bjh research paper

The Kg-antigen, RhAG with a Lys164Gln mutation, gives rise to haemolytic disease of the newborn

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Received 8 May 2020; accepted for publication 17 June 2020

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Summary

The Kg-antigen was first discovered in an investigation of a mother whose infant had haemolytic disease of the newborn (HDN). The antibody against the Kg-antigen is believed to be responsible for HDN. The Kg-antigen is provisionally registered under the number 700045, according to the Red Cell Immunogenetics and Blood Group Terminology. However, the molecular nature of the Kg-antigen has remained a mystery for over 30 years. In this study, a monoclonal antibody against the Kg-antigen and the recombinant protein were developed that allowed for the immunoprecipitation analysis. Immunoprecipitants from the propositus' red blood cell ghosts were subjected to mass spectrometry analysis, and DNA sequence analysis of the genes was also performed. A candidate for the Kg-antigen was molecularly isolated and confirmed to be a determinant of the Kg-antigen by cell transfection and flow cytometry analyses. The Kg-antigen and the genetic mutation were then screened for in a Japanese population. The molecular nature of the Kg-antigen was shown to be RhAG with a Lys164Gln mutation. Kg phenotyping further clarified that 0·22 % of the Japanese population studied was positive for the Kg-antigen. These findings provide important information on the Kg-antigen, which has been clinically presumed to give rise to HDN.

Introduction

Haemolytic disease of the newborn (HDN) is caused by an alloimmune condition that occurs in a second fetus around the time of birth.[1] In general, immunoglobulin molecules generated in the mother enter the fetal circulation by passing through the placenta. However, some of these molecules may induce an immune response against certain antigens on the red blood cells (RBCs), triggering hemolysis.[2] The severity of the immune response ranges from mild to severe, and can result in a fatal disease called hydrops fetalis. Known alloantigens that provoke this type of immune response include ABO, Rh, Kell, Duffy and the Kg-antigen.[1,2]

The Kg-antigen was discovered in 1989 during investigation of a case of HDN.[3] The propositus was the second Kg-positive child carried by the mother. At the time, the Kg-antigen was registered as a low-incidence antigen (700 series) and provisionally given the number 700045 in the Red Cell Immunogenetics and Blood Group Terminology, organized by the International Society of Blood Transfusion (ISBT).

In 2009, we reported a propositus with HDN.[4] The Kg-antigen was detected in the propositus but not in his mother. In addition, the Kg-antigen was further detected in his two elder brothers (twins) and his father. The father had one exchange blood transfusion in infancy, thus, it appeared that anti-Kg antibody-induced HDN may have occurred through two of the generations. Thus, the Kg-antigen is clinically believed to give rise to HDN.[4]

Over 30 years have passed since the Kg-antigen was discovered, however, the polymorphism for this antigen, as well as the determinant gene, have hitherto remained unknown. The molecular nature of the majority of other known antigens responsible for HDN and their determinant genes have been identified but not of the Kg-antigen. In this study, we detailed the antigen detected with the OSK46 monoclonal

First published online 23 July 2020
doi: 10.1111/bjh.16955

antibody, generated from a peripheral B lymphocyte of the propositus' mother and clarified the molecular nature of the antigen by mass spectrometry analysis. The Kg-antigen was found to be a membrane protein encoded by a widely known gene, *RHAG*. In addition, Kg-positive samples had a common mutation in the gene, namely, a missense mutation in the 3rd exon of the *RHAG* gene, which was further shown to be responsible for the Kg-antigen.

Methods

Samples and monoclonal antibodies

Blood samples were obtained from the family members after obtaining written-informed consent from the patients or patients' (<18 years) parents, and the approval by the ethics committee of Kindai University Nara Hospital, Nara, Japan. All blood samples in the study were obtained from blood donors and all protocols were approved by the ethics committee of Japanese Red Cross (Tokyo, Japan). All methods were performed in accordance with relevant guidelines and regulations. A monoclonal antibody against the Kg-antigen (OSK46) was generated using hybridoma technology with anti-Kg antibody-producing B cells from the propositus mother (II-3). In this process, immortal myeloma cells were hybridised and a hybridoma constitutively expressing a monoclonal antibody specific for the Kg-antigen was established. The Kg phenotyping was performed with an autoanalyser PK7300 system (Beckman Coulter, Inc., Brea, CA, USA) using the OSK46 monoclonal antibody after obtaining informed consent.

Preparation of recombinant OSK46 monoclonal antibody

To construct the expression vector for the OSK46 monoclonal antibody, we first prepared Ig cassette vectors, which consisted of pcDNA3·4-TOPO vector (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), containing the encoded cDNAs for human IgG1 and Igκ constant regions respectively (pcDNA3·4-Cg1 and pcDNA3·4-Ck1). We then isolated total RNA from the OSK46 hybridoma cell line. Nested reverse transcription polymerase chain reaction (RT-PCR) was performed using a SMART cDNA Library Construction Kit (Takara, Shiga, Japan), according to the manufacturer's protocol. The following primers were used for the nested RT-PCR: OSK46 IgH, 1st forward primer (SMART 1st): 5'-AAGCAGTGGTATCAACGCAGAGT-3' and 1st reverse primer: 5'-CTTGTCCACCTTGGTGTTGCT-3'; 2nd forward primer: 5'-GGGGCGGCCGCAGAGTGGCCATTACG GCCGGG-3' (SMART 2nd) and 2nd reverse primer: 5'-AAGCTGGTCGACGGCACGGTCACCA-3': OSK46 IgL, 1st forward primer: SMART 1st and 1st reverse primer: 5'-ACT-GAGGAGCAGGTGGGGGCACTTCTCCCT-3'; 2nd forward primer: SMART 2nd and 2nd reverse primer: 5'-GGGGTCGACCTAACACTCTCCCCTGTTGAAGCTC-3'. We amplified again the cDNAs encoding the variable region of each OSK46 IgH or IgL from the OSK46 cDNAs and cloned the PCR amplicons into Ig cassette vectors by using In-fusion HD Cloning Kit (Takara) (pcDNA3·4-OSK46-IgG1 and pcDNA3·4-OSK46-Igκ). Transient expression of the recombinant Ig protein was performed by using Expi293 Expression system (Thermo Fisher). We transfected Expi293F cells with both pcDNA3·4-OSK46-IgG1 and pcDNA3·4-OSK46-Igκ (each 15 μg to 9×10^7 cells) according to the manufacturer's protocol. Following the culture of the transfected cells for 7 days, the culture supernatants were centrifuged at $6{,}000 \times g$ for 15 min followed by dialysis with the buffer (20 mmol/l Tris-HCl, 150 mmol/l NaCl, 1 mmol/l EDTA, pH 7·5) for 16 h. We loaded the dialyzed solution onto a column of Protein A Sepharose Fast Flow (GE Healthcare, Totowa, NJ, USA) resins. Sample preparation on the column continued according to manufacturer-suggested protocols, resulting in the solubilized preparation of OSK46.

Proteomic approach for the identification of the Kg-antigen

We performed immunoprecipitation analysis according to standard serological testing protocols to identify candidate proteins carrying the Kg-antigen. Recombinant OSK46 (rOSK46) or human monoclonal antibodies against Di^b (HIRO-58),[5] K14 (HIRO-103),[6] IFC (HIRO-195; unpublished), and JK3 (HIRO-294; unpublished), along with the immunoglobulin G1 from human myeloma plasma (mIgG1; Athens Research & Technology, Athens, GA, USA), were incubated with Kg-positive erythrocyte ghosts for an hour at 37 °C. The erythrocyte ghosts were lysed using a lysis buffer [20 mmol/l Tris (pH 7·5), 150 mmol/l NaCl, 1% Na-deoxycholate, 1% Triton X-100, 0.1 % SDS, 5 mmol/l ethylenediaminetetraacetic acid (EDTA)] containing protease and phosphatase inhibitors. The protein complex bound with each of the antibodies was pulled down using protein A/G magnetic beads (Thermo Fisher Scientific). Proteins from each of the precipitates were identified and quantified using a mass spectrometer TripleTOF 5600 system in conjunction with ProteinPilot software, PeakView software, and the SWATH acquisition MicroApp (ABSciex, Framingham, MA, USA). The relative amount of each protein was calculated using the peak area in mass chromatogram and then compared among the precipitates.

Preparation and extraction of genomic DNA

Genomic DNA (gDNA) was isolated from 200 μl of whole blood by using a QIAsymphony automated nucleic acid purification system (QIAGEN , Hilden Germany).

PCR amplification and DNA sequence analysis

DNA sequence analyses were performed to search for a single nucleotide polymorphism in the *RH* genes, *RHD, RHCE*, and

RHAG. For each of the *RH* exons (1–10), we designed 29 intronic primer sets, which are shown in Table SIII. The target regions were amplified by PCR and the DNA products were confirmed via agarose gel electrophoresis. Subsequently, the DNA sequences of all of the *RH* exons were determined on a 3130 Genetic Analyser (Life Technologies/Applied Biosystems, Carlsbad, CA, USA) and were analysed in detail with GENETYX-MAC Ver. 19 (Genetyx Corporation, Tokyo, Japan).

Recombinant protein expression of the Kg-antigen

The cDNA was synthesised from reticulocyte mRNA extracted from an individual with wild-type *RHAG (RHAG*01)* and was amplified by PCR with a primer pair (RHAG-ENT1; 5'-CACCATGAGGTTCACATTCCCTC-3' and RHAG-R2; 5'-CTGATTGTCAAGTTATCTCGTC-3'). The obtained amplicons were then cloned into a plasmid vector (pENTR/SD/D-TOPO; Thermo Fisher Scientific). The DNA for *RHAG* with c.490A> C (*RHAG*490C*) was synthesised using a mutagenesis kit (Toyobo, Osaka, Japan) with *RHAG*01* plasmid DNA as the template. Either the *RHAG*01* or *RHAG*490C* DNA fragment was incorporated into the pEHX1·1 expression vector (Mammalian PowerExpress System; Toyobo,) and was transfected into CHO cells using the polyethylenimine (PEI) transfection method. Stable cell lines were established by limiting dilution technology and the obtained *RHAG*01*-CHO (wild-type) and *RHAG*490C*-CHO (Lys164Gln) cell lines were examined by flow cytometry (FC500; Beckman Coulter, Tokyo, Japan) to measure the expression of Rh-associated glycoprotein (RhAG) and the Kg-antigen on the cell surface with the anti-RhAG (LA18·18) and OSK46 antibodies, respectively.

PCR amplification and HRM analysis

A set of the specific primers for detecting the c.490A> C mutation in the 3rd exon of the *RHAG* gene were designed. The primers were 5'-acccaaatgctgatcatgaca-3' (him_RHAG_18730f) and 5'-agatttgctcccatataccg-3' (him_RHAG_18900r). gDNA was amplified from Kg-positive and -negative samples with the primer sets by using the Type-it HRM PCR Kit (QIAGEN) and the resultant PCR products were subjected to Rotor-Gene Q (QIAGEN) analysis. PCR amplification was performed with an initial denaturation at 95°C for 5 min, followed by 45 cycles at 95°C for 10 s, 52°C for 30 s, and 72°C for 10 s. The amplified gDNA fragments were acquired at the 72°C step.[7] Amplified gDNA was conjugated with a fluorescent dye and heated from 65°C to 95°C. The temperature was increased by 0.1°C at each step using the Rotor-Gene Q PCR cycler for the HRM test, which covers the full range of expected melting points. HRM curves were then analysed using Rotor-Gene Q software (QIAGEN). HRM curves were determined for each of the wild-type, heterozygote and homozygote samples.

Results

Serological analyses of the family members with the Kg-antigen

Nine samples were obtained from the family members (Fig S1) [4] and were phenotyped with the anti-Kg antibody purified from the serum of the propositus's mother (II-3), which was kindly provided by Saitama Medical University.[3] Six samples turned out to be Kg-positive, with the remaining three Kg-negative. We titrated the anti-Kg antibody, resulting in the detection of a high-titre in the mother's serum. The titre for the anti-Kg antibody increased to as high as 1,024 folds during her second pregnancy, probably because she had Kg-positive twins (III-1, 2) from her first pregnancy; the levels declined after delivery. The titre of the anti-Kg antibody has maintained the >256 folds level, even 900 days after the delivery (Fig S2).

Monoclonal antibodies against the Kg-antigen

The monoclonal antibody (OSK46; No.460M004) was confirmed to react against the propositus' father (II-2) and other Kg-positive RBCs, but not against II-3 or other Kg-negative RBCs. Since the OSK46 antibody was not considered sufficient in terms of specificity for immunoprecipitation analysis, we further prepared a recombinant antibody, rOSK46. The rOSK46 monoclonal antibody was confirmed to specifically react with Kg-positive RBCs, but not with Kg-negative RBCs (Table SI).

Biochemical analyses of molecular candidates for the Kg-antigen

We next attempted to identify the target protein of the anti-Kg antibody. From the results of the immunoprecipitation analysis with the rOSK46 monoclonal antibody, a number of proteins were detected in the precipitate from the erythrocyte ghosts. To identify candidates for the Kg-antigen, we compared the amounts of each of the precipitated proteins among the mIgG1, rOSK46, HIRO-58, HIRO-195, HIRO-103 and HIRO-294 antibodies (Table SII). Further comparison revealed that the rOSK46 antibody precipitated Rh proteins (RhD or RhCE) and RhAG) to an especially higher degree than the other antibodies studied (HIRO-58, HIRO-195, HIRO-103 and HIRO-294) (Fig 1). However, the separation of these three proteins using conventional immunoprecipitation analysis was difficult, probably because of the formation of a trimeric complex *in vivo*. Hence, these results suggested that the rOSK46 antibody precipitated either RhD, RhCE, or RhAG, whose molecular complex carried the Kg-antigen.

Mutation analyses of the molecular candidates for the Kg-antigen

We then searched for a mutation in the genes to find out the determinant for the Kg-antigen. Each of the 1–10 exons of

the *RHD* and *RHCE* genes were first amplified from the gDNA isolated from the propositus. All exons were shown to present as expected and both *RHD* and *RHCE* genes had intact DNA sequences without any mutations present. Subsequently, exons 1–10 of the *RHAG* gene were amplified and the amplicons were subjected to DNA sequence analyses in order to detect the presence of a mutation. Although all 10 exons of the *RHAG* gene were present, DNA sequence analyses identified a novel point mutation in the 3rd exon, namely, a doublet peak indicating a heterozygous c.490A> C base substitution (GenBank Accession Number; LC508243, dbSNP:rs144305805) in the Kg-positive sample (Fig S3). This c.490A> C mutation led to the amino acid substitution (Lys164Gln) in RhAG.

Molecular nature for the Kg-antigen

We performed flow cytometry analysis to confirm that the expression of the Kg-antigen resulted from RhAG with the amino acid substitution. First, we confirmed that the transfectants, *RHAG*490C*-CHO (Lys164Gln) and *RHAG*01*-CHO (wild-type), similarly overexpressed the RhAG polypeptides on the cell surface (Fig 2). While the expression of the Kg-antigen was not detected in *RHAG*01*-CHO cells, it was detected in *RHAG*490C*-CHO cells. These results clearly indicated that the Kg-antigen was derived from the single amino acid substitution of Lys164 to Gln in RhAG.

Mass screening of the Kg-antigen and the RHAG mutation

We next performed Kg phenotyping on a total of 61,362 samples from independent Japanese donors, primarily from the western Japan, with the OSK46 monoclonal antibody at routine examination appointments. One hundred and forty-two were found to be Kg-positive, with a frequency of 0·22 %. No significant indications suggesting any regional or sex related differences in the individuals with the Kg-antigen were detected (data not shown).

For high-resolution melting (HRM) analysis, gDNA samples were amplified with two specific oligonucleotide primers and were further analysed using a Rotor-Gene Q PCR cycler. Fig 3A shows the representative HRM curves, indicating different melting temperatures among the homozygous A/A, homozygous C/C and heterozygous A/C alleles (A; wild-type, C; c.490 A> C in the 3rd exon of the *RHAG* gene). In total, 200 samples were subjected to HRM analysis (Fig 3B, C). Using serotype analysis, we first confirmed that 100 samples were Kg-positive and the others were Kg-negative. We then examined these samples by HRM analysis and found them to be completely compatible with those of the predetermining serotype analysis. We also confirmed that the HRM analysis was compatible with the results of the DNA sequence analyses (data not shown).

Discussion

For over 30 years, the molecular nature of the Kg-antigen had been unknown and the determinant gene had remained unidentified. In this study, we applied a proteomic approach for identifying the molecular nature of this antigen. The Kg-antigen was first discovered in a case with HDN (Fig S1).[3,4] The propositus, a male newborn, was the third of three children.[4] Although there were no significant haematological abnormalities detected in general in the individuals with the Kg-antigen, the newborn displayed severe anaemia (haemoglobin 7·8 g/dl), a marked increase (510‰) in reticulocyte counts and total bilirubin of 14·2 mg/dl, which is consistent

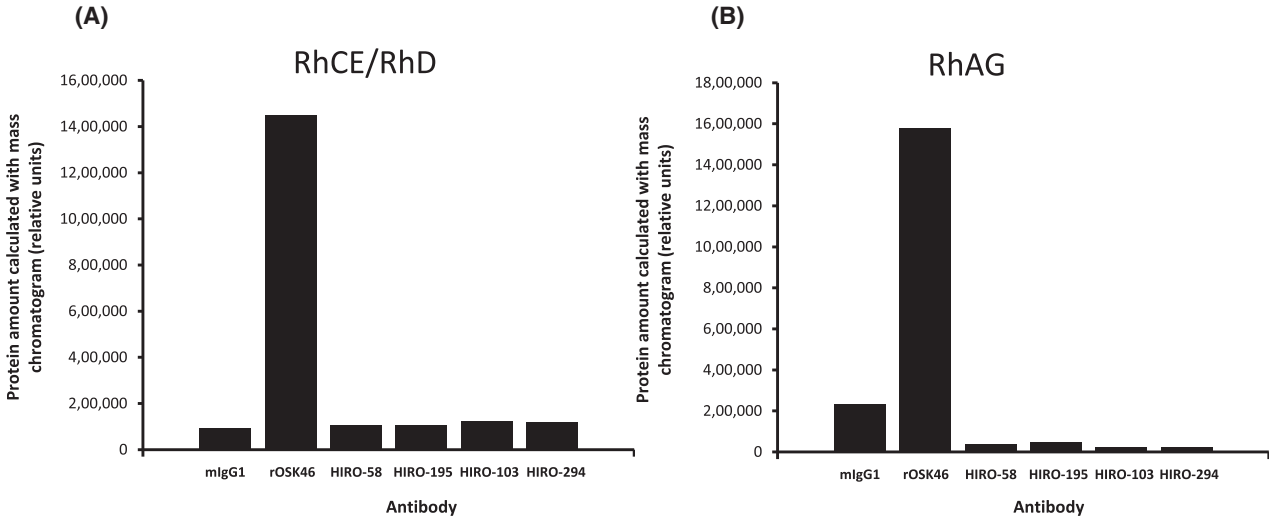

Fig 1. (A) Relative comparison of the protein amounts in the immunoprecipitants. Peak areas of RhD/RhCE and (B) RhAG were compared after total amounts of the protein in the immunoprecipitants were normalised. It was not clear whether either or both RhD/RhCE were detected because we could not distinguish these two proteins in the peptides assigned in mass spectrometry analyses.

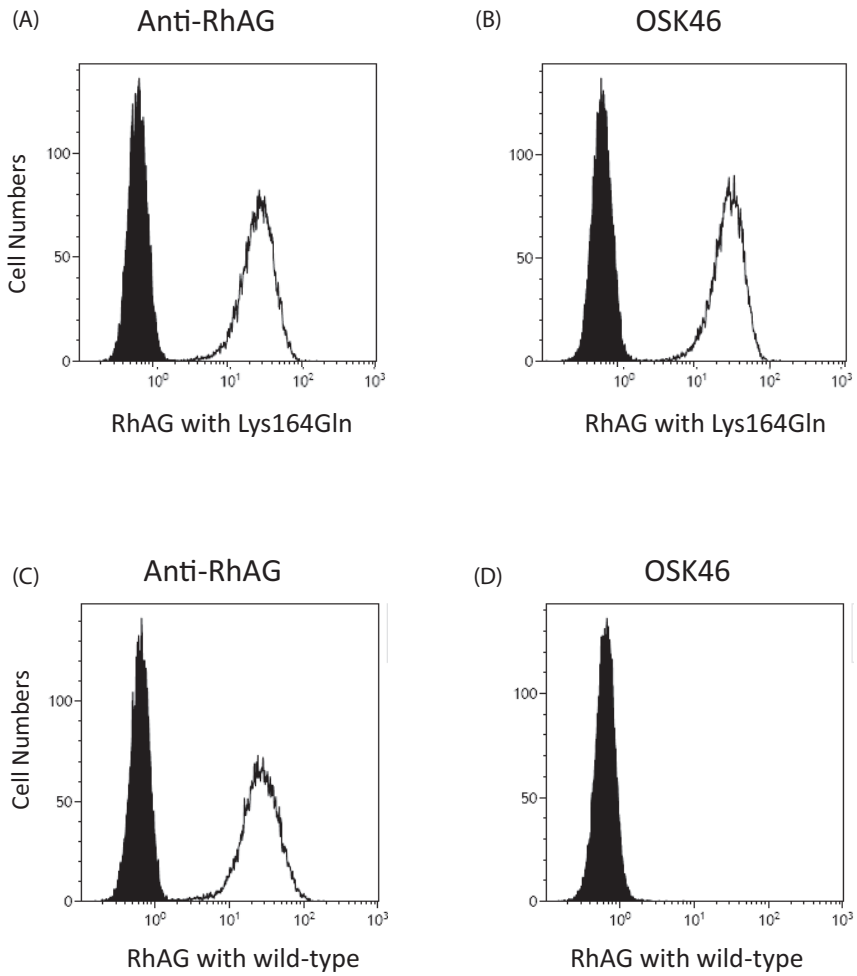

Fig 2. (A) and (B) Expression of wild-type RhAG and RhAG with a Lys164Gln mutation in the transfected CHO cells. *RHAG*490C*-CHO cells (Rh*AG* with Lys164Gln mutation; and (C) and (D) *RHAG*01*-CHO (RhAG wild-type;) cells were stained with the anti-RhAG and rOSK46 antibodies, respectively, and were analysed by flow cytometry. Untransfected CHO cells were added and were stained as negative controls.

with cases of severe haemolytic anaemia. Therefore, an exchange blood transfusion was performed. We performed a serotype analysis for all possible family members using serum from the propositus' mother and detected the Kg-antigen in 9 members of the family, among them, the propositus, his father and his two elder brothers (twins), but not his mother. His grandfather was also Kg-positive. The Kg-antigen was thus inherited through three generations in this family. Two of the cases with HDN were determined to be Kg-positive. Therefore, the Kg-antigen is clinically believed to give rise to HDN, although we could not currently provide further experimental evidence indicating that the Kg-antigen causes HDN through the antibody for the antigen. In addition, the anti-Kg antibody titre in the mother's serum was as high as 1,024 folds 2 months after delivery, according to antiglobulin methods,[4] indicating that the Kg-antigen is presumed to exert high immunogenicity. We then performed Kg-antigen screening in a Japanese population. From our results of the mass screening, the frequency of the Kg-antigen turned out to be 0·22%.

We have clarified here that the molecular nature for the Kg-antigen is a Lys164Gln mutation in RhAG. The RhAG protein has long been recognised as a homologue of RhD and RhCE and has been identified as a member of the Rh protein family, closely associated in the RBC membrane.[8,9] No developmental or other abnormalities in the individuals with the mutation in the *RHAG* gene were detected except for HDN. In 2010, a new blood group complex consisting of two antigens, Duclos and Ol^a, was found to be encoded by the *RHAG* gene.[10] The molecular nature of the Duclos and Ol^a antigens were determined to be Gln106Glu (c.316C> G in the 2nd exon) and Ser227Leu (c.680C> T in the 5th exon) mutations in RhAG respectively. In addition, a third antigen, DSLK, was reported from the UK. Furthermore, a homozygous c.490A> C (Lys164Gln) mutation, which was the same point mutation detected in our study, was reported in the

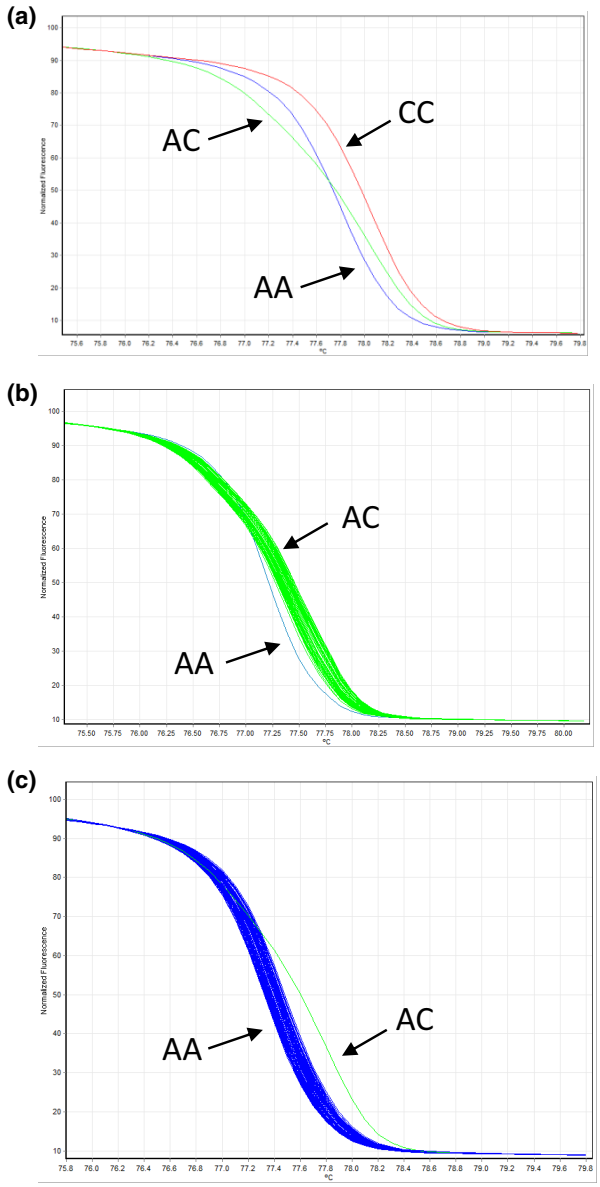

Fig 3. High resolution melting HRM) analysis. (A) The 3rd exon of the *RHAG* gene was amplified by PCR and analysed using HRM. Three types of HRM curves were obtained: curves (CC, AC, and AA) represent homozygous c.490C/C, heterozygous c.490A/C, and homozygous c.490A/A, respectively. (B) The HRM curves obtained from 100 Kg-positive samples. The curve AC indicated c.490A/C and curve AA was homozygous c.490A/A as a control. (C) The HRM curves obtained with Kg-negative 100 samples. The curve AA indicated homozygous c.490A/A and the curve AC was heterozygous c.490A/C as a control. [Colour figure can be viewed at wileyonlinelibrary.com]

3rd exon of the *RHAG* gene from a DSLK-negative individual.[10] However, it remained unclear how this *RHAG* mutation was associated with the DSLK antigen. Flow cytometry analysis of HEK293 cells expressing the RhAG protein with the Lys164Gln mutation and DSLK-negative RBCs showed the same reactivity with the monoclonal anti-RhAG (LA18·18) antibody.[10] Unfortunately, an anti-DSLK antibody reportedly failed to detect the cells expressing RhAG with the Lys164Gln mutation or wild-type cells by flow cytometry.[10] Therefore, we could not determine whether the Lys164Gln mutation resulted in the disappearance of the DSLK antigen. Ten years have passed since the DSLK antigen was discovered and the molecular nature of the antigen has remained unknown, even though a provisional number was assigned by ISBT for the DSLK-negative phenotype, to recognise the polymorphism. In this regard, the Kg-antigen may be antithetical to the DSLK antigen.

In our current study, the molecular nature of the Kg-antigen was shown to be *RHAG* with a Lys164Gln mutation. In addition, the Kg-antigen has been clinically presumed to give rise to HDN in the families we reported previously.[4] Severe immune responses against the Rh antigen, including the effector arm of the innate system, are known to be triggered in Rh-negative mothers carrying Rh-positive infants in a second pregnancy after a first pregnancy with an Rh-positive infant. The frequency of the RhD-negative status is 0·5% in the Japanese population, which is about 15% in the Caucasian population. RhD-negative women tend to develop anti-D antibody-sensitized pregnancy, with the prevalence of HDN at approximately 1 out of 500 pregnant women.[2] The clinical characteristics of HDN induced by the Kg-antigen appear to be similar to those of HDN induced by Rh incompatibility. However, the diagnostic system for the Kg-antigen has only been poorly established, which may have hampered the identification and prevention of HDN induced by the Kg-antigen.

The occurrence rate for HDN currently remains elusive in the second pregnancy of Kg-negative woman carrying a Kg-positive infant after her first pregnancy with Kg-positive one. Thus, we think that we first must detail the rate of occurrence of HDN and the severity in the second pregnancy with Kg incompatibility. When the occurrence rate is high enough and the clinical finding is significantly severe, we then have to practically consider the management of the pregnancy. Establishment of the clinical examinations for the Kg-antigen and also for the antibody against the Kg-antigen and the above described mutation analyses of the *RHAG* gene by using HRM analysis could help wider mass screening of the population with the Kg-antigen. This strategy is crucial for the prevention and therapeutic insight into HDN induced by the Kg-antigen.

Acknowledgments

We thank the laboratory staff at the Blood Center for performing the routine serological blood group determinations and the reagent production staff for preparing the OSK46 antibody. The authors also thank the blood group laboratory staff at the Japanese Red Cross Chu-Shikoku and Toukai-Hokuriku Block Blood Centers for performing the routine serological tests. The authors are grateful to Ms. Junko Takahashi, M.T for her help in the study.

Author contributions

M.T. designed the study, performed the genotyping and HRM analyses, and wrote the manuscript. T.H. contributed to the blood-type analysis and DNA collections; H. Takahashi and H. Tateyama to production of the monoclonal antibody (OSK46); T.M., H.A. and K.T. to production of the recombinant monoclonal antibody (rOSK46), and the proteomic approach; T.A. and K.O. to the proteomic approach; R.T., K.I. and K.O. to preparation of the recombinant protein and analysis; S.T. to collection of the samples, T.Y., T.K., Y.F., F.H., Y. Tani, and Y. Takihara to data analysis and discussion and Y. Takihara to the writing of the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no competing interest.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. The pedigree of the family with the Kg-antigen. We previously reported the family in reference 4. In this study, the expression of the Kg-antigen was further examined in additional family members. The arrow indicates the propositus with HDN (II-3). The Kg-antigen was detected in I-1, II-1, II-2, III-1, -2, -3 as indicated by closed squares and circles, respectively. An anti-Kg antibody was found in the serum from the propositus's mother (II-3). The occurrence of HDN, unconfirmed HDN and the clinical history for exchange-blood-transfusion (EBT) are indicated by HDN, HDN(?) and EBT, respectively. The upper row indicates the Rh blood types, while the lower row indicates the presence or absence of the Kg antigen

Fig S2. Time course of the titer for the anti-Kg (II-3) antibody after the delivery. The titer for the anti-Kg antibody was 1,024 folds just after the delivery. The levels declined to 512 and to 256 folds 400 and 863 days, respectively.

Fig S3. Comparison of the DNA sequences in a region of the 3rd exon of the *RHAG* gene. DNA sequence analysis indicated a heterozygous c.490A>C polymorphism (c.490 A/C) in the 3rd exon of the *RHAG* gene from the propositus with the Kg-antigen (a) and the wild-type (c.490 A/A) (b).

Table SI. Serologic tests with antibodies against the Kg-antigen.

Table SII. The list for the precipitated proteins by the mIgG1, rOSK46, HIRO-58, HIRO-195, HIRO-103, and HIRO-294 antibodies, which were analysed by using a mass spectrometer TripleTOF 5600 system.

Table SIII. PCR primers for the RHD, RHCE and RHAG genes.

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