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Age-Related Macular Degeneration Is Associated with the HLA-Cw*0701 Genotype and the Natural Killer Cell Receptor AA Haplotype

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Abstract

Purpose—To determine the association of human leukocyte antigen (HLA) C and its cognate killer cell immunoglobulin-like receptor (KIR) ligands with age-related macular degeneration (AMD).

Methods—HLA class I allele groups including the HLA-C principal alleles were genotyped in a cohort of 104 AMD cases and 93 controls by using the PCR-SSP (sequence-specific primers) method. This cohort was then genotyped for 16 KIR genes by PCR-SSP. Frequencies of the tested HLA/KIR alleles were then compared between patients with AMD and normal control subjects. HLA-C1, -Cw*07, and -Cw*0701 genotypes and their combinations with KIR genotypes/haplotypes were tested for association with AMD. Probabilities were obtained with a two-tailed χ^2 test and Bonferroni correction applied for multiple testing (P_c).

Results—The HLA-Cw*0701 allele, in combination with the inhibitory KIR AA haplotype was associated with AMD after logistic regression analysis ($P = 0.006$, $P_c = 0.036$, OR = 4.35, 95% CI = 1.41–13.44).

Conclusions—The HLA-Cw*0701 allele and KIR haplotype AA are associated with AMD. This genotype combination suggests that natural killer cells have a role in the pathogenesis of AMD. Replication studies are needed to confirm these novel HLA-KIR associations with AMD.

Age related macular degeneration (AMD) is the commonest cause of irreversible visual loss in the Western world, affecting approximately 25 million people worldwide.1,2 There are now multiple lines of evidence suggesting an important role for inflammatory events in the

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pathogenesis of AMD. Histologically, extracellular drusen deposits in the retina of patients with AMD have been shown to contain proteins that modulate the body's response to inflammation. These proteins include vitronectin, complement, and immunoglobulins.3 Inflammatory cells including macrophages,4–6 multinucleate giant cells,5,7,8 fibroblasts, and mast cells have been observed in association with Bruch's membrane in AMD donor eyes.9 Some characteristics of AMD have also been described in mice with macrophage defects.10 In addition, natural killer (NK) cells, which are lymphocytes of the innate immune system, have been described in subretinal neovascular lesions seen in patients with AMD.11 NK cells may therefore also be associated with the pathogenesis of AMD.

The human leukocyte antigen (HLA) system is essential for the immune regulation of self and foreign peptides via presentation of processed antigenic peptides to both CD4 helper and CD8 cytotoxic T lymphocytes. We previously reported the association of HLA with age-related macular degeneration (AMD).12 Individuals harboring the HLA-Cw*0701 allele were found to have an increased risk of AMD.12 Evidence from this study points to an important mechanism that may contribute to susceptibility for immune-mediated attacks on the RPE or endothelial cells in AMD. However, the precise nature of how this HLA association contributes to AMD is unknown.

A possible mechanism by which HLA class I molecules may be associated with AMD is via killer cell immunoglobulin-like receptors (KIRs). KIRs are regulatory molecules that are expressed predominantly by NK cells and also T cells.13 NK cells interact with HLA class I (A, B, C) ligands through their KIR receptors. Particularly relevant to NK recognition by KIRs are polymorphic HLA-C molecules. Through interaction with inhibitory KIRs, HLA-C molecules are able to modulate NK cell function.

Healthy cells are protected from spontaneous killing when they express an appropriate HLA class I ligand for an inhibitory KIR receptor expressed on NK cells. This observation corresponds with the reported phenotypic dominance of KIR-mediated inhibition over activation.14 However, aberrant or reduced levels of HLA class I expression can result in spontaneous destruction by NK cells. In this context, the expression patterns of HLA class I and II antigens in the choroid and sub-RPE deposits may be important.12 Notably, the presence of class II antigens in drusen and RPE cells15 and the apparent lack of class I antigens.

It follows that certain combinations of HLA-C and KIR gene variants may influence susceptibility to AMD. To test this hypothesis we analyzed HLA-C and KIR genotypes, both individually and in combination for association with AMD.

Methods

The study was approved by the Southampton Local Research Ethics Committee (approval no. 347/02/t) and adhered to the tenets of the Declaration of Helsinki. After informed, written consent, Caucasian subjects older than 55 years with a diagnosis of AMD and normal Caucasian control subjects older than 55 were recruited from general ophthalmology clinics at Southampton Eye Unit. Patients for the study underwent a detailed ophthalmic

examination to characterize AMD phenotypes. Stereoscopic fundus photographs and fluorescein angiograms were recorded with a digital retinal camera (model TRC50IX; Topcon, Tokyo, Japan). These photographs and angiograms were classified by a masked observer into geographic atrophy or choroidal neovascularization (CNV) subgroups. The CNV AMD group was further divided into occult, minimally classic, and predominantly classic CNV subgroups. General health was assessed, and care was taken to exclude patients who reported any infective illness in the preceding month. Information was also obtained about family history of AMD, relevant medical history, smoking history, ocular history; use of medications, vitamin or dietary supplementation, and body mass index (BMI). A 10-mL peripheral blood sample was collected from the patients, and DNA was then extracted by the salting-out method16 and stored at −20°C.

HLA and KIR Genotyping by PCR-SSP

HLA genotyping for principal HLA class I allele groups including the Cw allele was performed in 104 AMD cases and 93 age-matched healthy controls using PCR-SSP (sequence-specific primers)17,18 methodology as described previously for HLA typing.12 This group of patients and control subjects were then also genotyped for KIR genes.

The KIR locus contains polymorphic and homologous genes mapping to chromosome 19, region q13.4. KIR genotyping can be locus- or allele-specific. Locus-specific genotyping which detects the presence or absence of each gene in a given individual was used in this study. A genotyping kit (Pel-Freez KIR; Dynal Biotech, New Hyde Park, NY) was used to detect the presence or absence of 16 KIR genes (2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1, 2DP1, and 3DP1). This kit utilizes PCR sequence-specific primer (PCR-SSP) amplification methods published previously.19,20 The primer sets amplify 16 KIR alleles described by the international nomenclature committee of the World Health Organization (WHO; http://www.ebi.ac.uk/ipd/kir/).

Gel images of PCR products were photographed under ultraviolet transillumination and interpreted by two independent observers (SVG, AJL). Genotyping was validated by random repeat typing in 10% of cases and controls, for each of the HLA and KIR genotypes which showed a trend for significance ($P < 1.0$) for association with AMD in the study.

Statistical Methods and Analysis

Power calculations were performed with the Epi Info statistical package (ver. 3.4.1, 2007) provided by the Centers for Disease Control and Prevention (CDC; available at www.cdc.gov/epiinfo). These calculations were based on the number of cases and controls to be genotyped and the known frequency of specific HLA/KIR alleles. Power calculations were performed the 80% level to detect significant differences in HLA and KIR genotype frequencies between cases and controls at $\alpha = 0.05$. Allelic distributions for each locus were tested for conformity to Hardy-Weinberg equilibrium. Alleles at each locus were said to be in Hardy-Weinberg equilibrium if the observed homozygote and heterozygote frequencies did not differ significantly ($P > 0.05$) from those expected.

KIR and HLA genotype frequencies were compared in cases and controls by two-tailed probabilities calculated with the χ^2 test. Adjustment for multiple comparisons was made

using the Bonferroni method (i.e., probabilities for each comparison ≤ 0.05 were multiplied by the number of study comparisons: $n = 3$ in the HLA analysis and $n = 6$ in the combined HLA-C- KIR genotype analysis; see Table 5). Odds ratios were estimated using the approximation of Woolf, to avoid problems when critical entries were 0. All statistical analyses were performed with commercial software (SAS, ver. 9.1.; SAS Institute, Cary, NC).

Results

Baseline demographics of the unrelated Caucasian cohort recruited from a single clinic population at Southampton (104 cases of AMD and 93 normal controls) are described in Table 1. Genotyped frequencies were distributed in accordance with Hardy-Weinberg equilibrium for all HLA and KIR loci tested. The results from genotyping repeats performed in 10% of cases and controls were found to be 100% concordant with those obtained during initial genotyping. In total, genotyping data for 23 HLA-C principal allele groups were available for analysis after HLA class I genotyping. The allele frequencies for both the HLA and KIR genotypes in the control group were similar to those previously reported in the respective Caucasian populations.21–23

The allele frequencies of HLA-C alleles in our controls were similar to those reported in the U.K. population.23 Based on known KIR ligand specificity to a dimorphism on HLA-C allotypes,14 HLA-C alleles were initially grouped into C1 and C2 allele groups. The frequency of individuals with two copies of HLA-C1 alleles (C1C1), one copy of C1 (C1C2), and two copies of HLA-C2 alleles (C2C2) was similar between cases and controls (Table 2). HLA-C1 and -C2 allele distributions and the prevalence of HLA-Cw*07 principal allele groups with the exception of Cw*0701 did not show any significant difference between AMD cases and controls. The HLA-Cw*0701 allele was found to be strongly associated with the AMD group ($P = 0.03$, OR = 1.92).

Of the 16 KIR genes tested (Table 3), the frequency of KIR 2DL5 was found to be significantly more in the control group than in the AMD group (39.8% vs. 28.8%, $P = 0.006$, OR 0.45). This gene is a component of the B group of KIR haplotypes. None of the remaining 15 KIR genes showed any trend for significant association with either AMD or control groups. No significant associations with AMD were observed for the HLA-C1 allele group in combination with its known KIR ligands: KIR2DL2, KIR2DL3, and KIR2DS2 (Table 4). The remaining HLA-C1, -C*07, and -C*0701 allele combinations with KIR ligand alleles also did not show any significant association with AMD.

It has been noted that there is association with specific KIR haplotypes in the development of pre-eclampsia, in which this HLA-KIR genotype combination has been suggested to influence vascular remodeling.24 As KIR2DL5 is present on most of the B group haplotypes,13,14 we determined whether a haplotypic association was relevant for AMD or CNV formation in AMD.

The observed KIR genotypes were grouped into AA, AB, and BB haplotypes based on gene content. The KIR A haplotype is “inhibitory” and contains KIR2DS4 and KIR2DL4 as

activating KIRs and is the most common KIR haplotype, being present in approximately 30% of the Caucasian population. The remaining genotypes were categorized as B haplotypes. The Southampton cohort with KIR2DS4 and KIR2DL4 as the only activating receptors were thus designated AA and the remainder AB/BB (Table 3). There was no significant difference in the distribution of the AA and AB/BB genotypes between the AMD cases and controls.

To test the KIR–HLA haplotype model of Hiby et al.24 further, we performed univariate analyses. Variables tested in this analysis included combined frequencies of the C1 group of HLA alleles, the individual HLA alleles, Cw*07, Cw*0701, and the AA KIR haplotypes (Table 5). The combination of total HLA-Cw*07 alleles and KIR AA haplotypes correlated significantly with AMD status (OR = 3.36, $P = 0.003$, $P_C = 0.018$). The individual HLA-C*0701 allele and the KIR AA haplotypes in combination showed a strong association with AMD. The OR risk for AMD was significant at 4.35 ($P = 0.006$, $P_C = 0.036$) for this HLA-KIR allele combination.

To distinguish these independent variables, we performed multivariate analysis by logistic regression by using the binary logit model with backward elimination (Table 6). The significance of the HLA-Cw*0701-KIR AA paired association with AMD did not alter after this analysis. No significance was found with KIR 2DL5 and KIR AA genotypes in the regression model. Of interest, the effect of HLA-Cw*0701 when KIR AA genotypes were removed was found to be nonsignificant.

Discussion

The importance of defining associations between HLA and KIR genes in AMD lies in providing new insights into its basic pathogenesis and in helping to define an at-risk population for preventive measures to become applicable. Because of the critical role of HLA in regulating the immune response, most the HLA gene polymorphisms are known to result in functional amino acid substitutions in the expressed HLA molecules. These variant HLA molecules in turn affect the immune response to a vast range of antigens, with interindividual differences in immune responses to complex or simple antigens.25 Many HLA gene polymorphisms have been linked to susceptibility in a large number of immunologically mediated diseases affecting the retina and uvea of the eye, the skin, the gut, and the endocrine and joint systems.26 KIR gene models have now been proposed that genetically control levels of activation or inhibition.14 Many KIR genes and combinations of HLA class I-KIR genotypes that predispose to activating receptor-ligand interactions have now been linked to autoimmune inflammatory diseases including rheumatoid arthritis,27 psoriatic arthritis,28 and psoriasis vulgaris.29

Significant associations have been reported between HLA-DRB1*15 ($P_C = 0.000001$) and DQB1*06 ($P_C = 0.00001$) alleles and the development of CNV in presumed ocular histoplasmosis syndrome.30 These findings, along with the data from the present study, support the notion that certain HLA alleles are associated with CNV in various retinal diseases. Hence, further investigation of these three HLA alleles in a larger and/or different

cohort would be useful in determining the amount of risk contributed by them toward advanced AMD and CNV formation.

The combined HLA-Cw*0701 allele and KIR AA haplotype association with AMD status was highly significant in our Southampton cohort when tested by stepwise logistic regression analysis. The risk for disease was greater than fourfold when compared with controls for this paired HLA allele and KIR haplotype. The preponderance of inhibiting KIR 2DL5 in controls seems to be protective for AMD, whereas the presence of an appropriate HLA-C1 ligand interaction seems to render an individual more susceptible to AMD, perhaps because KIR2DL5 is a component of the B group of haplotypes. Therefore, its association with protection from AMD could be an inverse correlate of the susceptibility effect of the A group of haplotypes. Alternatively, KIR2DL5 itself or the B group of haplotypes which contain most of the activating KIR may be protective against this disorder.

Traditionally, activating KIR genotypes have been reported to be important in inflammatory conditions. A weak association of the activating receptor–ligand pair KIR2DS2:HLA-C 1 was observed in diabetes mellitus.31 A correlation with an activating KIR genotype was also found in the chronic inflammatory disease idiopathic bronchiectasis.32 This activating receptor association appears to be involved more in costimulation or activation of T cells rather than of NK cells.33

The underlying mechanisms by which KIR and HLA genes determine susceptibility to various ocular diseases has not been fully elucidated yet, and it should be stressed that a KIR-HLA association determined by a case– control study does not necessarily confirm KIR-HLA causality, but may reflect linkage disequilibrium (LD) with causal polymorphisms in other genes. The epistatic interactions of KIR with HLA make this less likely though. Although we cannot exclude LD with non KIR-HLA polymorphisms as causal in AMD, our data bear similarities to that observed in pre-eclampsia.24 In this disease the KIR-HLA genotype of the mother and fetus has been hypothesized to influence vascular remodeling in the placenta, leading to pre-eclampsia. This model, although highly speculative, could be relevant in AMD or, in particular, during CNV formation.

Linkage across the KIR locus is difficult to assess, as it is hard to separate haplotypes into true linkage groups, as we have genotyped both chromosomes simultaneously and because the KIR locus is so diverse (i.e., there are many different gene combinations especially in the telomeric part of the locus). This problem makes discerning a haplotype without typing the parents impossible. We tested for two copies of the common haplotype (AA), but were unable to distinguish the AB from the BB group of haplotypes.

It is important to note that the AA haplotype is a minimal KIR haplotype containing the inhibitory receptors for groups 1 and 2 HLA-C allotypes (KIR2DL1 and KIR2DL3) and the activating KIR2DL4 and KIR2DS4, the latter gene being nonfunctional in many individuals. 34 As the KIR2DL3–HLA-C interaction is considered one of weak inhibition, this haplotype is weakly inhibitory. This interaction may confer susceptibility to AMD via either NK cells or via weak inhibition of KIR-positive T cells, which are predominantly of the antigen-experienced effector–memory type.35

Because of the extreme polymorphic nature of the KIR and HLA gene regions, sampling variation between cases and controls can easily lead to both false-positive and -negative associations. Accordingly, a Bonferroni correction was applied in this study. Applying the correction for functionally well characterized KIR alleles permitted a realistic Bonferroni correction, rather than an overly conservative correction for all possible KIR alleles, which may cause even true-positive associations to be discarded.

This study had 80% power to detect significant differences in HLA and KIR genotype frequencies between cases and controls at $\alpha = 0.05$. For example, for an allele occurring at a frequency of 10% in the control population, detectable ORs are 2.3 for a predisposing allele and 0.5 for a protective allele with 95% confidence levels. The corresponding detectable ORs for KIR genes were 2.9 for a predisposing genotype and 0.2 for a protective genotype with 95% confidence levels. We may therefore not have detected smaller HLA and KIR associations with our sample sizes. It is also well recognized that the first report to describe an association between a gene and a disease often overestimates the fraction of disease caused by mutation in the gene in question.36 This emphasizes the need for replication studies to assess the HLA-KIR effect in further AMD populations.

In summary, this study is the first to establish HLA-C/KIR gene–mediated genetic susceptibility in AMD. The HLA-C*0701 allele in combination with inhibiting KIR haplotypes (AA genotype) is strongly associated with AMD. Continuing on from this hypothesis-generating study, further larger studies in different ethnic populations are needed to confirm these HLA-KIR associations with AMD. In addition, where possible, these studies should be complemented by functional studies to determine the contribution of these genes to the development of AMD. Dissecting the role of HLA and immune pathways in AMD may ultimately lead to opportunities to modulate these pathways by precise pharmacologic means and thus improve visual outcome in this devastating disease.

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Table 1

Basic Available Demographic Characteristics of the Southampton Study Population

	AMD Cases *n* = 104 (%)	**Controls *n* = 93 (%)**
Age		
Mean	76.5	73.1
Range	55–91	55–91
SD	7.8	10.2
Sex		
Female	67 (64.4)	58 (62.4)
Male	37 (35.6)	35 (37.6)
BMI	*n* = 86	*n* = 84
Mean	26.4	26.1
Range	17.3–40.5	17.7–36.9
SD	4.6	4.5
Smoking status	*n* = 86	*n* = 86
Ever smoked	49 (57.0)	34 (39.5)
Never smoked	37 (43.0)	52 (60.5)

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Table 2

HLA-C Allele Groups (C1, C2), Cw*07 and Cw*0701 Allele Frequencies in Southampton AMD and Control Groups

HLA-C Alleles	AMD Group *n* = 104 (%)	Control Group *n* = 93 (%)	*P*[*]	OR[†]	95% CI
C1C1	43 (41.3)	46 (49.4)	0.25	0.72	0.41–1.27
C1C2	50 (48.1)	34 (36.6)	0.10	1.60	0.91–2.84
C2C2	11 (10.6)	13 (14.0)	0.46	0.73	0.31–1.71
C1 alleles	136 (65.4)	126 (67.7)	0.38	1.16	0.82–1.64
C2 alleles	72 (34.6)	60 (32.3)	0.14	1.44	0.88–2.39
Cw*07 allele	63 (60.1)	46 (49.5)	0.11	1.57	0.89–2.76
Cw*0701 allele	43 (41.3)	25 (26.9)	0.03[‡]	1.92	1.05–3.50

[*] Two-sided *P* values using χ^2 test.

[†] OR according to the approximation of Woolf. χ^2 for trend testing all C groups = 0.23 (*P* = 0.62).

[‡] Significant results.

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Table 3

KIR Genotype and Haplotype Frequencies in Southampton AMD and Control Groups

KIR Genotypes	AMD Group *n* = 104 (%)	Control Group *n* = 93 (%)	*P*[*]	OR[†]	95% CI
2DL1	101 (97.1)	91 (97.8)	0.74	0.74	0.12–4.52
2DL2	50 (48.1)	48 (51.6)	0.24	0.87	0.50–1.52
2DL3	98 (94.2)	90 (96.8)	0.39	0.54	0.13–2.24
2DL4	103 (99.0)	93 (100.0)	0.34	1.90	1.67–2.17
2DL5	38 (36.5)	52 (55.9)	0.006[‡]	0.45	0.26–0.80
2DS1	36 (34.6)	43 (46.2)	0.09	0.61	0.35–1.09
2DS2	50 (48.1)	50 (53.8)	0.42	0.79	0.45–1.39
2DS3	25 (24.0)	32 (34.4)	0.10	0.60	0.32–1.12
2DS4	95 (91.3)	89 (95.7)	0.21	0.47	0.14–1.59
2DS5	27 (26.0)	28 (30.1)	0.51	0.81	0.44–1.52
3DL1	101 (97.1)	90 (96.8)	0.89	1.12	0.22–5.70
3DL2	104 (100.0)	93 (100.0)	—	—	—
3DL3	104 (100.0)	92 (98.9)	0.29	3.38	0.13–84.3
3DS1	30 (28.8)	34 (36.6)	0.24	0.70	0.38–1.28
2DP1	101 (97.1)	91 (97.8)	0.74	0.74	0.12–4.53
3DP1	104 (100.0)	93 (100.0)	—	—	—
AA	35 (33.7)	23 (24.7)	0.17	1.54	0.82–2.87
AB/BB	69 (66.3)	70 (75.3)	0.17	0.65	0.34–1.20

[*] Two-sided probabilities; χ^2 test.

[†] OR according to the approximation of Woolf.

[‡] Significant values.

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Table 4

HLA-C and KIR Receptor Paired Genotype Frequencies in Southampton AMD and Control Groups

HLA + KIR Genotypes	AMD Group *n* = 104 (%)	Control Group *n* = 93 (%)	*P*[*]	OR[†]	95% CI
C1 group + 2DL2	37 (35.6)	43 (46.2)	0.12	0.64	0.36–1.14
C1 group + 2DL3	87 (83.7)	77 (82.8)	0.87	1.06	0.50–2.25
C1 group + 2DS2	37 (35.6)	45 (48.4)	0.07	0.59	0.33–1.04
Cw*07 + 2DL2	27 (26.0)	27 (29.0)	0.62	0.86	0.46–1.60
Cw*07 + 2DL3	62 (59.6)	43 (46.2)	0.06	1.71	0.97–3.02
Cw*07 + 2DS2	28 (26.9)	29 (31.2)	0.51	0.81	0.44–1.50
Cw*0701 + 2DL2	19 (18.3)	14 (15.1)	0.54	1.26	0.59–2.68
Cw*0701 + 2DL3	18 (17.3)	24 (25.8)	0.14	0.60	0.30–1.20
Cw*0701 + 2DS2	20 (19.2)	15 (16.1)	0.57	1.23	0.59–2.59

[*] Two-sided *P*; χ^2 test.

[†] OR according to the approximation of Woolf.

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Table 5

HLA-C and KIR Genotype Paired Frequencies in Southampton AMD and Control Groups

HLA+ KIR Genotypes	AMD Group *n* = 104 (%)	Control Group *n* = 93 (%)	*P**	P_c†	OR‡	95% CI
C1 group + AA	31 (29.8)	23 (24.7)	0.42	2.52	1.29	0.68–2.43
Cw*07 + AA	25 (24.0)	8 (8.6)	0.003§	0.018§	3.36	1.43–7.89
Cw*0701 + AA	17 (16.3)	4 (4.3)	0.006§	0.036§	4.35	1.41–13.44
C1 group + AB/BB	67 (64.4)	70 (75.3)	0.09	0.54	0.59	0.32–1.10
Cw*07 + AB/BB	38 (36.5)	38 (40.9)	0.54	3.24	0.83	0.47–1.48
Cw*0701 + AB/BB	26 (25.0)	21 (22.6)	0.69	4.14	1.14	0.59–2.21

*Two-sided probabilities using χ^2 test.

†P_C indicates probabilities after application of Bonferroni correction factor ($n = 6$).

‡OR, using the approximation of Woolf.

§Significant values.

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Table 6

Multivariate Logistic Regression Analysis Showing the Effect of HLA-Cw*0701 and KIR 2DL5/AA Genotypes in AMD

HLA/KIR Genotypes	*P*	OR	95% CI
HLA-Cw*0701 with KIR AA	0.013*	4.35	1.41–13.44
HLA-Cw*0701 without KIR AA	0.308	1.32	0.95–2.50
KIR 2DL5	0.051	0.42	0.21–0.86
KIR AA	0.177	0.55	0.23–1.31

*Significant value. Analysis was performed by using the binary logit model with backward elimination.