Coexistence of inhibitory and activating killer-cell immunoglobulin-like receptors to the same cognate HLA-C2 and Bw4 ligands confer breast cancer risk

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Human leukocyte antigen (HLA) class I-specific killer-cell immunoglobulin-like receptors (KIR) regulate natural killer (NK) cell function in eliminating malignancy. Breast cancer (BC) patients exhibit reduced NK-cytotoxicity in peripheral blood. To test the hypothesis that certain KIR-HLA combinations impairing NK-cytotoxicity predispose to BC risk, we analyzed KIR and HLA polymorphisms in 162 women with BC and 278 controls. KIR-Bx genotypes increased significantly in BC than controls (83.3% vs. 71.9%, OR 1.95), and the increase was more pronounced in advanced-cancer (OR 5.3). No difference was observed with inhibitory KIR (iKIR) and HLA-ligand combinations. The activating KIR (aKIR) and HLA-ligand combinations, 2DS1+C2 (OR 2.98) and 3DS1+Bw4 (OR 2.6), were significantly increased in advanced-BC. All patients with advanced-cancer carrying 2DS1+C2 or 3DS1+Bw4 also have their iKIR counterparts 2DL1 and 3DL1, respectively. Contrarily, the 2DL1+C2 and 3DL1+Bw4 pairs without their aKIR counterparts are significantly higher in controls. These data suggest that NK cells expressing iKIR to the cognate HLA-ligands in the absence of putative aKIR counterpart are instrumental in antitumor response. These data provide a new framework for improving the utility of genetic risk scores for individualized surveillance.

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer-related deaths in women worldwide¹². Both innate and adaptive immune systems play a central role in preventing primary and recurrence of breast cancer. Natural killer (NK) cells, a subset of innate lymphoid cells representing 5–20% of peripheral blood mononuclear cells, mediate a fast-acting first-line defense against tumor transformation and viral infection. NK cells can lyse target cells quickly by direct cytotoxicity in an antigen-independent manner without the “priming” period required by T cells. NK cells also produce high levels of interferon-γ (IFN-γ) and a wide range of pro-inflammatory cytokines and chemokines, which contribute to the shaping of adaptive immune responses. The direct involvement of NK cells in controlling growth and metastasis of breast cancer was demonstrated by using T, B, and NK knock-out NOG mice and T and B knock-out NOD/SCID mice. A high level of natural cytotoxic activity of peripheral-blood lymphocytes was associated with reduced cancer risk. Women with breast cancer exhibit significantly reduced NK cell cytotoxicity in peripheral blood compared to healthy individuals.⁴⁻⁶ In contrast to other innate immune cells, NK cell population is highly heterogeneous and uses many specific germline-encoded repertoires of activating and inhibitory receptors to recognize target cells.¹¹ The KIR receptors are considered the key receptors that control human NK cell development and effector function.¹² Fourteen

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KIRs triggering either inhibition (3DL1-3, 2DL1-3, 2DL5) or activation (3DS1, 2DS1-5), or both (2DL4) have been identified. The KIR gene family displays a high degree of diversity determined by the variability in KIR gene content between haplotypes and allelic polymorphism of each gene13,14 (Fig. 1). Based on gene content, the KIR haplotypes are broadly classified into two groups: A and B15. Both A and B groups of haplotypes contain all four framework genes (KIR3DL3-3DP1-2DL4-3DL2) but differ substantially by the quantity and the quality of other KIR gene content. In addition to framework genes, group A haplotypes have a fixed set of four genes (KIR2DL3-2DL1-3DL1-2DS4) and encode inhibitory KIRs, 2DL1, 2DL3, 3DL1, and 2DL2, specific for all four HLA class I ligands, C1, C2, C1, Bw4, and A3/A11 respectively. Group B haplotypes have variable gene content and comprising one or more of the seven genes that are not part of the A haplotype (2DL2, 2DL5, 2DS1, 2DS2, 2DS3, 2DS5, 3DS1). While group A haplotypes contain only KIR2DS4 as an activating gene, group B haplotypes contain up to five activating KIRs—KIR2DS1, 2DS2, 2DS3, 2DS5, and 3DS1.

By interacting with specific self-HLA class I ligands, the inhibitory KIR receptors set the threshold for NK cell function, a maturation programming in NK cells termed licensing or education16,17. The licensing render the subsequent ability to survey, recognize and kill stressed target cells that have lost HLA class I molecules due to tumor transformation or viral infection. In the absence of inhibitory KIR-HLA interactions, NK cells become hypo-responsive or anergic. The ligand specificity for the activating KIRs remains elusive. Certain activating KIRs display a high degree of sequence homology with the corresponding inhibitory KIR in their extracellular Ig domains. Therefore these activating KIRs are expected to exhibit a binding specificity similar to their inhibitory counterpart. For example, KIR2DS1 and 2DL1 differ by only seven amino acids in their extracellular portion, and therefore, KIR2DS1 is known to bind weakly to HLA-C218–20. The KIR3DS1 that shares the highest sequence homologies with 3DL1 in their extracellular portion is shown to bind to HLA-Bw4 in a peptide-dependent manner24,25. The KIR2DL2 and 2DL3 bind to HLA-C1, but KIR2DS2, whose extracellular domain differs from KIR2DL2 and 2DL3 by only 3 and 4 amino acids, respectively, binds to HLA-A*11:01 complexed with a vaccinia viral peptide26. Activating receptor KIR2DS4 recognizes some C1- or C2-bearing HLA-C alleles, as well as the HLA-A3/11 epitope4,20. Using KIR-Fc fusion protein on a panel of 97 distinct HLA class I molecule-coated microbeads, specific alleles of the activating KIR2DS5 were shown to bind HLA-C2 allotypes26. The activating KIR2DS3 was not demonstrated to bind any HLA24,25.

Given that KIR genes at chromosome 19 and HLA genes at chromosome 6 are polymorphic and display significant variations, the independent segregation of these unlinked gene families produces extraordinary diversity in the number and type of KIR-HLA pairs inherited in individuals27,28. KIR-HLA variation affects the KIR repertoire of NK cell clones, NK cell maturation, the capability to deliver signals, and the NK cell response to human diseases29. To test the hypothesis that certain KIR-HLA combinations that impair NK cell cytotoxicity predispose to breast cancer risk, we analyzed a well-defined cohort of breast cancer patients and healthy controls from the native population of southern Iran.

**Results**

*Bx KIR genotypes are positively associated with breast cancer.* To test the possibility that KIR genes are involved in the risk of breast cancer, we determined the presence and absence of all 16 KIR genes and cognate HLA class I ligands in 162 patients with breast cancer and 278 healthy controls from the native population of southern Iran. A panel of 83 genotypes differing by KIR gene content were identified in this cohort of 440 native Iranians (Fig. 2). Sixty-one KIR genotypes were encountered in patients with breast cancer, while only 49 KIR genotypes were found in controls. Thirty-four KIR genotypes (41%) occurred exclusively in patients with breast cancer. Only three genotypes (#1, #40, and #42) occurred at significantly different frequencies between patients and controls. Two of them occurred at significantly low frequencies in patients compared to controls; AA genotype (genotype#40: 16.0% vs. 27.3%, p = 0.007, Odd ratio (OR) = 0.51, 95% confidence interval (CI) = 0.31–0.83) and the most common Bx genotype (genotype#1: 4.9% vs. 12.2%, p = 0.015, OR = 0.37, CI = 0.17–0.83) (Fig. 2). The decrease of genotypes#40 and #1 was more prominent in patients with the advanced stage of cancer (genotype#40: 6.8% vs. 27.3%, p = 0.007, OR = 0.19, CI = 0.06–0.65; genotype#1: 0% vs. 12.2%, p = 0.07, OR = 0.08, CI = 0.005–1.32). Genotype#42 occurred at significantly higher frequency in patients than controls (4.3% vs. 0.7%, p = 0.02, OR = 6.23, CI = 1.26–30.4), and the increase is pronounced in patients with the advanced stage of cancer (9.3% vs. 0.7%, p = 0.003, OR = 13.8, CI = 2.45–77.8).

**Figure 1.** Map of group A and B KIR haplotypes. Distinct KIR haplotypes carry quantitatively and qualitatively contrasting KIR gene content. Inhibitory KIR genes are depicted in white boxes. Activating KIRs are shown in dark boxes. Gray boxes represent pseudogenes or KIR genes with unclear function. HLA class I ligands for specific KIR are identified in dotted boxes. The centromeric and telomeric half are marked.
Figure 2. \(KIR\) gene content diversity in patients with breast cancer. Eighty-three distinct \(KIR\) genotypes were observed that differ from each other by the presence (shaded box) or absence (white box) of 16 \(KIR\) genes. The frequency (%F) of each genotype is expressed as a percentage and defined as the number of individuals having that specific genotype (N) divided by the number of individuals studied (n) in each group. The frequency of genotypes #1, #40, and #42 was significantly different between patients and controls and are marked by dark boxes. The C4 and T4 linkage groups are marked by red and blue boxes, respectively.
The Bx genotypes comprising 2–6 activating KIR genes, occurred more frequently in patients compared to controls (83.3% vs. 71.9%, \(p = 0.007\), OR = 1.95, CI = 1.19–3.18) (Table 1). The increase of Bx genotypes was prominent in patients with advanced stage of cancer (93.2% vs. 71.9%, \(p = 0.003\), OR = 5.33, CI = 1.6–17.72) (Table 1, Fig. 3). Particularly, the Bx genotypes that carry telomeric KIR3DS1-2DL5-2DS5-2DS1 gene cluster (i.e., T4 linkage group) occurred at increased frequency in patients with breast cancer compared to healthy controls (37% vs. 21.6%, \(p = 0.0005\), OR = 2.14, CI = 1.39–3.28), and the increase was further pronounced in patients with advanced stage cancer (45.5% vs. 21.6%, \(p = 0.001\), OR = 3.03, CI = 1.57–5.85). Contrariwise, the AA genotype decreased significantly in breast cancer patients compared to controls (16.7% vs. 28.1%, \(p = 0.007\), OR = 0.19, CI = 0.06–0.62) (Table 1, Fig. 3).

### Table 1. Frequency of KIR genotypes, linked gene groups, and genes in breast cancer patients and controls.

Carrier frequency (%F) of each genotype is expressed as a percentage and defined as the number of individual having the genotype (N +) divided by the number of studied (n) in the study group.

The Bx genotypes were increased in patients with breast cancer. Five of seven B haplotype-specific KIRs (2DL2, 2DL5, 3DS1, 2DS1, 2DS5) were significantly increased in patients with breast cancer compared to controls (Table 1). Three telomeric B haplotype-associated activating KIR genes, such as 3DS1, 2DS1, and 2DS5, were more prominently increased in patients with advanced-stage breast cancer (Table 1). Particularly, KIR2DS1 was significantly at higher frequency in patients with advanced-stage cancer compared to controls (70.5% vs. 36.3%, \(p = 0.0001\), OR = 4.18, CI = 2.09–8.35). Inversely, two of four A haplotype-specific KIRs were decreased in patients with breast cancer compared to controls: KIR2DL3 (74.1% vs. 90.6%, \(p = 0.000003\), OR = 0.29, CI = 0.11–0.52), and the decrease was pronounced in patients with advanced stage breast cancer (6.8% vs. 28.1%, \(p = 0.02\), OR = 0.41, CI = 0.19–0.89) (Table 1, Fig. 3).

**B haplotype-specific KIRs were increased in patients with breast cancer.** Five of seven B haplotype-specific KIRs (2DL2, 2DL5, 3DS1, 2DS1, 2DS5) were significantly increased in patients with breast cancer compared to controls (Table 1). Three telomeric B haplotype-associated activating KIR genes, such as 3DS1, 2DS1, and 2DS5, were more prominently increased in patients with advanced-stage breast cancer (Table 1). Particularly, KIR2DS1 was significantly at higher frequency in patients with advanced-stage cancer compared to controls (70.5% vs. 36.3%, \(p = 0.0001\), OR = 4.18, CI = 2.09–8.35). Inversely, two of four A haplotype-specific KIRs were decreased in patients with breast cancer compared to controls: KIR2DL3 (74.1% vs. 90.6%, \(p = 0.000003\), OR = 0.29, CI = 0.11–0.52), and the decrease was pronounced in patients with advanced stage breast cancer (6.8% vs. 28.1%, \(p = 0.02\), OR = 0.41, CI = 0.19–0.89) (Table 1, Fig. 3).

**KIR-HLA gene combinations in patients with breast cancer and controls.** No significant difference was observed in the frequencies of HLA-C1, C2, Bw4, and A3/11 ligands between breast cancer patients and controls (Suppl. Table 1). To examine whether KIR-HLA combinations confer risk for breast cancer, we compared the frequency of four well-characterized inhibitory KIR and cognate HLA class I ligand combinations (Table 2). No significant difference was found in the frequency of any inhibitory KIR and HLA class I ligand combinations between patients and controls. However, activating receptor and their putative HLA class I ligand combinations, such as KIR2DS1+C2 (52.4% vs. 26.9%, \(p = 0.001\), OR = 2.98, CI = 1.54–5.79) and
KIR3DS1 + Bw4+ (29.3% vs. 17.7%, \( p = 0.008, \text{OR} = 2.6, \text{CI} = 1.26–5.39 \)) were increased in patients with advanced breast cancer compared to controls (Table 2).

Coexistence of KIR2DL1 + C2+, and activating counterpart KIR2DS1+ was more frequent in patients with advanced breast cancer compared to controls (52.4% vs. 26.6%, \( p = 0.001, \text{OR} = 3.04, \text{CI} = 1.56–5.89 \)) (Fig. 3). In contrast, KIR2DL1 + C2+ without KIR2DS1 is more frequent in controls compared patients (45.5% vs. 27.8%, \( p = 0.0004, \text{OR} = 0.46, \text{CI} = 0.15–0.73 \)). The KIR3DL1 + Bw4+, and its activating counterpart KIR3DS1+ was more frequent in patients with advanced stage breast cancer compared to controls (29.3% vs. 15.6%, \( p = 0.033, \text{OR} = 2.23, \text{CI} = 1.05–4.72 \)).

**Table 2.** Carrier frequency of KIR+HLA class I ligand combinations in breast cancer patients and controls.
of peptide selectivity in its binding to HLA class I, indicating that the functional outcome of activating KIRs on tumor tissue with reduced HLA class I expression is not entirely permanent, and the functional activity of mature NK cells can be reset by new HLA environment in these patients. The molecular mechanism underlying the impaired NK cell cytotoxicity and antitumor effect in breast cancer is not identified.

The interaction of inhibitory KIR with specific cognate HLA class I ligand makes NK cells matured to acquire full effector function, developmental programming termed "licensing." In the absence of inhibitory KIR-HLA interactions, NK cells became hyporesponsive or anergic. The distribution of four inhibitory KIR-HLA class I ligand combinations is comparable between breast cancer patients and controls, indicating that the development of functionally active NK cells in patients might be similar to those of controls. However, NK licensing is not entirely permanent, and the functional activity of mature NK cells can be reset by new HLA environment in tumor tissue with reduced HLA class I expression, a mechanism that tumor develops to evade from adoptive immune response.

The activating KIR-based HLA class I-dependent licensing may also influence NK cell unresponsiveness to transformed cells. Although the mechanisms have not yet been identified, education by activating KIRs shares features with the hyporesponsiveness induced by chronic stimulation of other activating receptors expressed by NK cells. For example, chronic exposure to NKG2D ligands in mice renders NK cells hyporesponsive to target cells. A recent study found that the expression of NKG2D on blood NK cells was higher in breast cancer patients than the levels documented in healthy females. Similarly, when the ligand (m157) for the activating Ly49H is constitutively expressed, mouse Ly49H+ NK cells become hyporesponsive. NK cells expressing activating KIR2DS1 are hyporesponsive in the presence of self-HLA-C2 ligands and thus unable to mount an efficient response against breast cancer. This disarming model of licensing emphasizes the crucial role of activating KIRs 2DS1 and 3DS1 and interactions with their HLA class I ligands in developing anergic NK cells (disarmed), which are hyporesponsive and are not able to defend against the tumor. All patients with advanced cancer carrying 2DS1 + C2 or 3DS1 + Bw4 also had their inhibitory KIR counterparts 2DL1 and 3DL1, respectively. In contrast, the 2DL1 + C2 and 3DL1 + Bu4 pairs without their activating KIR counterparts were significantly higher in controls than patients. These data suggest that NK cells expressing inhibitory KIR to the cognate HLA class I ligands in the absence of putative activating KIR counterpart are instrumental in antitumor response.

It also remains possible that activating KIR receptors could recognize altered HLA class I complexes, e.g., specific HLA/peptide complexes. It is possible that the activating KIRs could directly bind neoantigens explicitly expressed on breast cancer cells, which may suppress cytolytic function but trigger cytokine release. Supporting this possibility, KIR2DS4 has been suggested binding to an unidentified protein expressed on melanoma-derived tumor cells, independently of HLA class I. Particularly, KIR2DS1 has been shown to displays a certain degree of peptide selectivity in its binding to HLA class I, indicating that the functional outcome of activating KIRs can be modulated by the nature of the presented peptide.

The human KIR genotypes can be simply divided into two groups, AA and Bx, with quantitatively and qualitatively contrasting KIR gene content. We found a striking association of Bx genotypes with breast cancer. Three of four prior studies suggested an association between breast cancer and B-haplotype-specific KIR genes. Consistent with our findings, Jobim et al. found a strong association between KIR2DL2 and Brazilian women with breast cancer. However, this study could not find any association with other KIR genes, such as KIR2DS2, 2DS3, and 2DL5, which are located at close proximity to 2DL2 with strong linkage disequilibrium. Jobim et al. also reported an association between KIR2DL2 + C1 in breast cancer, which was not observed in our study. Oztruk et al. also reported a strong association between KIR2DS1 and patients with breast cancer in Turkey, which is in agreement with our findings. However, they could not find an association with KIR2DS1-linked genes, such as 3DS1 and 2DS5.
Using a new cohort of breast cancer patients and controls from the Fars province, our collaborator Prof. Abbas Ghaderi and team recently reported an association between Iranian breast cancer patients and Bx KIR genotypes, centromeric Bx genotypes, and B-haplotypes carrying C4T4 motif (positive for seven KIRs: 2DL2, 2DS2, 2DL3, 2DL5, 3DL1, 2DS1, and 2DS3)30, which is in agreement with our findings. However, the study confirmed the association of individual B-haplotype-associated KIR genes only with breast cancers expressing estrogen receptors. Breast cancer positive for progesterone receptor or human epidermal growth factor 2 (HER2) were not associated with B-haploype-specific KIRs. Moreover, the HLA class I ligands were not analyzed in this study. In total contradiction to our findings, Alomar et al. reported a significant decrease in the frequencies of KIR2DS2, 2DS3, and Bx genotypes in 50 Saudi women with breast cancer compared to 65 controls37. HLA ligands were analyzed by only Turkish and Saudi studies. Inconsistent results observed between the studies are presumably contributed by multiple factors, including ethnic and population disparity in KIR and HLA genome, the differential composition of histologic breast cancer phenotypes, and small sample sizes.

The B-haplotype KIRs were correlated with an increased risk of other solid and hematological malignancies, including leukemia31, cervical neoplasia32, Hodgkin lymphoma33, gastric cancer34, head and neck squamous cell carcinoma35, urothelial bladder cancer36, colorectal adenocarcinoma37, systemic sclerosis38, and meningioma39. The B haplotype-specific KIRs, particularly those located at the telomeric half (3DS1, 2DS1, and 2DS5), were observed to be prominently increased in patients with an advanced stage of breast cancer. These results contrast with the classical view that activating NK cell receptors mediate spontaneous lysis of transformed cells and protect against the tumor40.

Given our study’s retrospective design, further investigations are warranted in a prospectively accrued patient population to substantiate our findings. The number of patients included in our study was insufficient to evaluate the impact of KIR-HLA combinations in tumors with different phenotypes, such as estrogen receptor-positive (ER+), progesterone receptor-positive (PgR+), and HER2+. Therefore, further systematic studies should be focused on determining the impact of combined KIR + HLA combinations using multivariate analysis. The limitation of our KIR-binding HLA epitope typing is its inability to discriminate HLA allotypes (e.g., Cw*05:01, Cw*02:02), which can differ in binding affinity61. In summary, our results provide a genetic basis for impaired NK cell antitumor activity in breast cancer. The KIR-HLA associations observed in this study provide further insight into genetic susceptibility to breast cancer, improving the utility of genetic risk scores for individualized screening and follow-up recommendations for earlier implementation of breast cancer risk-reduction strategies. Moreover, our results suggest that autologous activated NK cell clones with select KIR-HLA composition favoring antitumor activity could be a promising immunotherapeutic strategy against breast cancer.

Materials and methods

Study subjects and samples. A cohort of 162 women with breast cancer and 278 healthy controls from the southern part of Iran (Fars province) were included in this study. The patients were recruited at Faghih hospital, Shiraz University of Medical Sciences. The age-matched controls were collected from the same geographical area. The clinical and pathological characteristics were collected from patient medical records. Table 3 shows the distribution of clinicopathological characteristics of breast cancer. The breast cancer patients were categorized according to TNM staging62, and grouped as either early stage (0, I, and II) or advanced stage (III and IV) of disease. The study was reviewed and approved by the Medical Research Ethics Committee of Shiraz University of Medical Sciences and UCLA Institutional Review Board of human research protection. Genomic DNA was extracted from peripheral blood samples using either the standard salting-out method or by QIaamp blood kit (Qiagen, Hilden, Germany). The quality and quantity of DNA were determined by UV spectrophotometry, and the concentration was adjusted to 100 ng/μL. All DNA samples received at UCLA were de-identified and only marked as having been obtained from patients with breast cancer or controls. Informed consent was obtained from all subjects. Data obtained were Health Insurance Portability and Accountability Act (HIPAA) compliant, and the study adhered to the tenets of the Declaration of Helsinki. All methods were carried out in accordance with relevant guidelines and regulations.

KIR genotyping and genotype/haplogroup classification. The presence and absence of 16 KIR genes were determined using our previously developed duplex SSP-PCR typing method45. Ambiguous and unusual KIR genotypes were resolved by using the alternative SSP-PCR typing method27. Based on the presence and absence of KIR genes, we divided the study subjects into two groups: the AA and Bx genotype carriers. The AA genotype subjects carried only KIR3DL3-2DL3-2DL1-2DP1-3DP1-2DL4-3DL1-2DS4-3DL2 genes that are characteristic of A-haploype. The rest were regarded as Bx genotype carriers (AB heterozygous and BB homozygous carriers). Based on our previous linkage disequilibrium analyses, we determined the frequency of B-haplotype-specific KIR gene clusters46,48. One of them comprises KIR2DS2-2DL2-2DS3-2DS5B genes and is located at the centromeric half of the KIR gene complex (termed C4 linkage group). In contrast, another cluster contains KIR3DS1-2DL5A-2DS5-2DS1 genes and is located at the telomeric half of the complex (termed T4 linkage group).

HLA class I ligand typing by novel direct sequencing. We developed a novel direct DNA sequencing method to determine KIR-binding HLA-A, -B, and -C ligands. The procedure starts with gene-specific amplification of exon 2 and 3 of HLA-A, -B, and -C loci followed by direct sequencing of PCR amplicons (Suppl. Fig. 1, Suppl. Table 2). The primers amplify all common and well-documented HLA-A, -B, and -C alleles46. However, the HLA-B amplification excludes HLA-B*73:01. The reverse primers used in the HLA-B specific amplification (3Bln3-37R) binds to the intron-3 region from nucleotide 1028 to 1050. Since the HLA-B*73:01 allele has mutations at reverse primer annealing site at nucleotide 1032 from G to A and 1038 from G to C, the HLA-B*73:01 is not be amplified by HLA-B PCR. However, HLA-B*73:01 is amplified by HLA-C specific amplification. Gene-
specific PCR reaction (20 μL volume) comprised a final concentration of 1 × LT buffer II, 500 μM of each deoxyribonucleotide triphosphates (dNTPs), 0.3 μM of each forward and reverse primers to either HLA-A, -B or -C, 1.5 U of LT Tgo DNA polymerase (Roche Applied Science, Germany), and 100 ng genomic DNA. The PCR thermal cycling was performed in ABI 9700 GeneAmp PCR system (Applied Biosystems, USA) using the following thermal cycles: initial denaturation for 1 min at 94 °C; 12 cycles at 94 °C for 10 s, and 68 °C for 2.5 min; 20 cycles of 94 °C for 15 s, 63 °C for 30 s, and 68 °C for 2 min; and a final extension at 68 °C for 7 min. The PCR products (2 μL) were subjected to electrophoresis on 2% agarose gel to visualize specific bands with the expected size. The PCR amplicons were purified from unincorporated primers and dNTPs by digesting with ExoSAP-IT exonuclease-I (USB Corporation, Cleveland, OH) according to the manufacturer’s protocol and were used as a template in the sequencing reactions. Then the segments of exon 2 that encode the KIR-ligands were sequenced at both directions using the BigDye terminator V1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions (10 μL) comprise 2 μL of sequencing reagent premix, 1 μL dilution buffer, 0.3 μL of sequencing primer (10 pM/μL), and 2 μL of purified PCR amplicon. The following PCR thermal cycling profile was used: 25 cycles of 96 °C for 20 s, 53 °C for 20 s, 60 °C for 1 min, and soak at 4 °C. Once the cycling was completed, the sequencing reactions were precipitated using sodium acetate/EDTA buffer and ethanol to concentrate the reactions and to eliminate unincorporated fluorescent-labeled nucleotides. The precipitates were resuspended in 15 µL of Hi-Di deionized formamide (Applied Biosystems), denatured by heating at 95 °C for 2 min, and loaded into the ABI PRISM™ 310 capillary sequencer (Applied Biosystems). Finally, sequence analysis was performed using Assign SBT v3.5.1 software (Conexio Genomics, Western Australia), which can combine both forward and reverse sequences files to inspect and edit the electropherograms. The Assign program assigned the alleles by comparing the test sequences with a library of known HLA-A, -B and -C sequences downloaded from the international ImMunoGeneTics (IMGT-HLA Database (http://www.ebi.ac.uk/imgt/hla). The KIR-binding HLA class I ligands were deduced from the assigned alleles. We have validated this method by using a panel of 31 UCLA DNA standards that includes most core HLA class I types (Suppl. Table 3).

Data analysis and statistical methods. The percentage of each KIR gene in control and patient groups was determined by direct counting (individuals positive for the gene divided by individuals tested per popula-
tion × 100). Differences between the study groups in the distribution of each KIR genotypes, KIR genes, HLA ligands, and KIR-HLA combinations were estimated by the two-tailed Fisher Exact probability (P) test, and p < 0.05 was considered to be statistically significant. Odds ratio (OR) and 95% Confidence Intervals (CI) were calculated to determine the magnitude and statistical significance of associations.

Data availability
The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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E.A. and S.F. collected samples. E.A. performed KIR and HLA ligand typing. E.A., K.R. and S.B. performed the statistical analysis and drafted the manuscript. E.A., A.G., and R.R. conceived and designed the study, performed the interpretation, and edited the manuscript. The paper was reviewed and approved by all authors.

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The authors declare no competing interests.

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