnull and KELel frequency data are provided.

MATERIALS AND METHODS

Serologic investigations

In Innsbruck, serologic screening for KEL:1,-2 samples was performed by use of a gel matrix system containing polyclonal anti-KEL1 or anti-KEL2 (microtyping system, DiaMed-ID, Cressier, Switzerland). Screening in Vienna was performed employing an automated analyzer (Model PK-7200, Olympus, Vienna, Austria) with monoclonal anti-KEL1 (MS56, DiaMed), and two different polyclonal anti-KEL2 (DiaMed; Ortho, Neckargemünd, Germany) in the indirect antiglobulin test (IAT) with anti-human globulin–containing gel cards. In Graz, screening was performed with two different polyclonal anti-KEL1 (Immucor, Rödermark, Germany; Diagast, Loos, France) on an Olympus PK-7200 analyzer in a first step; in a second step all KEL:1 samples were tested with a gel matrix system containing polyclonal anti-KEL2 (DiaMed). Subsequently, KEL:1,-2 were confirmed by two different polyclonal anti-KEL2 (DiaMed; Biotest, Dreieich, Germany) in IAT with anti-human globulin–containing gel cards.

In all centers, all samples with discrepant phenotyping-genotyping results were further studied serologically with the following human polyclonal reagents in IAT in gel matrix (DiaMed): anti-KEL1 and anti-KEL2 (three different serum samples each from Biotest; Immucor and Ortho), anti-KEL3 and anti-KEL4 (two different serum samples each, DiaMed and Immucor), and anti-KEL6 and anti-KEL7 (DiaMed). RBC antibody screening and specification were performed by IAT in gel matrix (DiaMed), as was the direct antiglobulin test with polyspecific antihuman globulin (anti-IgG/-C3d). The presence or absence of minute KEL1 and KEL2 antigen expression was demon-

strated by adsorption of human polyclonal anti-KEL1 or anti-KEL2 reagents (Biotest) for 1 hour at 37°C with equal volumes of washed RBC samples in parallel with negative and positive control RBCs (of KEL:1,-2 and KEL:1,2 phenotype), extensive washing with cold phosphate-buffered saline, and subsequent acid elution (DiaCidel, DiaMed) for further antibody specification.

DNA isolation and polymerase chain reaction amplification procedures

Ethylenediaminetetraacetic acid–anticoagulated blood samples were subjected to DNA isolation with a DNA extraction kit (Nucleon BACC2, Amersham Biosciences, Freiburg, Germany).

Designations, sequences, and employed concentrations of all primers (Microsynth, Balgach, Switzerland) are given in Tables 1 and 2; the corresponding cycling conditions are given below. All polymerase chain reaction (PCR) procedures were performed on an automated thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Brunn am Gebirge, Austria). PCR amplicons were visualized by agarose gel electrophoresis and documented by digital imaging.

The final reaction volume of the *KEL**1/*KEL**2 genotyping PCRs with sequence-specific priming (PCR-SSP) was 10 µL, containing 50 mmol per L KCl, 1.5 mmol per L $MgCl_2$, 10 mmol per L Tris-HCl (pH 8.3), 0.01 percent gelatin, 5.0 percent glycerol, 100 µg per mL cresol red, 200 µmol per L each dNTP, 50 to 100 ng f genomic DNA (UV-quantitated), and 0.4 units of AmpliTaq DNA polymerase (Applied Biosystems). Control primers were added to check for correct negativity in case there were no *KEL*-specific amplification products in the respective reactions.[16]

Products of long-range PCR-SSPs LR-1 to LR-4 (Tables 1 and 2) were used for "allele-separating" DNA sequencing. Such separate amplification of complete *KEL**1 and *KEL**2 alleles from heterozygous genomic DNA was performed applying a long-template PCR system with a template buffer throughout all experiments (Expand and Template Buffer 3, respectively, Roche, Penzberg, Germany). Therefore, the *KEL**1/*KEL**2-discriminating single-nucleotide polymorphism at coding nucleotide 578 in exon 6 of the *KEL* gene (rs8176058) was used. Reaction LR-2 yielded a 5.403-bp *KEL**2-specific amplicon covering the region between the promoter (3′ end of the forward primer binds to nucleotide –950 in front of the A of the start codon ATG) and nucleotide 578, whereas LR-4 resulted in a 16.993-bp *KEL**2-specific PCR fragment encompassing the region from nucleotide 578 to nucleotide 2497 (298 bp after the stop codon TAA in exon 19). Preparative long-range PCRs for DNA sequencing had a total volume of 150 µL and were amplified in three portions of 50 µL each.

KEL gene fragments F-1 to F-6 (Tables 1 and 2) were used for confirmation of mutations found by DNA sequencing of the allele-separating long-range PCRs and the first sequencing round of all KELnull samples. The setup conditions were as described for the *KEL**1/*KEL**2 DNA typing reactions, but with 300 ng of DNA in a total volume of 150 µL and amplified in three portions of 50 µL each.

Mutated *KEL**2 alleles of heterozygous KELnull samples (Berlin [178] and Berlin [179]) were amplified separately for haplotype-specific DNA sequencing in the genomic regions concerned. For analysis of Berlin (179), heterozygous position nucleotide 1 of intron 8 was used to discriminate the two alleles in forward and reverse orientation, respectively. Consequently, PCR fragments Berlin-179-1 and 179-3 (Tables 1 and 2) represented two PCR fragments of the allele *KEL**2(W316X)null, one covering the region from nucleotide 162 in front of the 3′ splice site of intron 6 to nucleotide 1 of intron 8, the second from nucleotide 1 of intron 8 to nucleotide 125 of intron 10. Berlin-179-2 and 4 represented the same regions but of the second allele—*KEL**2(IVS8+1G>T)null—in the respective heterozygous KELnull sample. Both of the *KEL**2null alleles of sample Berlin (178) were sequenced separately from 50 bp in front of the intron 11-exon 12 border to 50 bp after the exon 16-intron 16 border. Analogously, the four PCR fragments Berlin-178-1-4 represented both separated *KEL**2null alleles of the respective KELnull sample; the complete coverage of these PCR fragments ranged from nucleotide 157 in front of the 3′ splice site of intron 11 to nucleotide 94 of exon 17. The setup conditions were as described for PCR reactions F-1 to F-6. Accordingly, both of the *KEL**2null alleles of sample Berlin (179) were sequenced separately from 50 bp in front of the intron 6-exon 7 border to 45 bp after the exon 10-intron 10 border.

DNA sequencing

Of all preparative PCRs described above, 10 µL was visually controlled by agarose gel electrophoresis, the remaining 140 µL were purified for DNA sequencing with a PCR purification kit (QIAquick, Qiagen, Hilden, Germany). DNA sequencing was performed at the Microsynth DNA service facility with dye terminator technology from Applied Biosystems. All sequences were analyzed with computer software (Generunner, Version 3.05, Hastings Software Inc., Hudson, NY; and Chromas, Version 1.41, Conor McCarthy, School of Biomolecular and Biomedical Science, Faculty of Science and Technology, Griffith University, Brisbane, Australia).

Cycling conditions

The cycling conditions for the *KEL**1/*KEL**2 genotyping reactions were initial denaturation step of 120 seconds at

TABLE 1. Primers used

Reaction	Size of amplicons (bp)	*KEL* exons amplified	Primer name	Primer sequence	Concentration (nmol/L)	PCR program
KEL*1	140	Part of 6	KEL+672-allK-F	CGCCAGTGCATCCCTCACC	200	KEL1, KEL2
			KEL+578-KEL1-F	GACTTCCTTAAACTTTAACCGCAT	200	KEL1, KEL2
KEL*2	141	Part of 6	KEL+672-allK-R	CGCCAGTGCATCCCTCACC	250	KEL1, KEL2
			KEL+578-KEL2-F	GGACTTCCTTAAACTTTAACCGCAC	250	KEL1, KEL2
Control	434	NA	HGH-352F	TGCCTTCCCAACCATTCCCTTA	100	KEL1, KEL2
			HGH-739R	CCACTCACGGATTTCTGTTGTGTTTC	100	KEL1, KEL2
LR-1	5403	1-5′part of 6	KEL-Prom-950F	GAGATCCTATGAGGTAGGCATAGGCAAG	300	LR-1, LR-2
			tKEL+578-KEL1-R	GACTCATCAGAAGTCTCAGCA	300	LR-1, LR-2
LR-2	5403	1-5′part of 6	KEL-Prom-950F	GAGATCCTATGAGGTAGGCATAGGCAAG	300	LR-1, LR-2
			tKEL+578-KEL2-R	GACTCATCAGAAGTCTCAGCG	300	LR-1, LR-2
LR-3	16993	3′part of 6, to 19	oKell-578-KEL1-F	GACTTCCTTAAACTTTAACCGAAT	300	LR-3, LR-4
			KEL-i19+276R	GGCCTTCAAACCCACCAGGTCAC	300	LR-3, LR-4
LR-4	16993	3′part of 6, to 19	II Kell-578-KEL2-F	GGACTTCCTTAAACTTTAACCGCAC	300	LR-3, LR-4
			KEL-i19+276R	GGCCTTCAAACCCACCAGGTCAC	300	LR-3, LR-4
F-1	1859	Promoter, 1, 2, 3, 4	KEL-Prom-433F	TAAACCTTTGTCGGTCTGGTGCTCC	300	F-1-6
			KEL-i4+101R	AATCCCACCTGGGATGGTGCAAA	300	F-1-6
F-2	1370	5,6	KEL-i4-149F	CTCGTAATGTTATGCCAGAATCAGGTTAG	300	F-1-6
			KEL-i6-567R	GCAGGCACAGGGAGCAGCATAGGC	300	F-1-6
F-3	2356	7, 8, 9, 10	KEL-i6-162F	TCTGGACTCTTCTTCTCATGCCCCTC	300	F-1-6
			KEL-i10+125R	CAACTTGCCTGCTTCTATGGAAAGCC	300	F-1-6
F-4	943	11	KEL-i10-249F	GATGATGCCTCCTAGAGGCCTTGC	300	F-1-6
			KEL-i11+533R	ATGGAGTTTCACTCTTGTCACCCAGG	300	F-1-6
F-5	2177	12, 13, 14, 15, 16, 17	KEL-i11-157F	CACTGCCTTCCTTTCTCCACAGATCC	300	F-1-6
			KEL-i17+94R	GCGAAGGGCAGAAAGCCATGCAAC	300	F-1-6
F-6	2174	17, 18, 19	KEL-i16-169F	GAACCTAAGGGAAGACCACAAGGGAG	300	F-1-6
			KEL-i19+159R	CCCGTACAGTTAATGACTTCCAGGCATAG	300	F-1-6
Berlin-179-1	550	7, 5′part of 8	KEL-i6-162F	TCTGGACTCTTCTTCTCATGCCCCTC	300	F-1-6
			KEL-s1179-i8+1GR	GGCCCCCAGTTCCAGGCAC	300	F-1-6
Berlin-179-2	550	7, 5′part of 8	KEL-i6-162F	TCTGGACTCTTCTTCTCATGCCCCTC	300	F-1-6
			KEL-s1179-i8+1TR	GGCCCCCAGTTCCAGGCAA	300	F-1-6
Berlin-179-3	1848	3′part of 8, 9, 10	KEL-i10+125R	CAACTTGCCTGCTTCTATGGAAAGCC	300	F-1-6
			KEL-s1179-i8+1GF	TGGTCACTATCGACCAGCTCACGG	300	F-1-6
Berlin-179-4	1848	3′part of 8, 9, 10	KEL-i10+125R	CAACTTGCCTGCTTCTATGGAAAGCC	300	F-1-6
			KEL-s1179-i8+1TF	GATGGTCACTATCGACCAGCTCATGT	300	F-1-6
Berlin-178-1	611	12, 13	KEL-i11-157F	CACTGCCTTCCTTTCTCCACAGATCC	300	F-1-6
			KEL-s1178+1477CR	AGGGACCCACATCGTTGTATTCCTG	300	F-1-6
Berlin-178-2	611	12, 13	KEL-i11-157F	CACTGCCTTCCTTTCTCCACAGATCC	300	F-1-6
			KEL-s1178+1477TR	AGGGACCCACATCGTTGTATTCCTA	300	F-1-6
Berlin-178-3	1600	3′part of 13, 14, 15, 16, 17	KEL-i17+94R	GCGAAGGGCAGAAAGCCATGCAAC	300	F-1-6
			KEL-s1178+1477CF	GAAGCCAGAGCTGGCCCCAC	300	F-1-6
Berlin-178-4	1600	3′part of 13, 14, 15, 16, 17	KEL-i17+94R	GCGAAGGGCAGAAAGCCATGCAAC	300	F-1-6
			KEL-s1178+1477CF	GAAGCCAGAGCTGGCCCCAC	300	F-1-6

TABLE 2. Primers used

SEQ reaction	*KEL* fragments sequenced with	Primer name	Primer sequence	Concentration (nmol/L)	PCR program
1	LR-1, LR-2, F-1	KYPro-212F	CCTGAGAAGCTGAGATAAAG	NA*	NA
2	LR-1, LR-2, F-1	KYe2+4F	TCTCCCTCCACTCACTTCAG	NA	NA
3	LR-1, LR-2, F-1	KYi2-71F	GCACCAACAGATTCATTCTC	NA	NA
4	LR-1, LR-2, F-2	KYi4-81F	AAAACCCATCTGATGAGAAC	NA	NA
5	LR-3, LR-4, F-2	KYi6+483R	AGGAATGTACGGGAGATAAG	NA	NA
6	LR-3, LR-4, F-3, Berlin-179-1, Berlin-179-2	KYi6-91F	TGCTTCTCTTCTGTCCAATC	NA	NA
7	LR-3, LR-4, F-3	KYi8-58F	TCTCACACCCAAGGGGAAGC	NA	NA
8	LR-3, LR-4, F-3, Berlin-179-3, Berlin-179-4	KYi10+74R	CCCTCCCTGAGAGAGAGATG	NA	NA
9	LR-3, LR-4, F-4	KYi10-204F	GAAGGATTTACTCAGCCAGG	NA	NA
10	LR-3, LR-4, F-5, Berlin-178-1, Berlin-178-2	KYi11-109F	TAGCAGCAGCTCCAGCCCAG	NA	NA
11	LR-3, LR-4, F-5, Berlin-178-3, Berlin-178-4	KYi13-97F	TGGATGCCTGCCTGTCAGGG	NA	NA
12	LR-3, LR-4, F-5	KYi14-89F	AGGGCAGGCAGCATGAACAG	NA	NA
13	LR-3, LR-4, F-5	KYi15-47F	GGTTGGAGAATTGGGGTCAC	NA	NA
14	LR-3, LR-4, F-5	KYi16-87F	ATGCTCCTGGGAGCTGATTC	NA	NA
15	LR-3, LR-4, F-6	KYi16-87F	GCCCACTTGACATCACCTCC	NA	NA
16	LR-3, LR-4, F-6	KYi18-317F	GCCCACTTGACATCACCTCC	NA	NA
17	S1179-3, S1179-4	KEL-i9+74R	CTGCCTTCCCCAAGGTTTCC	NA	NA
18	S1179-3, S1179-4	KEL-i9+712R	AATGGGCAGCTACCTCCCTC	NA	NA
19	S1178-7, S1178-8	KEL+1575R	ACCTGTGTTGGGGGTGAGGC	NA	NA
20	S1178-7, S1178-8	KEL-i17-84R	GCCATGCAACTGTACTTGTG	NA	NA

* NA = not applicable.

94°C, 10 cycles for 10 seconds at 94°C and 60 seconds at 65°C, and 20 cycles for 10 seconds at 94°C, 50 seconds at 61°C, and 30 seconds at 72°C. The cycling conditions for the long-range PCR-SSPs LR-1 and LR-2 were initial denaturation step of 120 seconds at 92°C; 10 cycles for 10 seconds at 92°C, 30 seconds at 65°C, and 8 minutes at 68°C; and 25 cycles for 10 seconds at 92°C, 30 seconds at 65°C, and 8 minutes plus an increment of 20 seconds at 68°C.

The cycling conditions for the long-range PCR-SSPs LR-3 and 4 were initial denaturation step of 120 seconds at 92°C; 10 cycles for 10 seconds at 92°C, 30 seconds at 65°C, and 15 minutes at 68°C; and 25 cycles for 10 seconds at 92°C, 30 seconds at 65°C, and 15 minutes plus an increment of 20 seconds at 68°C. All LR reactions had one final elongation cycle for 7 minutes at 68°C. The cycling conditions for the non–allele-separating sequencing strategy reactions F-1 to F-6 were initial denaturation step of 120 seconds at 94°C; 10 cycles for 15 seconds at 94°C, 90 seconds at 65°C, and 90 seconds at 72°C; and 20 cycles for 15 seconds at 94°C, 60 seconds at 61°C, and 120 seconds at 72°C.

Calculation of allele frequencies and statistical methods

*KEL**2null and *KEL**2el allele frequencies were calculated with the data obtained during the study and regionally obtained *KEL**1 and *KEL**2 allele frequencies. *KEL**2 alleles without detectable mutation but completely devoid of KEL2 antigen expression were considered *KEL**2null alleles. Three assumptions were used to establish equations for the calculation of wild-type *KEL**1 and *KEL**2 and cumulative *KEL**2null and *KEL**2el allele frequencies: 1) The observed KEL:1,2 phenotype frequency represented the actual frequency of *KEL**1/*KEL**2 heterozygous individuals. 2) The observed KEL:1,-2 phenotype frequency includes actual *KEL**1 homozygous and "hidden" *KEL**1/*KEL**2null and *KEL**2el heterozygous individuals. 3) The sum of the *KEL**1, *KEL**2, and cumulative *KEL**2null and *KEL**2el allele frequencies equals 100 percent of all *KEL* alleles. Confidence intervals (CIs) were calculated according to the Poisson distribution (cumulative number of rare *KEL**2null and *KEL**2el alleles per participating center).[17] Single *KEL**2null or *KEL**2el allele frequencies were calculated as the cumulative *KEL**2null and *KEL**2el allele frequencies of each participating center divided by their respective sum of *KEL**2null and *KEL**2el alleles observed.

Flow cytometry

RBC surface expression of KEL1, KEL2, KEL4, and KEL7 antigens as well as of total Kell glycoprotein was determined by indirect immunofluorescence and flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany), with the following primary reagents: human monoclonal anti-KEL1 (clone 601, Diagast; donated by K. Göttfert), human polyclonal anti-KEL1 (Immucor), anti-KEL2 (Biotest), anti-KEL4 (Immucor) and anti-KEL7 (DiaMed), and murine monoclonal anti-total Kell glycoprotein (anti-CD238, clone BRIC 68, International Blood Group Reference Laboratory, Bristol, UK). All polyclonal serum samples had been prepared for use by repeat adsorption onto antigen-positive group O RBCs, washing, and subsequent acid elution (DiaCidel, DiaMed). As sec-

TABLE 3. Serologic features of investigated KEL variant RBC samples*

Number	Sample†	IAT with anti-				Adsorption-elution with anti-		KEL1,2 phenotype	*KEL* *1/2 genotype
		KEL1	KEL2	KEL3	KEL4	KEL1	KEL2		
1	Innsbruck (70)	+	–	–	+	ND	–	KEL:1,-2	*KEL* *1/*KEL*2
2	Innsbruck (99)	+	–	–	+	ND	+	KEL:1,2el	*KEL*1/*KEL*2
3	Innsbruck (111)	+	–	–	+	ND	+	KEL:1,2el	*KEL*1/*KEL*2
4	Innsbruck (127)	+	–	–	+	ND	–	KEL:1,-2	*KEL*1/*KEL*2
5	Innsbruck (135)	+	–	–	+	ND	–	KEL:1,-2	*KEL*1/*KEL*2
6	Innsbruck (178)	+	w+	+	+	ND	ND	KEL:1,2weak	*KEL* *1/*KEL*2
7	Graz (25)	+	–/w+	–	+	ND	+	KEL:1,2el	*KEL* *1/*KEL*2
8	Graz (72)	+	–	–	+	ND	–	KEL:1,-2	*KEL*1/*KEL*2
9	Vienna (5)	+	–	–	+	ND	–	KEL:1,-2	*KEL*1/*KEL*2
10	Vienna (27)	+	–/w+	–	+	ND	+	KEL:1,2el	*KEL*1/*KEL*2
11	Vienna (32)	+	–	–	+	ND	–	KEL:1,-2	*KEL*1/*KEL*2
12	Vienna (40)	+	–	–	+	ND	–	KEL:1,-2	*KEL*1/*KEL*2
13	Vienna (89)	+	–	–	+	ND	–	KEL:1,-2	*KEL*1/*KEL*2
14	Vienna (98)	+	–	–	+	ND	–	KEL:1,-2	*KEL*1/*KEL*2
15	Bregenz	–	–	–	–	–	–	KEL:1,-2 (KELnull)	*KEL*2/*KEL*2
16	Salzburg	–	–	–	–	–	–	KEL:1,-2 (KELnull)	*KEL*2/*KEL*2
17	Berlin (178)	–	–	–	–	–	–	KEL:1,-2 (KELnull)	*KEL*2/*KEL*2
18	Berlin (179)	–	–	–	–	–	–	KEL:1,-2 (KELnull)	*KEL*2/*KEL*2

* ND = not determined. + = positive; – = negative; w+ = weak positive.
† Samples 1 through 14 were KEL:6,7; Samples 15 through 18 were KEL:6,-7. Anti-KEL5 (Anti-Ku) was found in Samples 16 through 18, whereas in all other samples no irregular RBC antibodies could be detected.

ondary reagents, R-phycoerythrin-conjugated goat anti-human IgG $F(ab')_2$ (Immunotech, Marseille, France) and R-phycoerythrin-conjugated rabbit anti-mouse IgG $F(ab')_2$ (Dako Cytomation, Glostrup, Denmark) were used.

Absolute numbers of KEL2 antigens per RBC were established with human antibody-binding standards (Quantum Simply Cellular, Bangs Laboratories, Fishers, IN) stained with adsorption-elution–purified polyclonal anti-KEL2 (Biotest) and fluorescein isothiocyanate–labeled Fab fragment goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Absolute KEL2 antigen densities were calculated according to instructions by Bangs Laboratories and required calculational correction for the influence of indirect immunofluorescence. This correction was achieved with values obtained from indirectly anti-D (RH1, CD240D) staining (with human monoclonal anti-D, clone P3×249, Diagast) of RBCs with known absolute D antigen density assayed in parallel.[15]

RESULTS

Presence of *KEL**2 alleles in 401 blood donor samples typed KEL:1,-2 by routine serology

We hypothesized that *KEL**2null and *KEL**2el alleles would much more frequently be encountered as mutants of the frequent wild-type *KEL**2 allele than from the rarer *KEL**1 allele. Therefore, the Austrian blood transfusion centers from Innsbruck, Graz, and Vienna performed a serologic screening for KEL:1,-2 (K+k–) individuals, collecting 177, 123, and 101 samples, respectively. These samples represented 2.55 percent of the expected total number of Austrian KEL:1,-2 individuals.

*KEL**1/*KEL**2 genotyping of these 401 KEL:1,-2 samples revealed discrepant *KEL**1/*KEL**2 heterozygosity in 14 cases (3.5%). Therefore, serologic KEL1/KEL2 antigen typing of these samples was repeated, including KEL2 antigen determination by adsorption-elution technique. Accordingly, 1 case with weak positivity for KEL2 in IAT, 4 cases positive for KEL2 only by adsorption-elution, and 9 cases without any detectable KEL2 antigen expression were identified (Table 3, Samples 1-14). Additionally, all samples were investigated for KEL3/KEL4 (Kp^a/Kp^b) and KEL6/KEL7 (Js^a/Js^b) antigen expression and irregular RBC antibodies (Table 3).

Sequencing of 14 *KEL**2 variant alleles reveals considerable genetic heterogeneity

To elucidate the molecular background of *KEL**2 allele–positive samples with absent or depressed KEL2 antigen expression, *KEL**2 allele–specific sequencing was performed. Because the start codon of a gene is by far more easy to define compared to its transcription initiation site, all mutations identified are given counting A of the start codon ATG as nucleotide 1 and taking accession number BC050639 as reference sequence.

Sample Innsbruck (178), the KEL1-positive sample with weak positivity for KEL2 in IAT, showed a C841T substitution predicting a regular *KEL**2 allele with an Arg281Trp amino acid exchange (allele *KEL**2(R281W)weak, *KEL**3) encoding the KEL3 (Kp^a)

TABLE 4. Novel and known *KEL2null, *KEL**2el, *KEL**2mod, and *KEL**2weak (allele of this study only) alleles***

Number	Nationality	Town or state	Phenotype	Number of alleles observed	Mutation (location)	KEL*2 allele	Reference	Accession
KEL*2null alleles								
1	Taiwan	Taipei	KELnull	2	IVS3+1G>C (i3)	(IVS3+1G>C)null	10	NA
2	Reunion Isl.	Not given	KELnull	14	IVS3+1G>A (i3)	(IVS3+1G>A)null	9	NA
	US	Seattle	KELnull	1	IVS3+1G>A (i3)	(IVS3+1G>A)null	9	NA
	Sweden	Linköping	KELnull	2	IVS3+1G>A (i3)	(IVS3+1G>A)null	12	NA
	Austria	Bregenz	KELnull	2	IVS3+1G>A (i3)	(IVS3+1G>A)null	This study	AM085124
3	Yugoslavia	Not given	KELnull	2	T246A (e4)	(C82X)null	9	NA
4	US (African)	Michigan	KELnull	2	C382T (e4)	(R128X)null	9	NA
	US (African)	North Carolina	KELnull	2	C382T (e4)	(R128X)null	9	NA
5	Japan	Not given	KELnull	1	(IVS5-2 A>G) (i5)	(IVS5-2 A>G)null	11	NA
6	US	New York	KELnull	1	C574T (e6)	(R192X)null	9	NA
7	Sweden	Umea	KELnull	2	903delG (e8)	(903delG)null	12	NA
8	Austria	Salzburg	KELnull	2	(IVS8+1G>A) (i8)	(IVS8+1G>A)null	This study	NA
	Austria	Innsbruck (127)	KEL:1,-2	1	(IVS8+1G>A) (i8)	(IVS8+1G>A)null	This study	AM085117
9	Germany	Berlin (179)	KELnull	1	(IVS8+1G>T) (i8)	(IVS8+1G>T)null	This study	AM085127
10	Germany	Berlin (179)	KELnull	1	G948A (e9)	(W316X)null	This study	AM085128
11	Portugal	Not given	KELnull	2	C1042T (e9)	(Q348X)null	9	NA
	Austria	Vienna (40)	KEL:1,-2	1	C1042T (e9)	(Q348X)null	This study	AM085122
12	US	Seattle	KELnull	1	G1208A (e10)	(S363N)null†	9	NA
	US	New York	KELnull	1	G1208A (e10)	(S363N)null†	9	NA
13	Austria	Innsbruck (70)	KEL:1,-2	1	C1216T (e11)	(R406X)null	This study	AM085114
	Austria	Vienna (98)	KEL:1,-2	1	C1216T (e11)	(R406X)null	This study	NA
14	Japan	Not given	KELnull	1	G1377A (e12)	(W459X)null	11	NA
15	Sweden	Uppsala	KELnull	2	C1420T (e13)	(Q474X)null	12	NA
16	Germany	Berlin (178)	KELnull	1	C1477T (e13)	(Q493X)null	This study	AM085125
17	Germany	Berlin (178)	KELnull	1	C1546T (e14)	(R516X)null	This study	AM085126
18	Austria	Vienna (5)	KEL:1,-2	1	C1678G (e15)	(P560A)null	This study	AM085120
19	Austria	Innsbruck (135)	KEL:1,-2	1	C2023T (e18)	(R675X)null	This study	AM085118
20	Israel (Arab)	Not given	KELnull	2	G2027A (e18)	(S676N)null	9	NA
*KEL**2el and *KEL**2mod alleles								
1	Austria	Graz (25)	KEL:1,2el	1	C306A, C1298T (e4,e11)	(D102E,P433L)el	This study	AM085123
2	US	NY(mod-3)	KEL:1,2mod	1	T986C (e9)	(L329P)mod	13	NA
3	US	Seattle/NY(mod-1)	KEL:1,2mod	1	G1088A (e10)	(S363N)mod†	9	NA
	US	NY(mod-2)	KEL:1,2mod	1	G1088A (e10)	(S363N)mod†	9	NA
4	US	NY(mod-3)	KEL:1,2mod	1	G1596A (e15)	(W532X)mod	13	NA
5	US	NY(mod-4)	KEL:1,2mod	1	C1719T (e16)	(G573G)el	9	AM085115
	Austria	Innsbruck (99)	KEL:1,2el	1	C1719T (e16)	(G573G)el	This study	AM085115
6	Austria	Vienna (27)	KEL:1,2el	1	A1763G (e16)	(Y588C)el	This study	AM085121
7	US	NY(mod-2)	KEL:1,2mod	1	A2030G (e18)	(Y677C)el	13	NA
	Austria	Innsbruck (111)	KEL:1,2el	1	A2030G (e18)	(Y677C)el	This study	AM085116
8	US	NY(mod-4)	KEL:1,2mod	1	G2107A (e19)	(G703R)mod	13	NA
KELweak allele								
1	Austria	Innsbruck (178)	KEL:1,2weak	1	C841T (e8)	*KEL**3	3	AM085119

* Position of mutation counting A of start codon ATG as nucleotide 1; i = intron; e = exon; NA = not available; NY, New York. NM_000420 taken as reference mRNA.

† The apparent discrepancy describing *KEL**2(S363N) allele as either "null" or "mod" derives from data given in the original publications.[9,13]

antigen. The presence of KEL3 is known to exert a suppressive effect on KEL2 and the other Kell system antigens on the same molecule.[3] The four cases positive for the KEL2 antigen only by adsorption-elution showed different mutations in their respective *KEL**2 sequences. Innsbruck (99) exhibited the single silent mutation C1719T, defining allele *KEL**2(G573G)el, with so far unexplained weakening of KEL2 antigen expression (Table 4).

In three of the nine KEL1-positive, *KEL**1/*KEL**2 heterozygous cases without detectable KEL2 antigen expression, no mutation could be identified. In the remaining six cases either *KEL**2 splice site or nonsense mutations were found (Table 4).

Sequencing of all mutations was repeated with the respective non–allele-separating sequencing strategy, that is, with amplification reactions F-1 to F-6. In all cases, the aforementioned mutations were confirmed, with heterozygous results at the respective positions (Fig. 1C).

Analysis of KEL blood group antigen expression of variant *KEL**2 allele RBCs

To define the immunohematologic characteristics associated with *KEL**2 variant alleles, the expression of different KEL blood group antigens was quantified by flow cytometry. As shown in Table 5, all KEL:1,2el samples

Panel	A	B	C
location *KEL*	nt919–nt924, i8 nt1–nt3	nt943–nt951	nt1714–nt1722
wild-type	Leu . Lys . **splic** C T C A A G **G** T G *	Asp . **Trp** . Leu G A C T G **G** T T G *	Phe . **Gly** . Ala T T T G G **C** G C T *
allele sample	*KEL**2(IVS8+1G>T) null, Berlin (179), 1st	*KEL**2(W316X) null,G948A Berlin (179), 2nd	*KEL**2(G573G)el, C1719T Innsbruck (99)
both parental alleles F-3, F-3, F-5	Leu . Lys . **---** C T C A A G **K** T G *	Asp . **---** . Leu G A C T G **R** T T G *	Phe . **Gly** . Ala T T T G G **Y** G C T *
mutated allele Berlin-179-2, 179-3, LR-4	Leu . Lys . **splic** C T C A A G **T** T G *	Asp . **STOP** G A C T G **A** T T G *	Phe . **Gly** . Ala T T T G G **T** G C T *

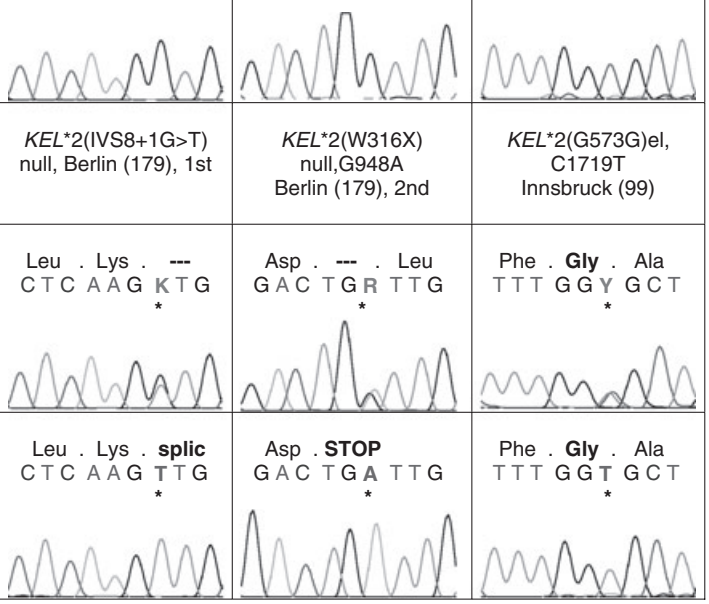

Fig. 1. Examples of DNA sequencing are shown for the three *KEL*-alleles: *KEL2(IVS81GT)null, *KEL**2(W316X)null, and *KEL**2(G573G)el. Panels depict chromatograms along with DNA and deduced amino acid sequences; *location of the mutation; splic = the exon 8, 3′ splice site; STOP = stop codon. In the top panels, the respective wild-type DNA-sequences are given. In the middle panels, heterozygous nucleotide positions are visible as a result of DNA-sequencing PCR fragments including the wild-type and mutated parental *KEL* alleles. K = G or T; R = A or G; and Y = C or T (PCR fragments F-3, F-3, and F-5, from left to right). In the bottom panels, the respective mutations are visible as a result of DNA-sequencing PCR fragments, only including the mutated parental allele (PCR fragments Berlin-179-2, Berlin-179-3, and LR-4, from left to right).**

showed markedly reduced KEL2 antigen expression of less than 4 percent of KEL:1,2 wild-type controls. Hence, because the mean (±standard deviation [SD]) absolute KEL2 antigen density of five KEL:1,2 controls amounted to 8090 (±600) KEL2 antigens per RBC, KEL:1,2el RBCs were calculated to express up to a maximum of 298 KEL2 antigens per cell. In comparison, the KEL:1,2weak sample had a considerably higher KEL2 antigen expression of 1897 sites per cell.

KEL:1,2el and KEL:1,2weak individuals expressed generally lower levels of KEL1 antigen than homozygous KEL:1,-2 controls but higher levels than heterozygous KEL:1,2 controls (Fig. 2A). Therefore, the reduced amounts of *KEL**2el or *KEL**2weak allele-encoded proteins at the cell membrane were associated with enhanced expression of *KEL**1-encoded molecules. Nevertheless, all studied KEL:1,2el and KEL:1,2weak RBC samples displayed a net reduction in surface Kell glycoprotein. This was indicated by reduced amounts of high-prevalence KEL7 (Js^b) and KEL4 (Kp^b) antigens as well as total Kell protein, compared to KEL:1,2 controls (Fig. 2B).

Screening for *KEL**1 alleles among 811 samples typed KEL:-1,2 by routine serology

To estimate the prevalence of *KEL**1 alleles with very weak or absent KEL1 expression, 811 Tyrolean samples phenotyped KEL:1,2 (K–k+) by serologic

TABLE 5. KEL2 (k) antigen expression of KEL2el, KEL2weak, and control RBC samples*

Sample	KEL1,2 phenotype	*KEL**2 allele	KEL2 antigen expression (%)
KEL:1,-2 controls (n = 5)	KEL:1,-2	no *KEL**2 allele	0.00 ± 0.37†
KEL:1,2 controls (n = 5)	KEL:1,2 (sgd)	*KEL**2 (one copy)	100.00 ± 7.42†
KEL:1,2 controls (n = 5)	KEL:1,2 (dgd)	*KEL**2 (two copies)	184.02 ± 12.12†
Innsbruck (99)	KEL:1,2el	*KEL**2(G573G)el	0.77
Innsbruck (111)	KEL:1,2el	*KEL**2(Y677C)el	ND
Innsbruck (127)	KEL:1,2el	*KEL**2(IVS8+1G>A)null	<0.74
Innsbruck (178)	KEL:1,2weak	*KEL**2(R281W)weak = *KEL**3	23.45
Graz (25)	KEL:1,2el	*KEL**2(D102E, P433L)el	3.68
Vienna (27)	KEL:1,2el	*KEL**2(Y588C)el	2.10

* Relative KEL2 antigen expression levels of KEL variant RBC samples were calculated after indirect immunofluorescence staining with polyclonal anti-KEL2. Mean fluorescence intensities of KEL:1,2 control samples were set as 100 percent. sgd = single gene dose; dgd = double gene dose; ND = not determined.
† Mean ± SD.

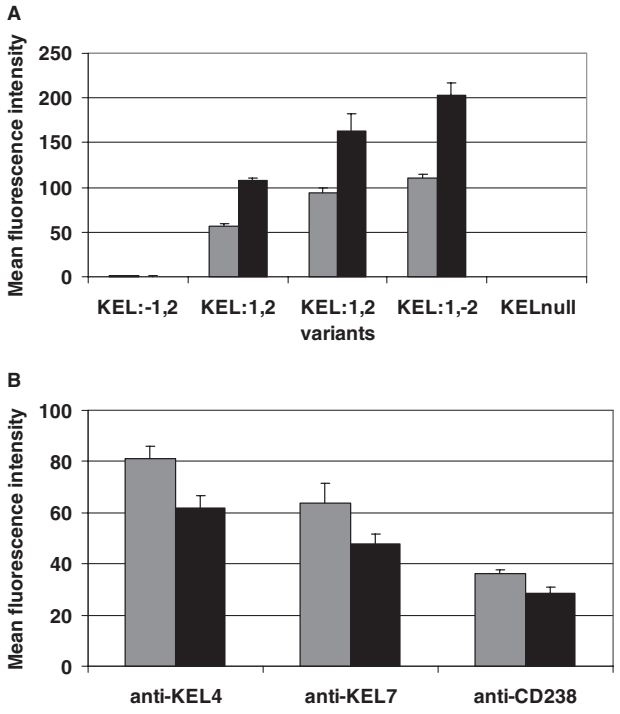

Fig. 2. (A) KEL1 (K) antigen expression of KEL variant and control RBCs. Mean fluorescence intensities (+SD) of 5 control samples each of KEL:1,2, heterozygous KEL:1,2, and KEL:1,-2 phenotype, in comparison to 13 KEL variant samples (9 KEL: 1,2null, 3 KEL:1,2el, and 1 KEL:1,2weak) as well as 1 KELnull sample (Bregenz) indirectly stained with monoclonal (▩) or polyclonal (■) anti-KEL1 after subtraction of isotype control are shown. Note baseline values of homozygous KEL2 and KELnull cells. (B) KEL4 (Kp^b) and KEL7 (Js^b) antigen and total Kell protein (CD238) expression of KEL variant and control RBCs. Mean fluorescence intensities of 6 KEL:1,2,-3,4,-6,7 (K+k+, Kp(a–b+), Js(a–b+)) control samples (▩) and of 13 KEL variant samples (9 KEL:1,2null, 3 KEL:1,2el, and 1 KEL: 1,2weak; ■) indirectly stained with polyclonal anti-KEL4, anti-KEL7, or monoclonal anti-CD238 after subtraction of isotype controls are shown. No specific fluorescence was recorded with KELnull samples indicating complete lack of KEL antigen expression (data not shown).

screening were *KEL**1/*KEL**2 genotyped. All samples investigated were homozygous for *KEL**2, possibly including variant *KEL**2 alleles undetectable by this genotyping approach. Hence, no *KEL**1 alleles (95% CI, 0-3.285) with very weak or without KEL1 expression were found in this series, resulting in a 95 percent CI of their allele frequency of 0 to 0.000078.

Sequencing of two Austrian and two German samples of KELnull phenotype

In Austria, only two KELnull individuals are known at present. Both individuals typed homozygous *KEL**2/*KEL**2 by *KEL**1/*KEL**2 genotyping. Their DNA samples were sequenced with the non–allele-separating sequencing strategy (PCR fragments F-1 to F-6). Interestingly sample "Salzburg" was found to be homozygous for the *KEL**2(IVS8+>A)null allele already identified in heterozygous form in sample Innsbruck (127) (Table 4).

To further assess the genetic heterogeneity of KELnull, two additional KELnull samples of German origin were included into this study. Both individuals typed *KEL**2/*KEL**2 by *KEL**1/*KEL**2 genotyping, and the separate DNA sequencing of parts of both parental *KEL* alleles (reactions Berlin-178-1 to Berlin 178-4 and Berlin-179-1 to Berlin-179-4) showed heterozygosity for two different *KEL**2null in both cases. The remaining part of the two parental *KEL* genes did not show any heterozygous positions when sequenced together (Table 4). Exemplary compound heterozygous *KEL**2null sequencing chromatograms are shown in Figs. 1A and 1B.

All sequences were deposited at EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/ Table 4). A summary of all KELnull and KELel alleles identified by us and reported by others so far, as well as of the *KEL**2weak (*KEL**3) allele observed in this study is presented in Table 4. Austria and its federal states' numbers and origins of variant KEL phenotypes are shown in Fig. 3.

Frequencies of *KEL**2null and *KEL**2el alleles

*KEL**1, *KEL**2, and cumulative *KEL**2null and *KEL**2el allele frequencies and their lower and upper 95 percent CIs were calculated for the three different blood transfusion centers representing the federal Austrian states of Tyrol, Styria, and Vienna (including Lower Austria and Burgenland). Dividing the cumulative *KEL**2null and *KEL**2el allele frequencies by the observed number of *KEL**2null and *KEL**2el alleles resulted in individual *KEL**2null and *KEL**2el allele frequencies. Weighting the number of inhabitants of areas studied, the respective frequencies for the "rest" of Austria and for total Austria were calculated. The cumulative *KEL**2null and *KEL**2el allele frequency for total Austria was 0.001055 (95% CI, 0.000412-0.002569; Table 6). By use of these cumulative *KEL**2null and *KEL**2el allele frequency data, the total calculated number of *KEL**2null/*KEL**2null homozygous, *KEL**2el allele homozygous, *KEL**2null/*KEL**2el heterozygous, and *KEL**1/*KEL**2el heterozygous individuals were calculated and are given in Table 6.

DISCUSSION

In this most comprehensive study on *KEL* variant genetics so far, *KEL**1/*KEL**2 genotyping of 401 Austrian samples routinely phenotyped KEL:1,-2 revealed *KEL**1/*KEL**2 heterozygosity in 14 cases. More detailed examination of these samples demonstrated markedly reduced or absent

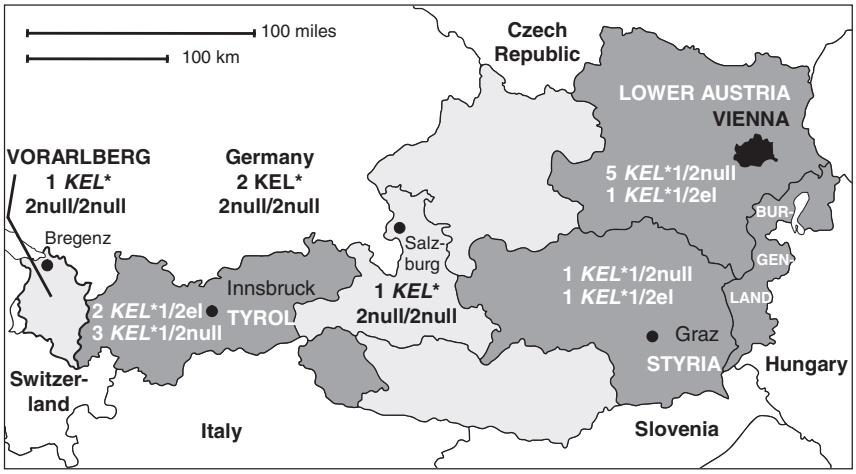

Fig. 3. Austria and its federal states' numbers and origins of variant *KEL* genotypes. Participating federal states are in dark gray. Cumulative numbers are given for federal states of Vienna, Lower Austria, and Burgenland.

TABLE 6. Frequency of the studied variant *KEL* alleles and genotypes deduced

Sample numbers, single allele and genotype frequencies (FRQ)	Innsbruck (Tyrol)	Graz (Styria)	Vienna*	"Rest" of Austria (weighted)	Total Austria (sum)
Number of inhabitants	683,317	119,0574	3,419,509	2,824,354	8,117,754
Number of KEL:1,2	126,176	70,429	49,313	NA	NA
Number of KEL:1,2	10,020	8,947	4,030	NA	NA
Number of KEL:1,-2	207	139	102	NA	NA
EXPECT "KEL:1,-2" per inhabitants	1,000	4,006	5,356	5,357	15,718
Number of "KEL:1,-2" investigated	177	123	101	NA	NA
Percent investigated of expected	17.71	3.07	1.89	0.00	2.55
Number of *KEL**2null alleles found	3	1	5	NA	9
Number of *KEL**2el alleles found	2	1	1	NA	4
*KEL**2 FRQ	0.961234	0.939631	0.959462	0.955231	0.955213
Lower (95% CI)	0.961588	0.940067	0.960213	0.955859	0.955852
Upper (95% CI)	0.960470	0.938319	0.957773	0.953746	0.953701
*KEL**1 FRQ	0.038211	0.059874	0.039297	0.043785	0.044297
Lower (95% CI)	0.038197	0.059847	0.039266	0.043757	0.043757
Upper (95% CI)	0.038241	0.059958	0.039366	0.043852	0.043854
*KEL**2null and *KEL**2el FRQ cumulative	0.000555	0.000495	0.001241	0.001038	0.001055
Lower (95% CI)	0.000215	0.000087	0.000521	0.000405	0.000412
Upper (95% CI)	0.001289	0.001723	0.002861	0.002526	0.002569
*KEL**2null or *KEL**2el FRQ single	0.000111	0.000247	0.000207	0.000204	0.000206
Lower (95% CI)	0.000043	0.000043	0.000087	0.000071	0.000073
Upper (95% CI)	0.000258	0.000862	0.000477	0.000535	0.000542
*KEL**2null/*KEL**2null per 1 million	0.11	0.06	1.07	0.59	0.61
Lower (95% CI)	0.02	0.00	0.19	0.09	0.30
Upper (95% CI)	0.60	0.74	5.68	3.36	10.39
*KEL**2el/*KEL**2el per 1 million	0.05	0.06	0.04	0.05	0.20
Lower (95% CI)	0.01	0.00	0.01	0.01	0.02
Upper (95% CI)	0.27	0.74	0.23	0.32	1.56
*KEL**2null/*KEL**2el per 1 million	0.15	0.12	0.43	0.33	1.03
Lower (95% CI)	0.02	0.00	0.08	0.05	0.15
Upper (95% CI)	0.80	1.48	2.27	2.08	6.64
*KEL**1/*KEL**2el per 1 million	16.98	16.26	29.63	19.08	19.31
Lower (95% CI)	6.57	6.82	5.18	6.74	6.74
Upper (95% CI)	39.43	37.54	103.32	49.85	49.85

* Cumulative numbers are given for federal states of Vienna, Lower Austria, and Burgenland.

KEL2 antigen expression in five and nine probands, respectively. In 11 of these cases as well as in 4 additionally studied KELnull samples, mutated *KEL**2 alleles were identified.

The immunohematologic properties of all investigated cases ruled out epistatic *XK* gene effects determining the McLeod phenotype characterized by globally reduced KEL antigen expression and complete absence of

XK protein.[2,18] All investigated *KEL**1/*KEL**2variant heterozygous samples displayed modestly increased KEL1 expression levels, compared to controls, whereas in four additional KELnull samples the complete absence of KEL antigens was confirmed both by adsorption-elution tests and by flow cytometry.

The allele-separating long-range PCR-SSP amplification-sequencing strategy proved very efficient to yield unambiguous DNA sequencing results and excluded cis-trans assigning difficulties deriving from *KEL**2variant heterozygosity. In three KEL:1,-2 but *KEL**1/*KEL**2 samples no causative weakening or inactivating *KEL**2 mutation could be identified. Obviously, further sites of phenotype-altering mutations in the promoter or intron regions of the *KEL* gene may be involved, in analogy to other blood group genes, such as known from the gene-silencing promoter mutation in the *FY* gene, or as yet unidentified gene-silencing mutations in certain *RHD* alleles.[19,20]

The majority of the mutated *KEL**2 alleles found in this study were novel, with five exemptions. *KEL**2(Q348X)null had previously been observed in a Portuguese KELnull individual and *KEL**2(IVS3+1G>A)null in KELnull individuals from different areas around the world; this *KEL**2null allele is by far the most frequent, with at least 19 individual observations.[9-12] The alleles *KEL**2(Y677C)el and *KEL**2(G573G)el had been found previously in a Kmod-2 and a Kmod-4 individual, respectively.[13] Finally, *KEL**2(R281W)weak is known as *KEL**3 determining the KEL3 antigen, with a well-established suppressive effect on KEL2 antigen expression in *KEL**1/*KEL**3 subjects.[3] Yazdanbakhsh and coworkers[21] found by immunoblotting of RBCs that the weakening of Kell antigens in the KEL3 (Kp^a) variant is due to a reduced amount of total Kell glycoprotein at the cell surface. The identification of these known *KEL**2variant alleles in this study validated our search strategy and demonstrated the accuracy of the applied amplification-sequencing procedures.

Eight novel *KEL**2null and two *KEL**2el alleles were discovered in this study, representing 67 and 33 percent of the total *KEL**2null- and *KEL**2el-encoding alleles, respectively. Allele *KEL**2(IVS8+1G>A)null is a splice site mutant of the *KEL**2 wild-type allele. Importantly, for other splice site mutant alleles, such as *KEL**2(IVS3+1G>C)null and *KEL**2(IVS3+1G>A)null, skipping of exon 3 and introduction of a downstream premature stop codon had been demonstrated.[9,10] In *KEL**2(IVS8+1G>A)null, skipping of 189-bp exon 8 would not disrupt the reading frame, but would instead cause an integral 63-amino-acid shortage of the respective Kell protein as analyzed in silico. Such potential peptide excision would not be expected to affect one of the six putative N-glycosylation sites or to alter the number of the 15 extracellular cysteine residues critical for correct protein folding.[2] Nevertheless, allele *KEL**2(IVS8+1G>A)null was also found in homozygous form in sample Salzburg, with classical KELnull attributes such as absent Kell protein expression and anti-KEL5 immunization. *KEL**2(IVS8+1G>A)null is the third most frequent *KEL**2null allele, with three observations so far. Exactly the same nucleotide is mutated in the novel *KEL**2(IVS8+1G>T)null allele found in KELnull sample Berlin (179) heterozygous with the nonsense mutant *KEL**2(W316X)null allele, with identical implications as for *KEL**2(IVS8+1G>A)null. Hence, 4 of the 20 *KEL*null alleles currently known are caused by two distinct exon 3-intron 3 and two exon 8-intron 8 border splice site mutations. *KEL**2(P560A)null together with the *KEL**2(S363N)null and *KEL**2(S676N)null are the only three *KEL**2null alleles currently known which are caused by missense point mutations.[9] Regarding our *KEL**1/*KEL**2(P560A)null heterozygous sample, the predicted substitution of proline might be a possible explanation for the complete lack of KEL2 expression, because this amino acid is known to have an essential role in determining local conformations of proteins.[22] Missense null alleles observed in the heterozygous state, however, need to be confirmed as unexpressed by either immunoblotting of RBC membranes or COS cell expression studies, which has not been done for *KEL**2(P560A)null in this study.

KELnull, KELel, and KELmod phenotypes are possibly within a continuous spectrum determined by gene dosage effects, as exemplified by individuals with compound heterozygosity or homozygosity for the *KEL**2(S363N)null allele, displaying a KELnull or KELmod phenotype, respectively.[9,13] Only adsorption-elution techniques as performed in our study might result in an appropriate distinction of the two phenotypes. Notably, *KEL**2(R675X)null would predict a Kell protein, shortened to 674 amino acids, which however is not expressed at all. Similarly, *KEL**2(W532X)el predicted to encode an even more truncated 531 amino acid protein and currently listed among other *KEL**2mod alleles may in fact be a *KEL**2null allele.[13] With respect to *KEL**2el alleles, our results indicate that the observed *KEL**2 mutations were associated with a markedly reduced insertion of the Kell glycoprotein into the plasma membrane, because of either translational disturbance or affected intracellular Kell trafficking. The applied sample screening strategies in KEL1,-2 or KEL-1,2 phenotypes were intended to deliver "heterozygous" KELnull and KELel samples, coexpressing either regular KEL1 or KEL2 from the other chromosome, respectively. Our study design, however, not only allowed for the efficient collection of these heterozygous KELnull/KEL1 and KELel/KEL1 samples but also for the first exact calculation of phenotype frequencies in a defined geographic region. Notably, the expected number of 5.47 Austrian (population, 8.1 million) KELnull individuals is much lower than the frequency estimates (1:15,000-1:25 000 individuals) reported earlier.[3] In comparison, 0.39 and 8.38 KELel individuals homozygous for *KEL**2el and compound *KEL**2null/*KEL**2el heterozygous, respectively, can be

expected in Austria. The sum of actual Austrian KELnull and all KELel individuals would not exceed 14.23 or 1 per 570,310 inhabitants.

Approximately 1 percent of all seemingly KEL:1,-2 Austrian individuals may be expected to carry an undetected and rudimentary expressed *KEL**2el allele. These *KEL**1/*KEL**2el heterozygous individuals are usually overseen and serologically mistyped as KEL:1,-2 despite expressing minute amounts of KEL2. Comparably low D antigen expression levels were found in extremely weak D and DEL blood donors whose RBCs were shown to induce anti-D immunization in D– recipients.[20,23,24] To date it is not clear whether minute KEL2 quantities encoded by *KEL**2el alleles could induce anti-KEL2 alloimmunization in KEL:1,-2 individuals, with implications for hemolytic transfusion reactions and hemolytic disease of the fetus and newborn. Similarly, KELnull individuals may be at risk for anti-RBC alloimmunization when transfused with blood of KELel phenotype. Therefore, exceedingly rare "KELnull" blood donors should be investigated on a molecular basis to confirm their true KELnull phenotypes (homozygous *KEL**2null/*KEL**2null) and to distinguish them from KELel individuals (heterozygous *KEL**2null/*KEL**2el or homozygous *KEL**2el/*KEL**2el). Analogously, before transfusing KEL:1,-2 girls and women of child bearing age with phenotype-matched blood, transfused material could be investigated for traces of KEL2. For both settings, adsorption-elution techniques for KEL2 antigen typing seem to be appropriate. Alternatively, nucleotide 578 *KEL**1/*KEL**2 genotyping appears to be useful for the identification of actual *KEL**1 homozygous donors and exclude carriers of KEL*2el alleles.

REFERENCES

1. Claperon A, Rose C, Gane P, et al. The Kell protein of the common K2 phenotype is a catalytically active metalloprotease, whereas the rare Kell K1 antigen is inactive. Identification of novel substrates for the Kell protein. J Biol Chem 2005;280:21272-83.
2. Westhoff CM, Reid ME. Review: the Kell, Duffy, and Kidd blood group systems. Immunohematology 2004;20:37-49.
3. Daniels G. Human blood groups. 2nd ed. Oxford: Blackwell Science; 2002.
4. Wagner T, Bernaschek G, Geissler K. Inhibition of megakaryopoiesis by Kell related antibodies. N Engl J Med 2000; 343:72.
5. Wagner T, Resch B, Reiterer F, Gassner C, Lanzer G. Pancytopenia due to suppressed hematopoiesis in a case of fatal hemolytic disease of the newborn associated with anti-K supported by molecular K1 typing. J Pediatr Hematol Oncol 2004;26:13-5.
6. Klein H, Anstee D. Blood transfusion in clinical medicine. 11th ed. Oxford: Blackwell Science; 2006.
7. Lee S, Wu X, Reid M, Zelinski T, Redman C. Molecular basis of the Kell (K1) phenotype. Blood 1995;85:912-6.
8. Race RR, Sanger R. Blood groups in man. 6th ed. Oxford: Blackwell Scientific Publications; 1975.
9. Lee S, Russo DC, Reiner AP, Lee JH, et al. Molecular defects underlying the Kell null phenotype. J Biol Chem 2001;276: 27281-9.
10. Yu LC, Twu YC, Chang CY, Lin M. Molecular basis of the Kell-null phenotype: a mutation at the splice site of human KEL gene abolishes the expression of Kell blood group antigens. J Biol Chem 2001;276:10247-52.
11. Koda Y, Soejima M, Tsuneoka M, et al. Heterozygosity for two novel null alleles of the KEL gene causes the Kell-null phenotype in a Japanese woman. Br J Haematol 2002;117: 220-5.
12. Wester ES, Storry JR, Schneider K, et al. Genetic basis of the K(0) phenotype in the Swedish population. Transfusion 2005;45:545-9.
13. Lee S, Russo DC, Reid ME, Redman CM. Mutations that diminish expression of Kell surface protein and lead to the Kmod RBC phenotype. Transfusion 2003;43:1121-5.
14. Körmöczi GF, Gassner C, Shao CP, Uchikawa M, Legler TJ. A comprehensive analysis of DEL types: partial DEL individuals are prone to anti-D alloimmunization. Transfusion 2005;45:1561-7.
15. Körmöczi GF, Förstemann E, Gabriel C, et al. Novel weak D types 31 and 32: adsorption-elution-supported D antigen analysis and comparison to prevalent weak D types. Transfusion 2005;45:1574-80.
16. Gassner C, Schmarda A, Kilga-Nogler S, et al. RHD/CE typing by polymerase chain reaction using sequence-specific primers. Transfusion 1997;37:1020-6.
17. Sachs L. Angewandte statistik. 8th ed. Berlin: Springer Verlag; 1997.
18. Ho M, Chelly J, Carter N, et al. Isolation of the gene for McLeod syndrome that encodes a novel membrane transport protein. Cell 1994;77:869-80.
19. Tournamille C, Colin Y, Cartron JP, Le Van Kim C. Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. Nat Genet 1995;10:224-8.
20. Gassner C, Doescher A, Drnovsek TD, et al. Presence of RHD in serologically D-, C/E+ individuals: a European multicenter study. Transfusion 2005;45:527-38.
21. Yazdanbakhsh K, Lee S, Yu Q, Reid ME. Identification of a defect in the intracellular trafficking of a Kell blood group variant. Blood 1999;94:310-8.
22. MacArthur MW, Thornton JM. Influence of proline residues on protein conformation. J Mol Biol 1991;218:397-412.
23. Flegel WA, Khull SR, Wagner FF. Primary anti-D immunization by weak D type 2 RBcs. Transfusion 2000;40:428-34.
24. Wagner T, Körmöczi GF, Buchta C, et al. Anti-D immunization by DEL red blood cells. Transfusion 2005;45:520-6.