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Characterization of rhesus macaque *KIR* genotypes and haplotypes

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Abstract Certain combinations of the killer immunoglobulin-like receptors (KIR) and major histocompatibility complex class I ligands in humans predispose carriers to a variety of diseases, requiring sophisticated genotyping of the highly polymorphic and diverse *KIR* and *HLA* genes. Particularly, *KIR* genotyping is challenging due to polymorphisms (allelic substitutions), genomic diversity (presence/absence of genes), and frequent duplications. Rhesus macaques are often used as important animal models of human diseases such as, e.g. AIDS. However, typing of rhesus macaque *KIR* genes has not been described so far. In this study, we report the identification of additional novel rhesus macaque *KIR* cDNA sequences and a sequence-specific *KIR* genotyping assay. From a cohort of four rhesus macaque families with a total of 70 individuals, we identified 25 distinct *KIR* genotypes. Segregation analyses of *KIR* genes and of two polymorphic microsatellite markers allowed the identification of 21 distinct *KIR* haplotypes in these families, with five to 11 segregating *KIR* genes per haplotype. Our analyses confirmed and extended knowledge on differential gene *KIR* gene content in macaques and indicate that rhesus macaque and human *KIR* haplotypes show a comparable level of diversity and complexity.

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Introduction

Killer cell immunoglobulin-like receptors (KIRs) are cell surface receptors of the immunoglobulin (Ig) superfamily expressed on the cell surface of natural killer (NK) cells and subsets of T lymphocytes (Gardiner 2008; Lanier 2008; Parham 2005). Mapping to the leukocyte-receptor complex in a head to tail fashion, *KIR* genes encode type I transmembrane glycoproteins with either two or three extracellular Ig-like domains as well as stem, transmembrane, and cytoplasmic regions (Kelley et al. 2005). Inhibitory KIRs have long cytoplasmic tails with immunoreceptor tyrosine-based inhibitory motifs (ITIM), whereas activating KIRs possess short tails lacking ITIMs and instead contain a positively charged amino acid in the transmembrane region that mediates interaction with immunoreceptor tyrosine-based activating motif-containing adaptor molecules (Feng et al. 2005; Lanier 1998; Long 1999). The nomenclature of *KIR* genes is based on these structural and functional features (Marsh et al. 2003).

A hallmark of *KIR* genes is their diversity and polymorphism as seen in substantial differences in gene content and numerous alleles of certain genes, respectively (Uhrberg et al. 1997; Valiante et al. 1997). Sixteen *KIR* genes are currently known in humans and numerous *KIR* genotypes and haplotypes have been defined (Gardiner 2008; Robinson and Marsh 2007; Vilches and Parham 2002). All human *KIR* haplotypes contain the *KIR3DL3* and *KIR3DL2* genes at the centromeric and telomeric end of the *KIR* locus, respectively, in addition to *KIR3DP1*,

KIR2DL4, *KIR2DL2/3*, and *KIR3DL1*/*KIR3DS1*, which are present on almost all haplotypes. According to their gene content, *KIR* haplotypes have been classified into either group A or B, with A haplotypes lacking activating *KIR* genes except *KIR2DS4*, and all other haplotypes grouped as B (Hsu et al. 2002; Uhrberg et al. 1997). Differential gene contents are usually created by non-reciprocal recombination (Martin et al. 2003; Norman et al. 2009; Uhrberg 2005; Wilson et al. 2000) and the extent is typically determined by polymerase chain reaction (PCR) involving sequence-specific primers (Houtchens et al. 2007; Martin et al. 2008; Uhrberg et al. 1997; Vilches et al. 2007). Remarkably, certain combinations of the highly variable KIRs and their HLA class I ligands predispose carriers to various infectious and autoimmune diseases and influence reproduction (Hiby et al. 2008; Johansson et al. 2006; Khakoo et al. 2004; Martin et al. 2007; Nelson et al. 2004; Parham 2005).

Despite the importance as non-human primate model of human infectious diseases (Bontrop and Watkins 2005), detailed knowledge of *KIR* haplotypic gene content and diversity is rather limited in rhesus macaques. Previously published reports point to substantial diversity of rhesus macaque *KIR* genes and haplotypes (Hershberger et al. 2001; Sambrook et al. 2005). In particular, rhesus macaques show extensive expansions of *KIR3D* genes, which are likely the result of co-evolution with *Mamu-A* and *Mamu-B* class I genes that are substantially expanded in rhesus macaques (Otting et al. 2007; Otting et al. 2008). Notably, the impact of *KIR* diversity on the outcome of infectious/autoimmune diseases in rhesus macaque disease models is not known. Furthermore, rhesus macaques possess different types of activating *KIR* genes compared to human and great apes (Blokhuis et al. 2009a; Hershberger et al. 2001).

Here, we established a rhesus macaque *KIR* genotyping based on 13 novel full-length and previously published *KIR* sequences. Typing of 70 animals from four families allowed to define 25 genotypes and 21 haplotypes. The number of distinguishable *KIR* genes in the tested families varies from five to 11 per haplotype.

Materials and methods

Animals

All rhesus macaques are housed in the facilities of the German Primate Center. Blood samples were obtained during regular veterinary inspections. All experiments were carried out in accordance with the German Animal Welfare Law, guidelines of the German Research Foundation, and the European Communities Council Directive (86/609/EEC). Families consist of single dominant males with multiple females and their offspring.

RNA extraction, cDNA library construction, and isolation of *KIR*-containing clones

Blood from 15 to 30 unrelated rhesus macaques was obtained and pooled into two large samples, respectively. Peripheral blood mononuclear cells were obtained by centrifugation using Ficoll-Hypaque 1.077 (Sigma) for 40 min at 600×*g*. The cell pellet was washed twice with 1× PBS followed by enrichment of CD16-positive cells using CD16 microbeads for isolation of NK cells from non-human primates (Miltenyi Biotec) according to the supplier's recommendations. Total RNA was isolated from enriched CD16-positive cells with the RNeasy Plus Mini Kit (Qiagen) and cDNA libraries were constructed with the Creator SMART cDNA Library Kit (Clontech) according to the supplier's recommendations. *KIR*-containing cDNA clones were isolated from the library by hybridization with a P^{32}-labelled rhesus macaque *KIR* PCR fragment. All *KIR*-containing clones were completely sequenced. KIR amino acid sequences were aligned using ClustalX (Thompson et al. 1997).

Genomic DNA extraction

The cellular fraction of blood samples (5-15 ml) was incubated in lysis buffer (155 mM NH_4CL, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 8.0) for 20 min to lyse erythrocytes and centrifuged for 10 min at 7 °C and 200×*g*. The pellet was washed with 10 ml lysis buffer and incubated over night at 37 C in 5 ml SE buffer (75 mM NaCl, 25 mM EDTA), 250 µl 20% SDS and 20 µl Pronase E. After adding 2 ml 5 M NaCl, the reaction was centrifuged for 10 min at 1,250×*g*. The DNA was precipitated with 14 ml 100% EtOH, washed with 5 ml 70% EtOH, and resolved in H_2O.

KIR-specific PCR-SSP typing

KIR genotyping was performed using sequence-specific primers (Table 1) and a 'hot start' PCR. Aliquots of 30 µl were set up by using 1 U Taq DNA polymerase (Biotherm), 3 µl 10× buffer, 5 mM dNTPs, and 50 ng DNA. *KIR* primers and internal control primers (actin, Table 1) were used in 0.16 pmol/µl and 0.06 pmol/µl concentration, respectively. PCR conditions are the same for all primers, except for the annealing temperature (Table 1): initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, annealing for 30 s, and elongation for 45 s at 72 °C, followed by a final elongation at 72 °C for 5 min.

Table 1 KIR typing and microsatellite primer sequences

No.	*KIR* gene(s)	Primers (5′→3′)	Anealing temp (°C)	Length (bp)	Domain
1	*KIR2DL04*001*	GGTCAGGACAAGCCCTTCTG ACCAGGGGGTTGCTGGGTG	62	269	D0
2[a]	*KIR3DL01*002*, FJ562109[b]	TATCGTCGTGGGCTTTACAAC TGACCTGTGACCATGATCGT	58	218	D0
3	*KIR3DL01* (**003*, **004*, **006*, **008 N*, **009 N*, **010*)	TGTCACTATCGTCGTGGGCTTT CCAGGGGTTCACTGAGTGCT	62	205	D0
4	*KIR3DL01*011*, FJ562110[b]	TGCCTCAGGGAGGACACGTA CCTGATCGCCAGGGGGTCG	62	242	D0
5	*KIR3DL02*001*	TCGTGGTGGGTTTAACAACTTC GGGAGTCGACCACTCAGTGA	62	175	D0
6	*KIR3DLW03* (**004*, **005*)	TACAAAGACGACAGAAGCCACA CCAGGGGGTTGCTGGGAGT	62	160	D0
7	*KIR3DL05*007*, FJ562120[b], FJ562121[b]	GGAGTCCACAGAAAACCTTC TCTCCAACAAGGTGCACGGA	58	155	D1
8	*KIR3DL06*001*	GGTGTCACTATCGTCGTGGC GTCCCTGCGTGTGCCTGG	60	142	D0
9	*KIR3DL07*001*	CCTACAATGTTCTTCAGATATG GGAGCTGACAACACATAGTC	58	204	D1
10	*KIR3DL07*002*	TATGAGAAACCTTCGCTCTCAT AAGCATCTGTAGGTTCCTCCA	60	221	D2
11[a]	*KIR3DL08**001	AAGACCTCCCTGTCTGCCCA GACCTGTGACCCTGATCACG	62	286	D0
12	*KIR3DL08* (**002*, **003*)	TCTCTCAGCCCAGCCGGGA GTAGTGGGTCACTCGGGTG	60	260	D2
13	*KIR3DL10*001*, FJ562113[b]	CCTGTCTGCCCGGCCTAGT CGTGTGCCGGGGTCACAGT	62	188	D0
14[c]	*KIR3DL10*002*, FJ562112[b]	CTCAGGGAGGACACGTGACC GTCCCTGCGTCTGCCGGG	62	166	D0
15	*KIR3DL11*001*, FJ562116[b]	TCGTCAGATACCGTGTTTGG GTCACTGGGAGCTGACAAG	58	201	D1
16	*KIR3DL11*002*	TCTCAGCCCAGCCGGGCCT TTTGACAGAAACGGGCAGTGG	62	272	D2
17	*KIR3DL20* (**002*, **003*)	CTTAGGCTCCCTGCAATGCCA GTCACTCGGGTGTGACCACA	62	138	D2
18	AF334646, AF334647[b]	TTAGGCTCCCTGCAGTGCCG GTCACTCGGGTGTGACCACA	62	137	D2
19	*KIR3DS01* (**00101*, **002*, **003*)	ACGGTGCAGGCAGGAGAGG GACCACTGGTAGGGTGCGGA	62	218	D2
20[c]	*KIR3DS02* (**001*, **008*)	GTCAGGACAAGACCTTCTTGTT CTGCGTGTGCCGGGGTCAT	62	207	D0
21	*KIR3DS03* (**00101*, **002*, **003*)	GGTGCCTCAGGGAGGACACA GGTCCCTGCGTCTGCCGGA	64	172	D0
22	*KIR3DS04*001*	GAAATCAGGAGAGACGGTCAT GATGTCCAGGGTGTCACTC	58	244	D1
23[c]	*KIR3DS05* (**00201*, **00202*, **003*)	GTCAACGGAACATTCCAGGA TGTGACAGAAACGGGCAGTG	60	132	D2
24	*KIR3DS06*004*	CCCAGGTCCCTTGGTGAAATT ACCTGTGATCACGATGTCCC	60	271	D1
25	*KIR3DSW07*001*	CCCTGGTGAAATCAGGAGAT ACCGTAGCATCTGTAGGTCT	58	197	D1
26	*KIR3DSW07*002*	AATCAGGAGAGACGGTCACA CTCTGCAAGGTCAGACGTCT	58	170	D1
27	*KIR3DSW08*005*	AAAACCTTCCCTCCTGGCCT CTGGGAGCTGACAACACATC	60	264	D1

Table 1 (continued)

No.	*KIR* gene(s)	Primers (5′→3′)	Anealing temp (°C)	Length (bp)	Domain
28	*KIR3DSW08*006*	CTGCCCGGCCCAGCGCTG CCGACATCTGTAGGTCCCTGT	64	203	D0
29	*KIR3DSW08*007*	TGTCACTATCGTCGTGGGCTTT CCGACATCTGTAGGTCCCTGT	64	153	D0
30	*KIR3DSW09*004*	CGGTCACCCTACAATGTTCC GAGTGAGTGACAGAACCGTAA	60	190	D1
31	*KIR3DSW09*005*	TGCAGCTCCCGGTGCTCGG GGTCACTCGGGTCTGACCAT	62	199	D2
32	Microsatellite I	CTTAATTCCTGAAGTCTCACTTGTAAA CATTTCTAGGTGAACCCATCC			
33	Microsatellite II	CTCCTGCTGGAATTCACTCG TTTCTGTGTGAGGGCTTGAG			
34	β-Actin (positive control)	ACGGGGTCACCCACACTGTGC CTAGAAGCATTGCGGTGGACGATG			

[a] Primer pairs 2 and 11are located over exon-intron boundaries

[b] In those cases where an official designation is not yet available, we included the database accession number

[c] Primer pairs 14, 20 and 23 recognise besides alleles of one locus also recombinants of different *KIR* genes

All PCR products were analysed by agarose gel electrophoresis.

Microsatellite analysis

Short tandem repeats were identified in the sequenced *KIR* haplotype (Sambrook et al. 2005) by manual inspection. Flanking primers (Table 1) were designed and used in a PCR consisting of an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 59 °C for 30 s, and 45 s at 72 °C, and a final extension step for 5 min at 72 °C. 6-FAM and HEX-labelled PCR products were analysed in an ABI3130xl sequencer (Applied Biosystems) along with the Genescan 400 HD ROX size standard (Applied Biosystems). Allele sizes were calculated with Gene Mapper v4.0 (Applied Biosystems).

Database accession numbers

Rhesus macaque *KIR* cDNA sequences reported here have been assigned database accession numbers FN424249-FN424261.

KIR gene nomenclature

At the KIR Polymorphism Workshop in Berkeley in 2009, it became obvious that due to substantially increasing numbers of sequences, a robust and sustainable nomenclature system for macaque *KIR* genes and alleles must urgently be introduced to avoid further confusion. As information of *KIR* gene content was available only for a single haplotype at that time, a nomenclature of macaque *KIR* genes was worked out by a committee (see Immuno Polymorphism Database (IPD) database at http://www.ebi.ac.uk/ipd/kir/). In order to install a nomenclature even in the absence of genotype and haplotype data, sequences of *KIR* genes/alleles were assigned based on clustering in phylogenetic trees. In those cases where an assignment of a certain *KIR* sequence as either an allele of a known gene or a distinct gene was not obvious, a "W" (workshop) was introduced in the gene symbol to account for its preliminary name. The same rules of the human *KIR* gene nomenclature (Marsh et al. 2003) were applied to designate macaque *KIR* genes. A report of the committee on *KIR* gene nomenclature in macaques will be published soon and will soon be available on the IPD website. Therefore, the new nomenclature is already applied in this study to avoid repeated and short-term renaming.

Results

Identification of novel rhesus macaque *KIR* cDNA sequences

Two groups of unrelated animals of Indian origin were chosen to establish two cDNA libraries from enriched NK cells. Altogether, 13 full-length KIR cDNA clones were isolated from both libraries and completely sequenced. All isolated *KIR* cDNA clones show nucleotide substitutions compared to already known rhesus macaque *KIR* sequences (not shown), thus representing novel sequences. The sequences were sent to the IPD and received official designations (Table 2). These cDNA sequences code for

Table 2 Official *KIR* gene names

KIR cDNA	Gene name	Database acc. no.
Clone 6	*KIR3DLW03*004*[a]	FN424253
Clone 12	*KIR3DLW03*005*	FN424256
Clone 5	*KIR3DL05*007*	FN424252
Clone 2	*KIR3DL11*002*	FN424250
Clone 21	*KIR3DL11*003*	FN424259
Clone 26	*KIR3DL11*004*	FN424261
Clone 23	*KIR3DS05*003*	FN424260
Clone 13	*KIR3DS06*004*	FN424257
Clone 15	*KIR3DSW07*002*	FN424258
Clone 9	*KIR3DSW08*006*	FN424254
Clone 10	*KIR3DSW08*007*	FN424255
Clone 1	*KIR3DSW09*005*	FN424249
Clone 3[b]	*KIR3DL11*	FN424251

[a] W denotes workshop designation

[b] Clone 3 likely represents an alternatively spliced transcript of *KIR3DL11*

seven inhibitory and six activating KIR3D molecules (Fig. 1). Characteristic features of primate KIR3D molecules were found: the inhibitory KIRs show two ITIMs in their cytoplasmic region, whereas the activating KIRs lack these motifs and display a charged amino acid in the transmembrane region, which is in all cases an arginine residue (Fig. 1). Thus, our data confirm and extend previous findings by others that macaque activating KIRs have arginine instead of a lysine residue (Bimber et al. 2008; Blokhuis et al. 2009a; Hershberger et al. 2001; Sambrook et al. 2005). The deduced amino acid sequences of some KIRs are nearly identical, e.g. clones 6 and 12 differ by a single amino acid and clones 21 and 26 differ at two positions (Fig. 1), indicating that these cDNA sequences represent alleles of two distinct *KIR* genes. According to assignment of the Rhesus Macaque KIR Gene Nomenclature Committee (see "Materials and methods" section), clones 6 and 12 belong to the *KIR3DLW03* gene (alleles *004 and *005) and clones 21 and 26 are derived from the *KIR3DL11* gene (alleles *003 and *004). cDNA clone 2 is also assigned to *KIR3DL11* (allele *002), but shows 12 and 14 amino acid substitutions to *3DL11*003* and *3DL11*004*, respectively, and obviously is a more distantly related allele. Clones 9 and 10 represent a further pair of allelic sequences, which differ by six amino acid residues and were assigned the names *KIR3DSW08*006* and *KIR3DSW08*007*.

KIR cDNA clone 3 carries a 150 bp deletion in the exon encoding the D2 domain and most likely represents a product derived from usage of cryptic splice sites of the *KIR3DL011* gene and is similar to transcript variant 4 of a *KIR3DL* gene described by Hershberger et al. 2001.

Determination of *KIR* genotypes

Based on multiple alignment of our new (Fig. 1) and of already known *KIR* sequences, we identified sequence-specific substitutions that were exploited to establish specific primers (Table 1). For *KIR3DL11* alleles **003* and **004*, it was not possible to establish specific primers that would allow unambiguous priming. Therefore, these two alleles were not tested in our analysis. The various primer pairs allow for discrimination of rhesus macaque *KIR* sequences at different levels: some primers allow detection of alleles (e.g. primer pairs 9 and 10), while others detect distinct genes (e.g. primer pair 8). Altogether, a set of 31 primer pairs were established (Table 1) and used to type a panel of four families with a total number of 70 animals. We identified 25 *KIR* genotypes, revealing ten to 16 specific PCR products out of 31 reactions (Table 3). Whereas *KIR* genes *2DL4*, *3DL11*, *3DL20* (*2DL5*), and *3DSW08* were found in all studied rhesus macaque individuals, we did not detect any monkey carrying *3DL06*, *3DL07*, *3DS06*, or *3DSW07*. It should be noted that *KIR2DL5* likely represents an alternatively spliced product of the *KIR3DL20* gene, which shares closer relationship with human *KIR2DL5* only in one Ig domain-encoding exon (Bimber et al. 2008; Rajalingam et al. 2004; Sambrook et al. 2005).

We used the primer set only for analysis of presence/absence polymorphisms of *KIR* genes. However, this set can also be exploited for transcription studies of individual *KIR* genes because all primer pairs were located in exons, except primer pairs 2 and 11 that are specific for *KIR3DL01* and *KIR3DL08*, respectively, and are located over exon-intron boundaries.

Determination of *KIR* haplotypes

Having established *KIR* genotypes, we analysed four rhesus macaque families and followed the segregation of *KIR* sequences in the offspring to determine *KIR* haplotypes. An example of a pedigreed family is shown in Fig. 2. A total of 21 different haplotypes were identified, with numbers of segregating *KIR* genes varying between five and 11 (Table 4). In accord with the previously noted diversity of rhesus macaque *KIR* genes (Hershberger et al. 2001; Sambrook et al. 2005), only *KIR* haplotypes 15 and 16 were found in more than one family (not shown). Nevertheless, it was possible to identify common features: genes *KIR2DL4*, *KIR3DL11*, *KIR3DL20* (*KIR2DL5*), and *KIR3DSW08* were found in all haplotypes studied here, suggesting that these could represent framework *KIR* genes. Three of these are three-domain-*KIRs*, further emphasising the diverse nature of lineage II *KIR* genes in rhesus macaques.

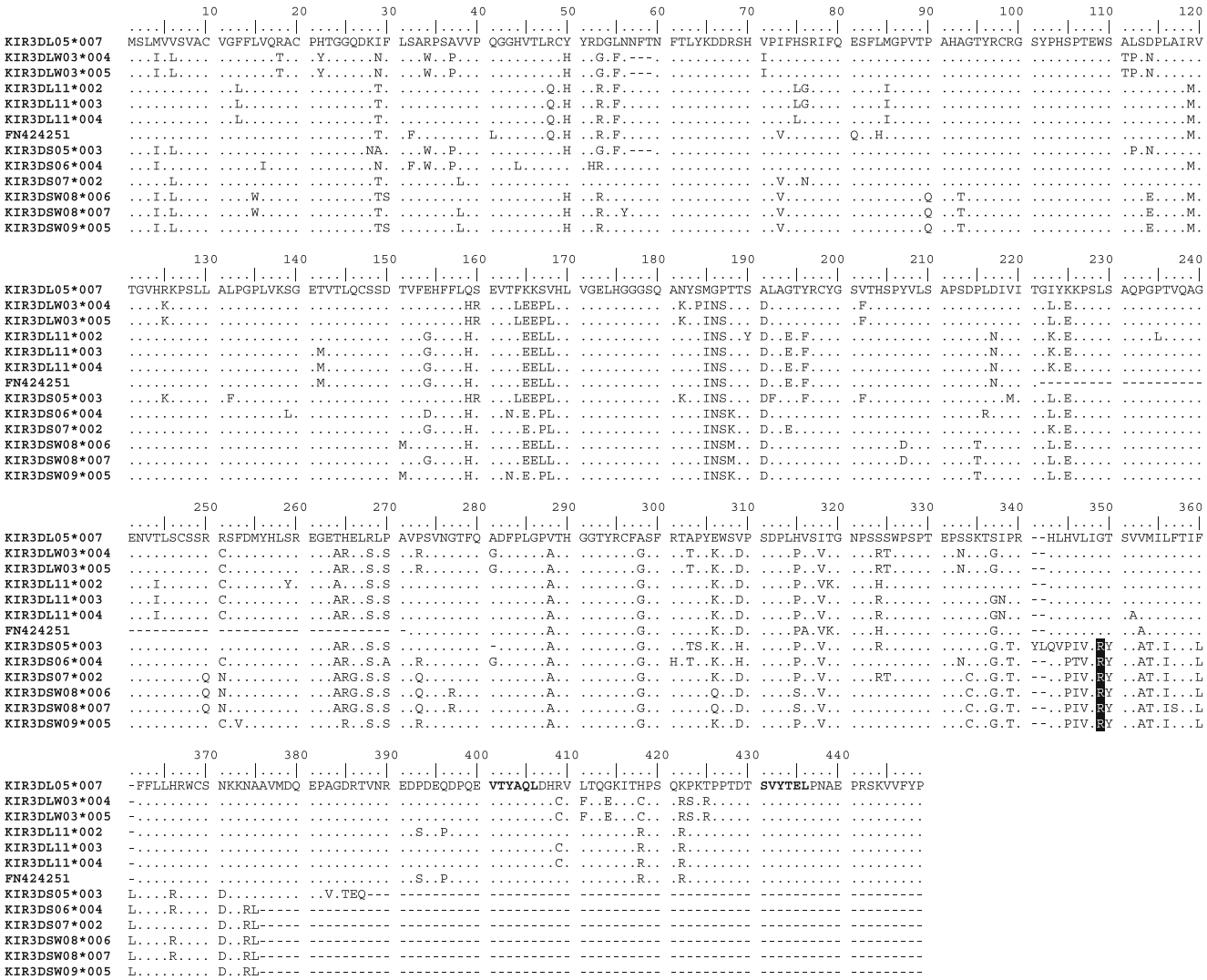

Fig. 1 Comparison of deduced amino acid sequences of the newly identified Indian rhesus macaque *KIR3D* cDNA sequences. Identical amino acid residues are indicated by a *dot*, *dashes* denote introduced gaps to maximise homology. Immunoreceptor tyrosine-based inhibitory motifs (*ITIM*) are shown in bold, and the positively charged arginine residues in the transmembrane region of activating KIRs are marked in *black*. KIR clone 3 (see also Table 2) represents an alternatively spliced transcript of *KIR3DL11* and has the DDBJ/EMBL/GenBank database accession number FN424251

In several cases, we noticed a specific PCR product, but could not observe segregation in the offspring (indicated by question mark (?) in Table 4). Thus, the studied families are not informative in these cases and more families need to be typed. It should be noted, however, that rhesus macaque families consist of a single dominant male breeding with several females and, therefore, only few offspring of a distinct pair are available for segregation studies. Interestingly, *KIR* haplotypes 2 (*3DL10*), 7 (*3DL01*, *3DSW08*), 8 (*3DL11*), 10 (*3DL10*), and 11a (*3DL10*) showed presence of two 'allelic' sequences on a single haplotype, indicating duplication of the corresponding *KIR* gene. However, another explanation with similar probability is that these sequences do not represent alleles, but belong to different *KIR* genes that co-segregate in the offspring. If this would be the case, the nomenclature of genes *3DL01*, *3DL10*, *3DL11*, and *3DSW08* demands respective revision. In any case, further haplotype analyses with additional rhesus macaque families are needed to clarify this point.

We noticed nine microsatellite markers in the completely sequenced *KIR* haplotype (Sambrook et al. 2005). Two of them turned out to be polymorphic in the studied animals and were used to confirm the segregation and haplotype analyses. One microsatellite marker is located 6 kb 5′ of *KIR2DL4*, the other maps outside the *KIR* region approximately 30 kb 3′ of the *FCAR* gene and is located in the *NLRP7* gene. Both microsatellite markers were not used in the analysis of cynomolgus macaques published by Bimber et al. 2008. Especially, the latter microsatellite shows a remarkable degree of polymorphism (Table 4). We used the

Table 3 *KIR* genotypes

Genotype no.	*001	*002, FJ562109	*003, *004, *006, *008N, *009N, *010	*011, FJ562110	*001	*004, *005	*007, FJ562120, FJ562121	*001	*001	*002	*001	*002, *003	*001, FJ562113	*002, FJ562112	*001, FJ562116	*002
	KIR2DL04	KIR3DL01			KIR3DL02	KIR3DLW03	KIR3DL05	KIR3DL06	KIR3DL07		KIR3DL08		KIR3DL10		KIR3DL11	
1	+	+		+			+				+		+	+		+
2	+	+		+			+				+		+	+		+
3	+	+		+									+	+		+
4	+	+		+	+								+	+		+
5	+	+		+	+						+					+
6	+	+		+	+						+		+	+		+
7	+	+		+	+		+				+		+	+		+
8	+	+		+	+						+		+	+		+
9	+	+	+				+					+	+		+	+
10	+	+		+			+						+	+		+
11	+	+	+	+			+					+	+			+
12	+	+					+						+	+	+	+
13	+	+	+				+					+	+	+		+
14	+	+		+	+		+				+		+	+		+
15	+			+	+		+					+	+			+
16	+			+			+						+		+	+
17	+	+			+	+	+						+	+		+
18	+	+		+			+						+	+		+
19	+	+		+		+	+						+	+		+
20	+	+		+		+	+						+	+		+
21	+	+		+	+	+	+						+	+		+
22	+	+		+		+	+						+	+		+
23	+	+				+	+						+	+		+
24	+				+	+	+						+	+		+
25	+	+			+	+	+						+	+		+

Table 3 (continued)

*002, *003 KIR3DL20	AF334646, AF334647	*00101, *002, *003 KIR3DS01	*001, *008 KIR3DS02	*00101, *002, *003 KIR3DS03	*001 KIR3DS04	*00201, *00202, *003 KIR3DS05	*004[a] KIR3DS06	*001 *002[a] KIR3DSW07	*005 *006 KIR3DSW08	*007	*004 *005 KIR3DSW09
	+		+			+			+	+	
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	+		+						+	+	
	+		+			+			+	+	

[a] We could not obtain any PCR product for *KIR* genes *3DS6*004, 3DSW07*002* and *3DSW09*005* from the samples analysed in this paper, but the respective primers work on positive controls (diluted cDNA clones).

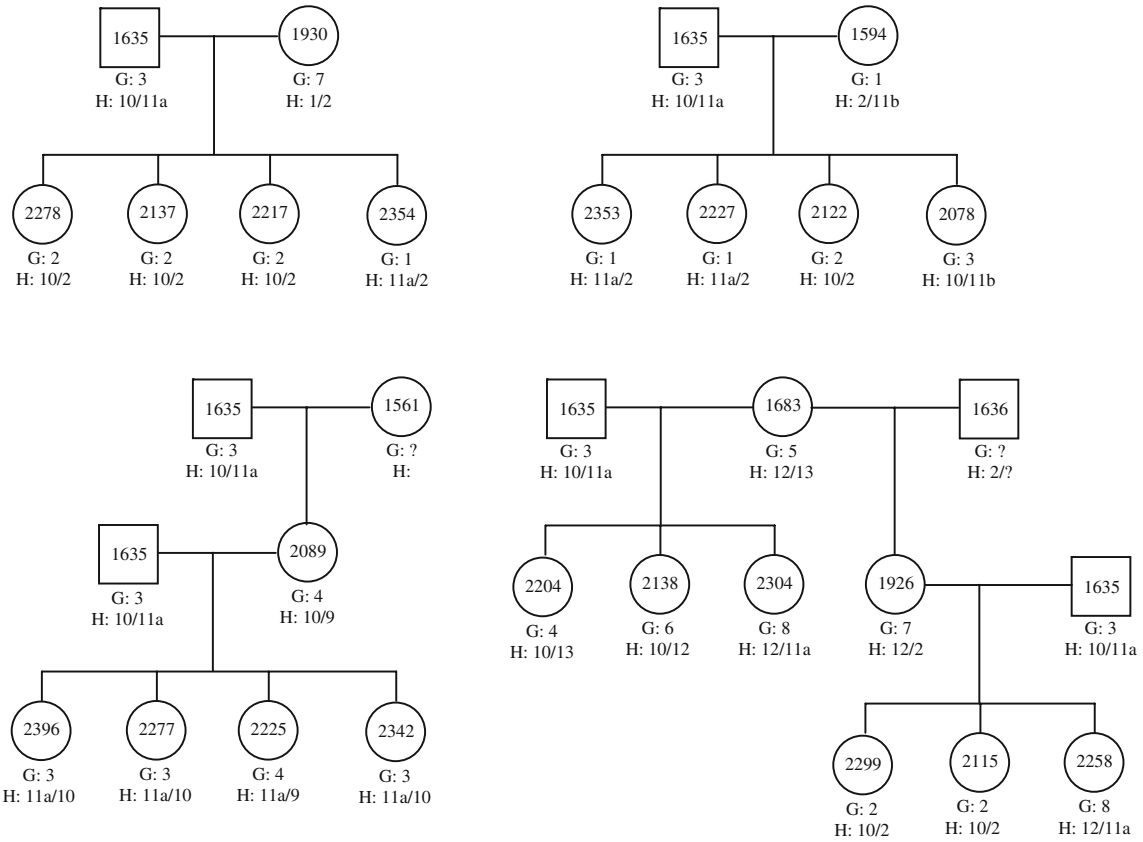

Fig. 2 Pedigree of one of the analysed rhesus macaque families. Animal identification numbers are indicated. *G* and *H* denote *KIR* genotype and *KIR* haplotype, respectively. DNA of rhesus macaque individuals 1561 (died in 2002) and 1636 (delivered to another institution in 1997) is no longer available and their *KIR* haplotypes were partially inferred from offspring

two polymorphic microsatellite markers as additional tools to follow the segregation of maternal and paternal *KIR* haplotypes in the offspring. Notably, subgroups of haplotypes 11, 15, and 16 were identified. Within these haplotypes, differences in *KIR* genes were not obvious, but the members of a subgroup differ in their microsatellite markers. It remains to be shown whether differences between these haplotypes are only seen in those microsatellites, or can be found in *KIR* genes, too.

Discussion

Rhesus macaques serve as important non-human primate models of human infectious and autoimmune diseases, and in transplantation studies. Combinations of *KIR* and (major histocompatibility complex) *MHC class I* genotypes are known to influence these human diseases. However, knowledge of rhesus macaque *KIR* genotypes and methods to determine those were not available until now. Here, we report the establishment of a PCR-based sequence-specific *KIR* genotyping in the rhesus macaque and its usage for determination of *KIR* haplotypes in family studies. The typing resulted in identification of 25 genotypes and 21 haplotypes among 70 rhesus macaques from four families, emphasising the considerable diversity of *KIR* genes in rhesus macaques. Detailed knowledge of rhesus macaque *KIR* genotypes and haplotypes will be important for evaluation of non-human primate animal model studies of those human diseases where contributions of individual KIRs and their specific MHC class I ligands play important roles in determination of disease susceptibility and resistance. However, data on specific KIR and MHC class I interactions are still not available for the rhesus macaque.

Based on 25 genotypes in segregating families, we were able to identify 21 *KIR* haplotypes (Table 4). The number of segregating *KIR* genes per haplotype can vary between five and 11 in the analysed cohort. Differential *KIR* gene content and duplication of *KIR2DL4* were previously observed in rhesus macaques (Blokhuis et al. 2009b; Sambrook et al. 2005) and duplications were also found in cynomolgus macaques (Bimber et al. 2008). Our study extends these data of differential gene content and suggests that inhibitory and activating *KIR3D* genes might be duplicated on some haplotypes. In an accompanying paper in this issue of Immunogenetics, (Blokhuis and colleagues

Table 4 *KIR* haplotypes

Haplotype no.	*001	*002, FJ562109	*003, *004, *006, *008 N, *009 N, *010	*011, FJ562110	*001	*004, *005	*007, FJ562120, FJ562121	*001	*001	*002	*001	*002, *003	*001, FJ562113	*002, FJ562112	*001, FJ562116	*002	*002, *003
	KIR2DL04[a]	*KIR3DL01*			*KIR3DL02*	*KIR3DLW03*	*KIR3DL05*	*KIR3DL06*	*KIR3DL07*		*KIR3DL08*		*KIR3DL10*		*KIR3DL11*[a]		*KIR3DL20*[a]
1	+	?[b]		?	+		?				?		?	?		+	
2	+	+		?			+				+		+	+		+	
3	+			+			?						?			+	
4	+				+		?					+	?			+	
5	+	+					?						?	+		+	
6	+			+	+		?				+		?			+	
7	+	+	+				?					+	?			+	
8	+						?						?		+	+	
9	+	+		?	+								?	?		+	
10	+			?									+	+		+	
11a	+	+		?									+	+		+	
11b	+	+		?									?	?		+	
12	+	+		?	+						+					+	
13	+	+		?	+											+	
14	+	?		+		?							?	?		+	
15a	+	?				+	?						?	?		+	
15b	+	+				?	+						+	?		+	
15c	+	+				?	+						?	?		+	
16a	+	?			+	?	?						?	?		+	
16b	+	+			?	?	+						+	?		+	
17	+	?		+			?						?	?		+	
18	+	?		+			?						?	?		+	
19	+	?		+		+	?						?	?		+	
20	+				+	?								?		+	
21	+				+	?	+						+	?		+	

Table 4 (continued)

Haplotype no.	AF334646, AF334647 *KIR3DL20*[a]	*00101, *002, *003 *KIR3DS01*	*001, *008 *KIR3DS02*	*00101, *002, *003 *KIR3DS03*	*001 *KIR3DS04*	*00201, *00202, *003 *KIR3DS05*	*004 *KIR3DS06*	*001 *002 *KIR3DSW07*	*005 *006 *KIR3DSW08*	*007	*004 *005 *KIR3DSW09*	Microsatellite I	Microsatellite II
1	+	+	?			?			+	?		128	174
2	+		+			+			+	?		128	174
3	+					+			+	?		134	162
4	+					+			+	?		128	208
5	+					+			+	?		128	192
6	+	+	+		+				+	?		134	172
7	+			+					+	?		130	168
8	+								+	?		130	170
9	+		?		+	?			+	?		128	194
10	+		+		+	+			+	?		134	162
11a	+		+			+			+	?		134	172
11b	+		?			?			+	?		128	162
12	+	+							+	?		122	174
13	+								+	?		128	194
14	+					?			?	+		128	168
15a	+		+	+		?			?	+		128	172
15b	+		+	+		+			?	+		128	212
15c	+		+	+		+			?	+		128	194
16a	+		+			?			?	+		130	212
16b	+		?			+			?	+		128	210
17	+		+	+		?			?	+		128	170
18	+		+	+		?			?	+	+	128	192
19	+					?			?	+	+	128	168
20	+		?						?	+		128	210
21	+		?						?	+		128	210

[a] *2DL04*, *3DL11*, *3DL20*, and *3DSW08* were observed in every genotype (see Table 3), but segregation was not found. These genes might represent potential KIR framework genes

[b] Question mark (?) indicates presence of specific PCR product, but segregation was not observed in offspring of the analysed families

2010) also obtained evidence for *KIR* gene duplications as they found three cDNA sequences derived from the same *KIR* gene in a single animal. Such duplications, in particular recent ones, can result in complicated genetic situations as for example the same *KIR* gene sequence can be either derived from an allele or from a distinct gene, making sequence-specific genotyping technically demanding. Future studies involving complete sequencing of rhesus macaque *KIR* haplotypes will significantly contribute to identification of recombinant *KIR* genes and recent duplications, but also of *KIR* gene fusions that can result from deletions (Abi-Rached et al. 2010).

The previously described rhesus macaque *KIR* haplotype carries only five *KIR* genes: *3DL20*, *1D*, *2DL4*, *3DL10*, and *3DL01* (Sambrook et al. 2005). Interestingly, this sequenced haplotype does not contain typical activating *KIR* genes, which are present in all haplotypes identified in this study. Furthermore, it contains only two (*KIR3DL20* and *KIR2DL4*) of four framework genes identified here. Thus, the sequenced *KIR* haplotype is obviously rather uncommon. In humans, *KIR* haplotypes are assigned to either group A or group B, which differ considerably in both number and type of *KIR* genes (Hsu et al. 2002; Uhrberg et al. 1997). Although also rhesus macaque *KIR* haplotypes strikingly vary in gene content of both inhibitory and activating *KIR* genes, we could not detect clear differences in our cohort that would allow for clear-cut discriminations similar to the human group A and B haplotypes.

Our study describes the first sequence-specific typing approach of *KIR* genes in a macaque species. The advantage of this method is its speed and cost-effectiveness, making it ideal for high-throughput screening of large cohorts. Due to the short sizes of PCR products, also samples obtained by non-invasive methods (e.g. faeces) may be typed, which would allow sampling from free-living macaques. The disadvantages are that no novel alleles or genes are detected. In addition, recombinant genes might lead to misinterpretation of the obtained genotyping data. However, as with all complex genotyping, the method will constantly be improved upon knowledge of further rhesus macaque *KIR* sequences. The newly developed genomic sequencing technologies (second and third generation sequencing) are suitable to sequence entire rhesus macaque *KIR* haplotypes, resulting in substantially improved knowledge of *KIR* genes and allotypes and improved sequence-specific *KIR* genotyping. All these efforts will contribute to make association studies of *KIR* and *MHC* genotypes possible in rhesus macaque disease models.

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