Maternal natural killer cell immunoglobulin receptor genes and human leukocyte antigen-C ligands influence recurrent spontaneous abortion in the Han Chinese population

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Abstract. The underlying mechanism of recurrent spontaneous abortion (RSA) has remained elusive for many years. Several previous studies have suggested that the killer cell immunoglobulin receptor (KIR) gene family is associated with RSA, however, it is not clear exactly how. The present study detected KIR and human leukocyte antigen-C (HLA-C) genes in 110 Han Chinese women with unexplained RSA and 105 Han Chinese healthy females. The aim of the present study was to determine if certain genotypes were more susceptible to the occurrence of miscarriage. The frequency of KIR genes and different KIR haplotypes in the 2 groups demonstrated no statistical differences. However, in women who had miscarried ≥3 times, the frequency of KIR3DL1 was significantly reduced and the BB haplotype frequency was significantly higher compared with the control group. HLA-C2C2 was significantly increased in the KIR AB and KIR BB groups in the RSA groups compared with the control group. The women in the RSA group who had a homozygous HLA-C2C2 had a significantly higher frequency of the 2DS1 gene compared with the control group. The reduction of inhibitory gene and increased activation combinations may induce the activation of uterine natural killer cells, which may reduce the probability of fetal survival. To the best of our knowledge, the present study is the first report demonstrating the association between maternal KIR and HLA-C genes and RSA in women of a Han Chinese ethnicity. The present study revealed that females who miscarry ≥3 times may be used as selection criteria for RSA and so may exhibit higher research value.

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Introduction

Recurrent spontaneous abortion (RSA) is defined by the American Society for Reproductive Medicine (ASRM) as ≥2 consecutive spontaneous abortions, whereas the Royal College of Obstetricians and Gynecologists and the European Society for Human Reproduction and Embryology, specify it as the loss of ≥3 consecutive fetuses prior to 24 weeks of pregnancy with the same partner. Statistically, 15-25% of pregnancies result in miscarriage and <5% of women will experience two miscarriages, only 1% of women will experience the loss of a fetus ≥3 times (1,2). The causes of RSA are considered to include cytogenetic abnormalities, anatomic irregularities, endocrine disorders, infection, autoimmunity, atypical blood clotting, sperm quality and environmental factors (2). However, in ~50% of RSA cases no clearly defined etiology is identified (3).

During pregnancy fetal trophoblast cells infiltrate into the maternal uterine blood vessels and release blood into the intervillous space (4). The outer layer of the chorionic villi (syncytiotrophoblast) is bathed directly in maternal blood, which enables the fetus to easily acquire nutrients (5). However, the exposure of trophoblast cells may lead to potential allogeneic immune responses by the mother. Typically, uterine immune responses allow the placenta to acquire maternal supplies and also prevent excessive invasion (4). Disruption of the normal balance between the itinerant trophoblast cells and the uterine tissues they colonize during placentation, may result in various clinical problems (4). The hypothesis that maternal-fetal immune disorders lead to miscarriage may be traced back to the 20th century when Medawar postulated that the fetus may be considered as an allograft to the mother, and that the absence of a maternal immune response is what allows embryonic implantation (6).

Natural killer cells (NKs) are the third largest granular cells and they account for 10-15% of the total lymphocytes in the blood (7). NKs are activated by external stimuli and

they serve a vital role in immune regulation and defense (5). A number of uterine NKs (uNKs) gather in the endometrium prior to placentation and they make up 75% of all uterine decidua lymphocytes in early pregnancy (8). At 20 weeks of pregnancy the uNKs begin to decline and they disappear by the third trimester (9). Tissue NKs differ from circulating NKs as their phenotype and function are modified by the local microenvironment (10,11). A total of 90% of peripheral blood NK cells (pbNKs) are $CD56^{dim}$ cells, which highly express CD16 and possess clear cytotoxic activity (12). uNKs are primarily $CD56^{bright}$ cells, which lack cytotoxicity and instead secrete various cytokines, which serve an important role in adjusting the invasion of trophoblastic cells (13,14).

NK activity depends on the integration of signals from numerous germline-encoded activating and inhibitory receptors, which are primarily from three families: C-type lectin-like receptors, immunoglobulin (Ig)-like transcripts and killer cell Ig-like receptors (KIRs) (15,16). KIRs are encoded by an array of highly polymorphic genes located on chromosome 19q13.4, which produce wide variations in KIR structure and combination (17). KIRs are classified according to their structure and function. Each KIR molecule consists of two or three extracellular Ig domains (2D and 3D molecules, respectively), a transmembrane section and a short (S) or long (L) intracellular tail (18). Each inhibitory KIR receptor contains an immune receptor tyrosine inhibition motif in its cytoplasmic domain, which may interact with SH2-containing protein-tyrosine phosphatase-1 in the cytoplasm, and inhibit the activation of the NK cell (19). Each activating KIR, possesses a short charged cytoplasmic tail, which combines with the immune receptor tyrosine activation sequence or DAP12 adapter protein, thus enhancing activation signals and gene expression in NKs (16,20). To the best of our knowledge, 15 KIR gene loci and two pseudogenes have been identified to date, among which a marked linkage disequilibrium exists (21). Two common haplotypes, designated A and B, have been defined based on the number of KIR genes present. The A haplotype is less variable and is recognized by the presence of a minimum of 6 inhibitory receptor genes (KIR-2DL1, -2DL3, -2DL4, -3DL1, -3DL2 and -3DL3) as well as the only activating receptor gene (KIR2DS4) (22). The B haplotype has a more variable and numerous gene content, which differs due to the various possible combinations of activating receptor genes (23). There are three distinct groups of KIR genotype in humans, which are KIR-AA, -AB and -BB. At present, >550 different KIR genotype IDs have been described, which has greatly enriched the understanding of the KIR gene family (allelefrequencies.net/kir6001a.asp; update in 2015).

KIRs combine with human leukocyte antigen (HLA) ligands on the surface of target cells and are closely associated with virus infection, malignancy, autoimmune diseases, organ transplantation and the process of pregnancy (24-27). In recent decades, an increasing number of studies have indicated that KIRs may be associated with RSA. Certain previous studies consider a maternal KIR-AA homozygous genotype as a protective factor, which inhibits the activation of uNKs and prevents the fetus from being rejected (28-30). However, others have reported that the KIR-B haplotype promotes uNK activation and cytokine secretion, which is conducive to trophoblastic cell implantation into the endometrium and thus promotes a successful pregnancy (31,32). It is possible that some specific combinations of KIR genes may serve a role in the autoimmune mechanisms underlying recurrent miscarriage. Individuals from different regions and ethnic groups often have large differences in the frequencies of KIR genotypes. The frequency of KIR-AA ranges from 1.5% in the Australian aborigines to 67.9% in the China Yunnan Province Nu (33,34). As numerous factors may lead to miscarriage, it is necessary to consider these while analyzing the association between KIR and spontaneous abortion. Considering the geographical and ethnic differences that are observed in the KIR and HLA-C genes, the present study investigated only those of a Han Chinese ethnicity. The current study detected the KIR and HLA-C genes of 110 unexplained RSA and 105 healthy females. The aim of the present study was to determine if certain KIR genotypes influence the occurrence of spontaneous miscarriage.

Materials and methods

Study subjects and samples. In the present study the ASRM definition of RSA (as defined above) was adhered to. Couples with ≥2 recurrent spontaneous miscarriages prior to 20 weeks of pregnancy and no live births were recruited from Henan Provincial People's Hospital (Henan, China) between September 2015 and September 2016. The parental chromosomes were karyotyped. At day 3, the follicle-stimulating hormone, luteinizing hormone and testosterone levels were measured in the females in a clinical laboratory at the Henan Provincial People's Hospital (Henan, China) and the results were reported to us, an ultrasound examination or hysteroscopy was performed prior to the current study, semen quality was measured and a thyroid function test was performed in order to rule out abnormal thyroid function, which is related to miscarriage [thyroid stimulating hormone (TSH), triiodothyronine (T3), thyroxine (T4), free triiodothyronine (FT3), free thyroxine (FT4), anti-thyroid peroxidase antibodies (anti-TPO) and antithyroglobulin antibody (anti-TG)] (35). The blood glucose level and level of immune antibodies, including antiphospholipid, endometrial, sperm, anti human chorionic gonadotropin, anti zona pellucida and anti ovarian antibodies were measured and a TORCH-IgM (TOX-IgM, RV-IgM, CMV-IgM and HSV-IgM) screen was performed. Studies have demonstrated that TORCH-IgM antibodies have a strong association with the occurrence of RSA (36). Mycoplasma and chlamydia infection were tested for and blood coagulation function (PT, ARTT, FIB, TT) and D-dimer tests were performed identify if patients met inclusion criteria. The hypercoagulation of blood is associated with the occurrence of miscarriage (37).

Of the 188 patients tested who had normal chromosomes, 78 were excluded because the suspected reasons for their miscarriage were identified. Consequently, there were 110 couples of Han Chinese ethnicity that met the aforementioned conditions and were recruited into the experimental (RSA) group (age, 20-39 years). Each of the females enrolled in the study had no history of poisonous or teratogenic chemical exposure, lived a healthy lifestyle and had never fallen pregnant with a previous partner. The control group consisted of 105 healthy females (age, 19-39 years) recruited from the Henan Red Cross Blood

Center between November 2015 and September 2016, who had given birth to a minimum of 1 healthy child and had no history of miscarriage. The 2 groups had the same ethno-geographic origin and there were no differences in ages. All participants were informed about the study and written informed consent was obtained. Ethical approval for the present study was obtained from the Board of Ethics Committee of the Henan Provincial People's Hospital. Blood samples (5 ml) taken from all female participants were preserved in tubes containing EDTA at 4˚C prior to genetic evaluation.

DNA isolation. Maternal genomic DNA was isolated from 5 ml of blood using the TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd., Beijing, China). The quantity and quality of the DNA samples was detected using a NanoDrop™ 2000 spectrophotometer at wavelengths of 230, 260 and 280 nm (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The experiment procedure was conducted according to the manufacturer's protocol.

KIR genotyping. KIR genotyping was performed on maternal genomic DNA using a KIR Genotyping kit (cat. no. 54410D; Invitrogen; Thermo Fisher Scientific, Inc.), which detected the presence or absence of KIR genes using polymerase chain reaction (PCR)-sequence specific primers. Formulations of locus specific primers from the kit were used to amplify genomic DNA. The reaction buffer was mixed with the genomic DNA sample and Taq DNA polymerase (cat. no. M8296; Promega Corporation, Madison, WI, USA) in a 96-well thermal tray. Thermocyling was performed as follows: Initial denaturation of 95˚C for 1 min; 30 cycles of denaturation at 94˚C for 20 sec, annealing at 63˚C for 20 sec and extension at 72˚C for 90 sec. 2% agarose gel preparation was performed as follows: 2 g of agarose powder was poured into a 250 ml conical flask and 100 ml of 0.5X tetrabromoethane was dissolved in the flask. The microwave oven was heated to boiling and ethidium bromide (cat. no. 15585011; Thermo Fisher Scientific, Inc.) (6 μl) was added. The flask was shaken and the mixture was poured into the template and cooled for 30 min. When the thermocycling process was complete, 5 μl PCR products were loaded onto a 2% agarose gel using a pipette. Electrophoresis was carried out in the electrophoresis tank, which was filled with 0.5X tetrabromoethane. The gel was run at a voltage of 150 V for 20 min. Following electrophoresis, the gel was placed under UV light to observe and record the results.

HLA-C genotyping. For HLA-C1 and -C2 genotyping, the same primers were used as previously reported by Tajik *et al* (38). The PCR system in each reaction was 12 μl in total [60 ng genomic DNA (1 μl), 2X Taq PCR Master mix (Bio Basic, Inc., Markham, ON, Canada) (5 μl), nuclease-free water (5 μl), forward (0.5 μl) and reverse (0.5 μl) primers]. PCR conditions were: Initial denaturation for 2 min at 94˚C; 10 cycles of 10 sec denaturation at 94˚C and 60 sec annealing and elongation at 65˚C; 20 cycles of 10 sec denaturation at 94˚C, 50 sec annealing at 61˚C and 30 sec elongation at 72˚C; with a final extension step at 72˚C for 10 min. When the thermocycling process was complete, a total of 6 μl loading buffer (cat. no. B648314; Sangon Biotech Co., Ltd., Shanghai, China) was mixed with the amplification products and electrophoresed on 2% agarose gels. 2% agarose gel preparation was performed same as the above method and ethidium bromide was used as a DNA stain. The gel was run at a voltage of 150 V for 25 min. Following electrophoresis, the gel was placed under UV light (WD-9403C; Beijing Liuyi Biotechnology Co., Ltd., Beijing, China) to observe and record the results.

Statistical analysis. The percentage of KIR and HLA-C genes in the present study was determined by direct counting. The frequency of the two populations of KIR haplotypes (A and B) and HLA-C alleles (C1 and C2) were obtained using the Hardy-Weinberg principle ($p^2+2pq+q^2=1$) (39). All data were statistically analyzed using Chi-square analysis, Pearson Chi-square continuity correction and Fisher's exact test, on SPSS version 17 software (SPSS, Inc., Chicago, IL, USA). $P \leq 0.05$ was considered to indicate a statistically significant difference. Data are presented as numbers and percentages. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated.

Results

Carrier frequency of KIR genes in the RSA and control group. A total of 19 KIR genes were genotyped in all participants: 2DL1, 2DL2, 2DL3, 2DL4, 2DL5A, 2DL5B, 2DS1, 2DS2, 2DS3, 2DS4FUL, 2DS4DEL (2DS4 allele with a 22 base pair deletion), 2DS5, 3DL1, 3DL2, 3DL3, 3DS1, 2DP1, 3DP1FUL and 3DP1DEL (absence of exon 2 and its flanking intron sequences) (Table I and Fig. 1). The 4 framework genes (conservative gene positions fixed and present in each person's KIR gene), KIR2DL4, 3DL2, 3DL3 and 3DP1FUL or 3DP1DEL were present in all of the samples. The KIR pseudogene KIR2DP1 was present in all samples from the normal group, but only 99.1% of the samples from the experimental RSA group. In all participants, it was identified that compared with the activating KIR genes, the inhibitory KIR genes had aT higher frequency. Among the inhibitory KIR genes, KIR2DL1 demonstrated the highest frequency, as it was present in 99.1 and 100% of the experimental and control groups, respectively. The other most frequent inhibitory KIR genes were KIR2DL3 and KIR3DL1, which were present in ≥91.8% in each group. Apart from KIR2DS4 positive genotypes (KIR2DS4FUL, KIR2DS4DEL or combined genotypes), the frequency of the activating genes was <40%. KIR2DS2 demonstrated the lowest observed carrier frequency of any activating gene, as it was present in 18.2% of the experimental group and 16.2% of the control group. No statistically significant differences were identified between the frequency of each KIR gene in the experimental group and the control group. Additionally, no significant differences were identified in the frequency of the inhibitory and activated KIR genes in each group.

The experimental group was subsequently divided into 2 smaller groups depending on the number of miscarriages undergone by each participant. The frequency of the KIR3DL1 gene was significantly reduced in women who had aborted ≥3 times compared with the control group (P=0.025; OR, 0.277; CI, 0.085-0.904; Table II). However, there was still no significant difference identified between the frequency of the inhibitory and activating KIR genes in these 2 groups.

Table I. Comparison of the frequency of KIR genes in the RSA (n=110) and control (n=105) groups.

KIR gene		RSA group		Control group		P-value	Odds ratio	95% confidence interval
		n	%	n	%			
Inhibitory	2DL1	109	99.1	105	100.0	1.000	0.991	0.973-1.010
Inhibitory	2DL2	21	19.1	17	16.2	0.591	1.221	0.589-2.532
Inhibitory	2DL3	108	98.2	105	100.0	0.497	0.982	0.956-1.008
Inhibitory	2DL4	110	100.0	105	100.0	1.000	-	-
Inhibitory	2DL5A	41	37.3	37	35.2	0.757	1.095	0.615-1.950
Inhibitory	2DL5B	10	9.1	10	9.5	0.922	0.954	0.367-2.477
Activating	2DS1	44	40.0	40	38.1	0.783	1.083	0.614-1.912
Activating	2DS2	20	18.2	17	16.2	0.708	1.151	0.552-2.401
Activating	2DS3	26	23.6	23	21.9	0.774	1.102	0.568-2.135
Activating	2DS5	31	28.2	23	21.9	0.304	1.401	0.736-2.666
Inhibitory	3DL1	101	91.8	101	96.2	0.190	0.442	0.127-1.543
Inhibitory	3DL2	110	100.0	105	100.0	1.000	-	-
Inhibitory	3DL3	110	100.0	105	100.0	1.000	-	-
Activating	3DS1	42	38.2	35	33.3	0.470	1.238	0.694-2.210
Pseudogene	2DP1	109	99.1	105	100.0	1.000	0.991	0.973-1.010
Activating	2DS4.FUL	81	73.6	80	76.2	0.672	0.871	0.459-1.651
Activating	2DS4.DEL	56	50.9	47	44.8	0.388	1.277	0.733-2.227
Pseudogene	3DP1.FUL	15	13.6	8	7.6	0.168	1.914	0.751-4.874
Pseudogene	3DP1.DEL	109	99.1	105	100.0	1.000	0.991	0.973-1.010

RSA, recurrent spontaneous abortion; KIR, killer cell immunoglobulin-like receptor.

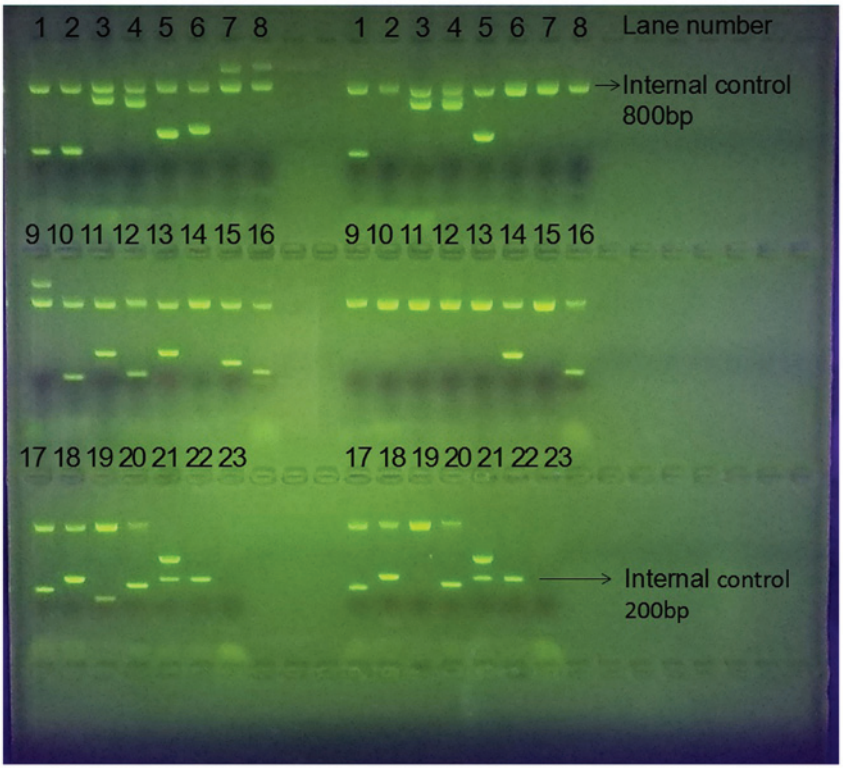

Figure 1. Representative agarose gel electrophoresis results. The DNA of two participants was separated by agarose gel electrophoresis to reveal their KIR genes. Each individual occupies 23 lanes consisting of 22 experiment lanes and one blank control lane (some genes were assigned duplicate lanes). In each experiment lane, there is an internal control polymerase chain reaction fragment (800 bp in lanes 1-20 and 200 bp in lanes 21-22). The bright bands in the experiment lanes correspond to the KIR genes. The two individuals' positive KIR genes are 2DL1, 2DL3, 2DL4, 2DS4FUL, 3DL1, 3DL2, 3DL3, 2DP1, 3DP1DEL (on the left) and 2DL1, 2DL3, 2DL4, 2DS4FUL, 2DS4DEL, 3DL1, 3DL2, 3DL3, 2DP1, 3DP1DEL (on the right). KIR, killer cell immunoglobulin-like receptors; bp, base pairs.

Table II. Comparison of the frequency of KIR alleles in women who had miscarriage ≥3 times (n=40) and the control group (n=105).

KIR genes		Patients		Control group		P-value	Odds ratio	95% confidence interval
		n	%	n	%			
Inhibitory	2DL1	39	97.5	105	100.0	0.340	0.975	0.945-1.006
Inhibitory	2DL2	6	15.0	17	16.2	0.815	0.913	0.425-1.960
Inhibitory	2DL3	39	97.5	105	100.0	0.340	0.975	0.945-1.006
Inhibitory	2DL4	40	100.0	105	100.0	1.000	-	-
Inhibitory	2DL5A	14	35.0	37	35.2	0.976	0.991	0.555-1.772
Inhibitory	2DL5B	2	5.0	10	9.5	0.220	0.501	0.164-1.537
Activating	2DS1	15	37.5	40	38.1	0.930	0.975	0.550-1.727
Activating	2DS2	6	15.0	17	16.2	0.815	0.913	0.425-1.960
Activating	2DS3	8	20.0	23	21.9	0.741	0.892	0.451-1.763
Activating	2DS5	12	30.0	23	21.9	0.191	1.528	**0.807-2.894**
Inhibitory	3DL1	35	87.5	101	96.2	**0.025**[a]	**0.277**	0.085-0.904
Inhibitory	3DL2	40	100.0	105	100.0	1.000	-	-
Inhibitory	3DL3	40	100.0	105	100.0	1.000	-	-
Activating	3DS1	14	35.0	35	33.3	0.800	1.079	0.601-1.935
Pseudogene	2DP1	39	97.5	105	100.0	0.340	0.975	0.945-1.006
Activating	2DS4.FUL	28	70.0	80	76.2	0.323	0.729	0.389-1.366
Activating	2DS4.DEL	17	42.5	47	44.8	0.743	0.911	0.521-1.593
Pseudogene	3DP1.FUL	5	12.5	8	7.6	0.249	1.737	0.673-4.481
Pseudogene	3DP1.DEL	39	97.5	105	100.0	0.340	0.975	0.945-1.006

[a]P<0.05 patient group vs. the control group. RSA, recurrent spontaneous abortion; KIR, killer cell immunoglobulin-like receptor.

KIR genotypes. The KIR genotypes were classified into three groups (AA, AB and BB) in accordance with the following rules: i) The four framework genes KIR2DL4, 3DL2, 3DL3 and 3DP1 were present in all haplotypes; ii) the A haplotype contained at least six loci encoding inhibitory receptors (KIR3DL3, 2DL3, 2DL1, 3DP1, 2DL4, 3DL1 and 3DL2) and only one activating receptor (KIR2DS4) (40); and iii) if activating receptors characteristic of the B haplotypes were detected, the participants were designated as having either an AB genotype or a BB genotype.

In the present study, the RSA group was revealed as having 19 different genotypes, whereas the control group only had 17 (Fig. 2). The frequency of KIR genotypes was similar in the RSA and control group. The most frequent genotype identified in all participants (50.9% of the RSA group and 53.3% of the control group) consisted of the 3DL1, 2DL1, 2DL3, 2DS4, 2DL4, 3DL2, 3DL3, 2DP1 and 3DP1 genes, which corresponds with an AA genotype (genotype ID 1; Table III). The KIR AB genotypes accounted for 40.0 and 42.9% of patients in the RSA and control groups, respectively. The frequency of KIR BB genotypes was revealed as 9.1% of patients in the RSA group, compared with 3.8% of patients in the control group (P=0.127; OR, 2.534; CI, 0.740-8.679). The haplotypes may also be subdivided into centromeric and telomeric contents: Cen-A (2DL3 and 2DL1), Tel-A (3DL1 and 2DS4), Cen-B (2DS2, 2DL2, 2DL5B and 2DS3) and Tel-B (3DS1, 2DL5A, 2DS5 and 2DS1). Comparing the control group with the RSA group, no significant differences were identified between the centromeric and telomeric classifications exhibited.

When the women who had miscarried ≥3 times were compared with the control group, the rate of the BB haplotype was significantly higher (P=0.025; OR, 3.617; CI, 1.107-11.818; Table IV). Additionally, the frequency of the Tel-BB haplotype was also significantly higher in the women who have aborted ≥3 times compared with the control group (P=0.025; OR, 3.617; CI, 1.107-11.818).

Frequency of HLA-C alleles in RSA patients and controls. The frequencies of the HLA-C alleles C1 and C2, in the RSA and control groups were analyzed. No significant differences were identified between the two groups (Tables V and VI). The present study also analyzed the distribution of the HLA-C genotypes of KIR AA, AB and BB in the 2 groups (Tables V and VI). The frequency of HLA-C2C2 in KIR AB and KIR BB was significantly higher in the RSA group compared with the control group (P=0.024 for KIR AB; P<0.001 for KIR BB; Table V and P=0.014 for KIR AB; P<0.001 for KIR BB; Table VI). The co-existing frequency of KIR2DS1-C2 genes in the RSA group was slightly higher than in the control group, however it was not statistically different (20.9 vs. 18.1%, P=0.617). The patients in the RSA group who had a homozygous HLA-C2C2 had a significantly higher frequency of the 2DS1 gene compared with the control group (45.5 vs. 0.0%, respectively; P<0.001; Fig. 3).

Table III. Frequency of the KIR genotypes carriers in the RSA (n=110) and the control (n=105) groups.

	RSA group		Control group				
KIR genotype	n	%	n	%	P-value	Odds ratio	95% confidence interval
KIR							
AA	56	50.9	56	53.3	0.734	0.908	0.521-1.582
AB	44	40.0	45	42.9	0.677	0.887	0.505-1.558
BB	10	9.1	4	3.8	0.127	2.534	0.740-8.679
Cen							
AA	71	64.5	74	70.5	0.365	0.760	0.420-1.377
AB	37	33.6	31	29.5	0.533	1.209	0.665-2.198
BB	2	1.8	0	0.0	0.497	0.982	0.956-1.008
Tel							
AA	65	59.1	62	59.0	0.989	1.004	0.571-1.764
AB	36	32.7	39	37.1	0.514	0.824	0.460-1.475
BB	9	8.2	4	3.8	0.190	2.261	0.648-7.889

RSA, recurrent spontaneous abortion; KIR, killer cell immunoglobulin-like receptor; Cen, centromeric; Tel, telomeric.

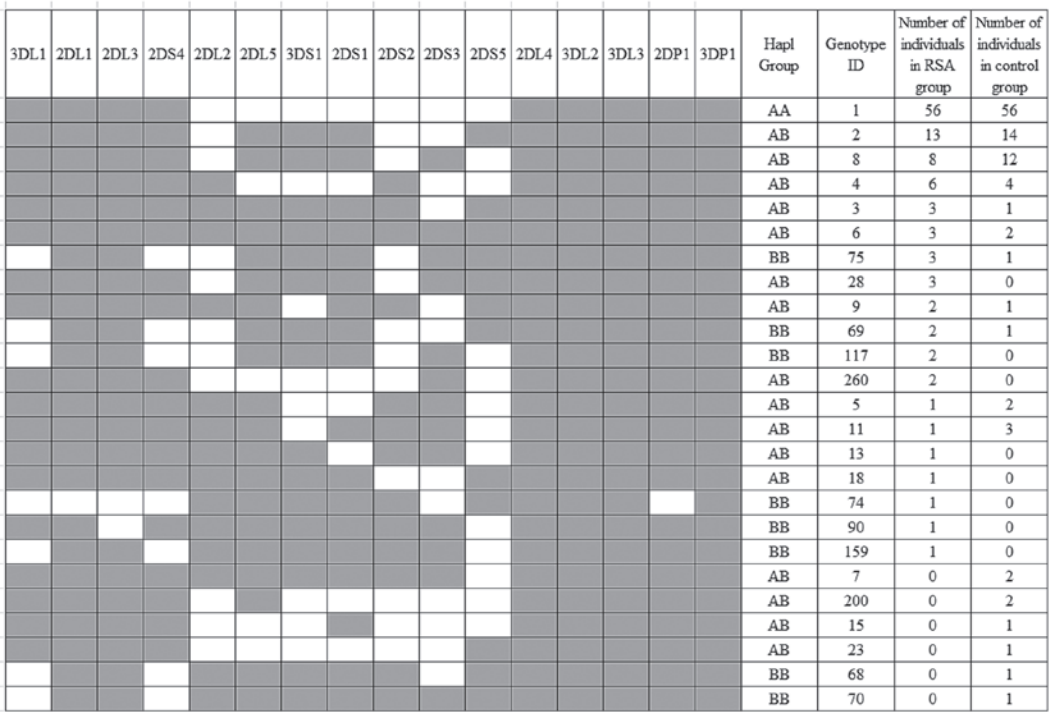

Figure 2. Distribution of KIR genotypes in the RSA and control group. A total of 16 KIR genes were used to distinguish KIR A and B haplotypes. The gray boxes represent the presence of the KIR gene and the white boxes represent their absence. A total of 19 and 17 KIR genotypes were identified in the RSA and control groups, respectively. KIR, killer cell immunoglobulin-like receptor; RSA, recurrent spontaneous abortion.

Discussion

During a normal pregnancy, a fetus grows in the maternal womb for nearly 40 weeks as an allogeneic entity without causing immune rejection. Immune dysfunction at the maternal-fetal interface may be associated with RSA (41). Previous studies have revealed that uNKs serve an important role in regulating the invasion of trophoblastic cells (13,14). During pregnancy a large number of uNKs converge in the maternal decidua and express KIRs (8). Meanwhile, the placental villous trophoblastic cells express HLA-C, -E, and -G molecules, which predominantly combine with uNK receptors (42-44). As fetal trophoblast cells make direct contact with the uterine tissues, appropriate recognition and immune responses in the maternal-fetal interface are essential for fetal growth and to prevent excessive placenta invasion or allogeneic rejection (4). Human uNKs secrete abundant cytokines and angiogenic growth factors, which serve a prominent

Table IV. Frequency of KIR genotypes carriers in women who had miscarriage ≥3 times (n=40) and the control group (n=105).

	RSA group with ≥3 miscarriages		Controls				
KIR genotype	n	%	n	%	P-value	Odds ratio	95% confidence interval
KIR							
AA	22	55.0	56	53.3	0.809	1.071	0.614-1.868
AB	13	32.5	45	42.9	0.129	0.641	0.360-1.140
BB	5	12.5	4	3.8	0.025[a]	3.617	1.107-11.818
Cen							
AA	27	67.5	74	70.5	0.646	0.869	0.477-1.583
AB	12	30.0	31	29.5	0.938	1.024	0.559-1.878
BB	1	2.5	0	0.0	0.246	0.975	0.945-1.006
Tel							
AA	25	62.5	62	59.0	0.612	1.158	0.656-2.044
AB	10	25.0	39	37.1	0.064	0.565	0.308-1.038
BB	5	12.5	4	3.8	0.025[a]	3.617	1.107-11.818

[a]P<0.05 patient group vs. the control group (P=0.025). RSA, recurrent spontaneous abortion; KIR, killer cell immunoglobulin-like receptor; Cen, centromeric; Tel, telomeric.

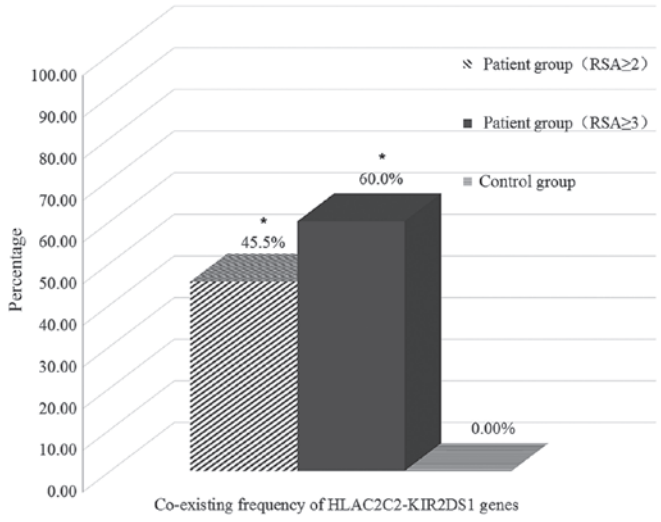

Figure 3. Co-existing frequency of C2C2-KIR2DS1 genes in the RSA and control groups. The women in the RSA group who had a homozygous HLA-C2C2 had a significantly higher frequency of the 2DS1 gene compared with the control group. In women who had aborted ≥3 times this frequency increased to 60%. *P<0.001 vs. the control group. RSA, recurrent spontaneous abortion; KIR, killer cell immunoglobulin-like receptor; HLA, human leukocyte antigen.

role in trophoblast cell invasion and placentation (13,14). Changes to the number or function of uNKs may lead to the development of an abnormal uterine local environment, which makes it difficult for the fetus to survive (45). Several previous studies have demonstrated that certain deleterious HLA-KIR pairings affect the secretion of cytokines and the expression of receptors, which may result in a range of pregnancy complications, including pre-eclampsia, fetal growth restriction and RSA (27,31,46).

A recent study demonstrated that significant differences exist in the frequency of KIR3DL1, 2DS4, 2DS1 and 2DS5 genes in 10 Han Chinese populations form different regions in China (47). This suggests that investigators who have considered the differences between ethnic groups, but have not considered the differences in geographical regions, may form mistaken conclusions when analyzing the correlations between KIR and disease. In the present study, the variation of KIRs in different ethnic and geographical groups was fully considered and all participants recruited to the study were from the same area and of the same ethnicity with no other regional migration known for three generations. The KIR and HLA-C genes present in the Han Chinese normal women and patients with RSA were detected, and it was investigated whether certain genotypes were more susceptible to RSA. To the best of our knowledge, the present study is the first time an association has been demonstrated between the uNK KIR and HLA-C genes in women of Han Chinese ethnicity with RSA. As it was not possible to collect all of the aborted fetuses from patients with RSA, there is a possibility that chromosomal aneuploidy in the fetus may have led to miscarriage, although this is unlikely to have been the cause in all cases. A previous study evaluated the karyotypes of the products of conception in women with RSA and revealed the frequency of abnormal chromosomes did not differ from the whole population (48).

The mechanism by which NK cells develop a tolerance to normal self-tissues depends mainly on the 'missing self' mechanism (49). In a normal situation, inhibitory KIRs interact with specific cognate HLA ligands that generate inhibitory signals and thereby prevent NK cells from being activated (50). The interactions of activation receptors with their self-specific ligands may result in NK cells hyporesponsiveness or reduced expression of cognate receptors (51). A previous study revealed that many NK cells with anti-HLA-C2 reactivity were present

Table V. Comparison of the frequency of HLA-C genotypes in the RSA (n=110) and control (n=105) groups.

KIR genotype	HLA-C genotype	RSA group		Control group		P-value
		n	%	n	%	
Total no. of genotype carriers in each group	C1C1	53	48.2	48	45.7	0.723
	C1C2	46	41.8	50	47.6	0.409
	C2C2	11	10.0	7	6.7	0.399
KIRAA	C1C1	25	44.6	21	37.5	0.307
	C1C2	25	44.6	28	50.0	0.444
	C2C2	6	10.7	7	12.5	0.691
KIRAB	C1C1	24	54.5	25	55.6	0.876
	C1C2	17	38.6	20	44.4	0.405
	C2C2	3	6.8	0	0.0	0.024[a]
KIRBB	C1C1	4	40.0	2	50.0	0.155
	C1C2	4	40.0	2	50.0	0.155
	C2C2	2	20.0	0	0.0	<0.001[a]

[a]P<0.05 patient group vs. the control group. Pearson Chi-square continuity correction was performed. RSA, recurrent spontaneous abortion; KIR, killer cell immunoglobulin-like receptor; HLA-C, human leukocyte antigen-C.

Table VI. Comparison of the frequency of HLA-C genotypes in women who miscarried ≥3 times (n=40) and the control group (n=105).

KIR genotype	HLA-C genotype	RSA group with ≥3 miscarriages		Controls		P-value
		n	%	N	%	
Total no. of genotype carriers in each group	C1C1	19	47.5	48	45.7	0.799
	C1C2	16	40.0	50	47.6	0.279
	C2C2	5	12.5	7	6.7	0.164
KIRAA	C1C1	9	40.9	21	37.5	0.622
	C1C2	11	50.0	28	50.0	1.000
	C2C2	2	9.1	7	12.5	0.439
KIRAB	C1C1	8	61.5	25	55.6	0.397
	C1C2	4	30.8	20	44.4	0.047[a]
	C2C2	1	7.7	0	0.0	0.014[a]
KIRBB	C1C1	2	40.0	2	50.0	0.155
	C1C2	1	20.0	2	50.0	<0.001[a]
	C2C2	2	40.0	0	0.0	<0.001[a]

[a]P<0.05 patient group vs. the control group. Pearson Chi-square continuity correction was performed. RSA, recurrent spontaneous abortion; KIR, killer cell immunoglobulin-like receptor; HLA-C, human leukocyte antigen-C.

in HLA-C1 homozygous and heterozygous healthy individuals with 2DS1, but not in HLA-C2 homozygous donors (52). This suggests that the functions of NK cells *in vivo* are critically regulated. A self-stabilization mechanism may exist in NK cells, allowing them to maintain homeostasis by self-regulating, however, when interference forces are above the self-regulation ability, referred to as the 'threshold effect', an immune attack will be activated and external factors are required to restore homeostasis (53).

In the present study, it was demonstrated that the KIR AA haplotype frequency of the two groups was similar, which was in line with the results reported by other studies in Han Chinese populations (54,55). No statistical significances were identified in the frequency of KIR genes in the RSA group compared with the control group. No significant difference was identified in the frequency of HLA-C alleles between the patients with RSA and the controls. A similar conclusion was reached by Christiansen *et al* (56). In the present study, the frequency

of the KIR3DL1 gene was significantly reduced in women who had aborted ≥3 times (n=40) compared with the control (n=105). The inhibitory KIR3DL1 gene is one of the most polymorphic KIR genes (57). The ligand for the KIR3DL1 receptor is the Bw4 epitope present on several HLA-B molecules, which is not expressed by fetal trophoblast cells (58). In patients with pulmonary artery hypertension, the expression of 3DL1 is lower and its function is disrupted, suggesting a central role for KIRs in the occurrence and development of immune-associated vascular diseases (59). The decreased frequency of the KIR3DL1 gene in patients who have miscarried ≥3 times may lead to the weakened inhibition of NK cells, which contributes to the imbalance of steady state and works against fetal survival. The different significance results that were identified between women who had miscarried ≥3 times and those who had not, suggests that this should be considered as a selection criteria when miscarriage diseases are being researched. A previous study also demonstrated that there was a decreased expression frequency of the uNK KIR2DL1 receptor in women with RSA who carried a C2 epitope (10). In the present study, the high rate of HLA-C2C2 in KIR AB and KIR BB individuals may decrease the expression of the KIR2DL1 receptor and simultaneously generate strong activation effects, which breaks the self-adjustment ability of NKs and activates NK-mediated immunological rejection of the fetus.

Komlos *et al* (60) first demonstrated that couples sharing common HLA antigens had a significantly higher percentage of repeated miscarriages compared with the control group. This led to further research on the subject and certain studies identified that KIRs were associated with RSA, however, there are disagreements over the underlying mechanisms. Varla-Leftherioti *et al* (61) revealed there was a lower frequency of inhibitory KIRs (inhibitory 2DL1, 2 and 3) in the peripheral blood and decidual tissue of women suffering multiple miscarriages, compared with fertile females. Other studies identified inhibitory genotypes as more common in the control subjects and activating genotypes as more common in patients with RSA (29,30,46,62). The frequency of the KIR2DS1 gene has been demonstrated to be significantly higher in patients with RSA compared with healthy controls (63). However, the frequency of activating KIR genes demonstrated no significant differences between the two groups in the present study. The KIR2DS1 gene combined with a specific ligand may generate a strong activation effect in NKs (64). The activation of KIR2DS1+uNKs, stimulated by fetal HLA-C2, may form soluble products, including granulocyte-macrophage colony-stimulating factor, which enhances the migration of primary trophoblast cells (65). A recent study also indicated that the co-expression of KIR2DS1 and HLA-C2 may be associated with RSA (66). In the present study, the co-existing frequency of KIR2DS1-C2 genes in patients with RSA was slightly higher than in the control groups (20.9 vs. 18.1%). However, the women in the RSA group who had a homozygous HLA-C2C2 had a significantly higher frequency of the 2DS1 gene compared with the control group (45.5 vs 0.0%; P<0.001). Furthermore, in women who had aborted ≥3 times this frequency was at 60.0%. This supports the hypothesis that strong activation effects surpass the self-regulation of NKs and alter their secretory activity into cell toxicity, which may ultimately lead to spontaneous miscarriage.

Previous research reported that the frequency of the KIR BB genotype was significantly lower and the KIR AB genotype was significantly higher in the RSA group compared with the control cohort (29). Additionally, infertile patients who carry the KIR A haplotype experienced fewer pregnancy losses following euploid single-embryo transfer compared with KIR B haplotype carriers (28). In the Han Chinese population, the KIR AA genotype has a high prevalence, which may reach up to half of the total number of the population in some areas (47), however, no difference was identified in the frequency of the KIR AA genotype between the two groups in the present study. The rate of the BB genotype was significantly higher in women who had aborted ≥3 times compared with the control group. Alternatively, certain previous studies have suggested that the maternal AA genotype is associated with an increased risk of pregnancy complications and the activation of uNK cells is a generic mechanism promoting trophoblast invasion into the decidua (27,31,32,46,67). The 2DL1 gene in all AA individuals represents the highest inhibitory influence when interacting with HLA-C2, which is characterized by a lack of appropriate cytokines to enforce uterine arterial remodeling, thus leading to poor placentation and ultimately fetal loss (32,46,58,68). In the present study, these tendencies were not identified.

In summary, many viewpoints have been published on how RSA is associated with individual genetics, but the underlying molecular basis of the disease has not yet been determined. To date, a variety of therapeutic regimens have been used in the clinical treatment of RSA, with varying results (6,69,70). Meta-analyses have concluded that paternal cell immunization, third-party donor leukocytes, trophoblast membranes and intravenous immunoglobulin trials did not improve the live birth rate or have a beneficial effect in women with previous unexplained recurrent miscarriage (69,71-73). A full understanding of the underlying pathogenesis of RSA is required prior to the development of an effective treatment plan, which may assist patients in completing successful pregnancies. The results of the present study revealed that in patients with RSA (miscarried ≥3 times), the frequency of inhibitory genes was reduced while activated gene combinations increased when compared with the control group. The self-activation of the maternal NK cells or the reduction of the activation threshold may result in NK cells becoming susceptible to irritability, which may be associated with the occurrence of RSA. The experimental results provide an insight for further study on miscarriage induced by maternal-fetal interface immune disorders. In addition, the results may have clinical therapeutic significance, helping select suitable fertilized eggs for women who are at high risk of recurrent spontaneous abortion and reduce the activation of uNKs and the incidence of pregnancy complications. Comprehensive analysis of large groups is required to obtain useful results. This is dependent on concerted efforts from multiple regions due to the varied nature of KIR genes throughout different ethnicities and locations.

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