# Impact of HLA Class I Antigen, Killer Ig-like Receptor and FCGR3A Genotypes in Breast Cancer Susceptibility and Tumor stage

**Angelica Canossi** ( angelica.canossi@cnr.it )
CNR Institute of Translational Pharmacology https://orcid.org/0000-0002-2790-905X
**Anna Aureli**
CNR Institute of Translational Pharmacology
**Tiziana Del Beato**
**Giorgio Novelli**
**Oreste Buonomo**
**Piero Rossi**
**Adriano Venditti**
https://orcid.org/0000-0002-0245-0553
**Franco Papola**
**Giuseppe Sconocchia**
CNR Institute of Translational Pharmacology

**Article**

**Keywords:** Breast cancer, Killer cell immunoglobulin-like receptors (KIR), HLA-C, Immunoglobulin G fragment C receptor (FCGR3A), genetic risk

**Posted Date:** July 21st, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-1860861/v1

# Abstract

The identification of clinical pathological role of genes regulating NK cell function, including HLA, KIR and FCGR3A, in breast cancer (BC) may be a challenge. The objective of this study was to determine whether these genes have an impact on the BC susceptibility and progression. In this study, 47 BC Italian patientswere genotyped by PCR-SSP typing (KIR) and PCR-SBT method (HLA-C and FCGR3A). HLA-C allele analysis showed that the HLA-C*07:02:01 (p = 0.04, OR = 4.867) and HLA-C*04:01:01 alleles (p = 0.04 OR = 2.364) were predisposing to tumor progression, and instead the HLA-C*05:01:01 allele was protective against disease (p = 0.019 OR = 0.087). In addition, we highlighted a significant reduction of the KIR2DS4ins in BC patients (p = 0.022) and an increased coexistence of KIR2DL1 and KIR2DS1 genes in advanced BC patients compared to early stages (p = 0.002). The concurrent lack of KIR2DL2 and KIR2DS4 genes in the presence of HLA-C2 alleles was significantly associated with increased susceptibility to BC (p = 0.012 OR = 5.020) or with lymph node involvement (p = 0.008 OR = 6.375). We identified different combinations of the FCGR3A-48/158 haplotypes and KIR genes in BC patients compared to controls. Our study provides evidence that the combined analysis of these biomarkers might help predict genetic risk scores for tailored screening of BC patients.


# 1 Introduction

Breast cancer (BC) is the most frequent malignancy in women worldwide and it represents the second most common cause of cancer deaths (1). Besides genetic mutations, diet, age, environmental contaminants and steroid hormones are involved in the pathogenesis of the disease. BC is characterized by a strongly inflammatory microenvironment supported by immune cells, cytokines and growth factors. A high proportion of Natural killer (NK) cells and neutrophils and a lower frequency of cytotoxic T cells and CD4 + cells were found in estrogen receptor (ER)-positive BC (2). NK cells represent the first-line defense against transformed cells of whom, class I human leukocyte antigens (HLA) and killer Ig-like receptors (KIRs) regulate their function. NK cell cytotoxic activities are regulated by opposing signals delivered by different surface receptors (KIR), which can activate or inhibit NK cell cytotoxic responses according to their interaction with HLA class I antigens on the surface of tumor cells.

KIRs are transmembrane proteins encoded by multiple genes mapping to chromosome 19q13.4 (3, 4). Generally, two types of KIRs can be identified: the first type has a long intracellular tail (KIRL), while the second type is characterized by a short intracellular tail (KIRS). The former are inhibitory, the latter stimulatory. Worldwide population variation in the KIR loci and the relationship between KIR genes and their HLA ligands were evaluated in the KIR anthropology component of the 15th International Histocompatibility Workshop, also with our contribution (5).

The HLA-C antigens are the best-known KIR ligands. They can be divided into two groups on the basis of polymorphisms at amino acid positions 77 and 80 in the α1-domain of the heavy chain: the C1 group carries a serine residue at position 77 with an asparagine at position 80, and the C2 has an asparagine at position 77 and a lysine residue at amino acid 80 (6, 7).

A relationship between KIR genotype and cancer has been recently proposed in different studies (9–12). Inhibitory KIRs, interacting with HLA class I antigens, regulate the NK cell cytotoxicity, including antibody-dependent cellular cytotoxicity (ADCC). The extent of this process relies on the binding affinity of the Fc portion of the IgG antibody to the CD16 receptor.

CD16A (FCGR3A gene) is a polymorphic activating receptor expressed on the surface of NK cells. It induces molecular signals that lead to the activation of the NK cell killing machinery resulting in the elimination of antibody-opsonized target cells (13). Changes in the antibody affinity for CD16 and/or CD16 gene polymorphisms can modulate the ADCC response as a function of the different IgG binding affinity of a valine or a phenylanine polymorphism at amino acid position 158 of the molecule (14–18). In addition to FCGR3A-158V/F, the 48L/R/H polymorphisms has also been extensively studied. *de Haas et al*. reported that both arginine and histidine have an enhanced binding to the IgG1, IgG3, and IgG4 (19) .

The importance of steroid hormones as prognostic markers in BC development is widely shown. About a fifth of women with BC (15–20%) over-expresses the human epidermal growth factor receptor 2 (HER2), that is often associated with a more aggressive profile and decreased survival (20). However, the use of HER2-targeted therapies, such as trastuzumab (Herceptin) in addition to chemotherapy, has been successfully included in the treatment of BC (21–23). Several studies have shown that NK and tumor-specific T lymphocytes strongly influence tumor development and response to anti-HER mAbs treatment.

Given that, the FCGR3A allele polymorphism might be a factor contributing to the anti-tumor activity of the antibody and it in association with KIR genes and their HLA-C ligands could help to understand their impact to BC susceptibility and staging.

## 2 Materials And Methods

# 2.1 Study design, location and subjects.

A cohort of forty-seven Italian patients with breast cancer (46 females and 1 male, mean age: 64 years, ranging from 45 to 86) were enrolled in the Division of Medical Oncology of Tor Vergata University Hospital in Rome. Histopathological assessment (pTNM), tumor stage, expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 status were determined by using immunohistochemical detection. The BC patients were mainly categorized according to TNM staging and grouped as early-stage (0-II) or advanced stage (III-IV). The study was approved by the Internal Review Board (IRB) of the Tor Vergata University Hospital, Rome, Italy (number 133/10). Written informed consent was obtained from all the subjects. We used a control group composed of 39 women (FCTR, mean age: 62.7 years) in addition to a mixed group of 66 healthy subjects (MCTR: 66.0 years). The reason for this choice follows from the assumption that, although BC rarely affects men (0.5-1% of all BC patients), its diagnosis is often delayed and is associated with worse outcome. As regards the case-control analysis of HLA-C allelic contribution, we considered another historical group of 76 healthy individuals, already typed for bone marrow transplantation. All subjects were ethnicity matched, unrelated, and randomly selected, with no cancer and no history of any immunological disease.

# 2.2 DNA extraction and KIR genotyping

DNA was isolated from 1 ml of cryopreserved peripheral blood cell sample using the DNA Blood Midi kit (*Qiagen, Hilden Germany*) by spin columns, according to the manufacturer's protocol. DNA purity and concentration were analyzed using an ultraviolet-visible (DU 530 spectrophotometer, Beckman Coulter Life Sciences, Brea, CA-US). KIR genotyping was performed by using a polymerase chain reaction with sequence-specific primers (PCR-SSP) for 16 KIR genes (KIR2DL1, 2DL2, 2DL3, 2DL4, 2DL5A, 2DL5B,2DS1, 2DS2, 2DS3, 2DS4del and 2DS4ins, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1) and 2 pseudogenes (2DP1,3DP1) (*KIR Typing Kit, MACS Molecular, MiltenyiBiotec, Bergisch-Gladbach, Germany*), with a genomic DNA control for contamination, a β-actin positive control, and negative control. PCR products were analyzed on a 2% agarose gel electrophoresis containing ethidium bromide and photo-documented on an ultraviolet transilluminator (Fig. 1). KIR genotyping is utilized for the analysis of gene content and the categorization of A/B haplotypes, as well as for prediction of NK cell reactivity in autologous and allo NK cell-based immunotherapy. The A haplotype contains several inhibitory KIR genes and only one activating KIR (KIR2DS4). Conversely, B haplotype displays one or more activating KIR genes (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1).

# 2.3 HLA-C allele typing by PCR-SBT

HLA-C typing was performed by a polymerase chain reaction (PCR) sequence-based typing (PCR-SBT) using the AlleleSEQR HLA-C Plus Sequence-Based Typing (SBT) Kit (Abbot Molecular, Des Plaines, Illinois, USA), which analyzes the allelic polymorphisms in exons 2–4 of HLA-C gene. Allele assignments were evaluated using Assign™ SBT software (Conexio Genomics, Fremantle, Western Australia). These HLA-C alleles were also considered as belonging to C1 (HLA-C*01,03,07,08,09,10,12,14) and C2 (HLA-C*02,04,05,06,15) subsets, depending on the presence of Asparagine (Asp) or Lysine (Lys) at position 80, respectively. The study of the interactions between a specific KIR and subsets of HLA-C allotypes (C1 and C2) was established according to the review scheme of Campbell KS et al. (24).

# 2.4 FCGR3A genotyping by sequence-based typing (SBT)

The genotyping of FCGR3A-158G/T (V/F) and FCGR3A-48A/T/G (L/R/H) was performed on genomic DNA by PCR-SBT technique using primers previously described[17]. Briefly, PCR reactions were set up with 250 ng of genomic DNA per 50 µl reaction, and PCR products were purified and sequenced using Big Dye Terminator v1.1 Cycle Sequencing Kit on an ABI Prism 3130 Genetic Analyzer (PE Applied Biosystems, Foster City, CA). Typing was obtained by alignment of the processed sequences and the reference human CD16A gene on dedicated software.

# 2.5 Statistical analysis

Two-tailed Pearson $X^2$ test or Fisher's exact test were used for comparison of the gene frequencies of the patient group with controls, as appropriate. A p value $\leq 0.05$ was considered to be statistically significant and Bonferroni correction for multiple testing was examined. The odds ratio (OR) and its 95% confidence intervals (CI) were calculated. The association of KIR genes and HLA-C ligands with BC status was assessed by the binary logistic regression analysis. Comparison between groups and correlation between variables were examined by parametric (t-test/one-way ANOVA, Pearson's correlation), and non-parametric tests (Kruskal Wallis, Friedman test for repeated measures, and Spearman's test), as appropriate.The SPSS statistical package, Version 13 for Windows, was used for data and statistical analysis. FCGR3A-48 and 158 allele and haplotype frequencies were assessed by an expectation-maximum (EM) algorithm. For multilocus genotypic data, the maximum likelihood was estimated via the EM algorithm when the gametic phase is not known. The Hardy–Weinberg equilibrium was calculated by the Guo and Thomson exact test. The analysis of was performed using the Arlequin V.3.0 population genetics software.

# 3 Results

## 3.1 Patients

Forty-seven BC patients with different histological diagnoses of early or advanced and metastatic cancer were considered for this study. Patients were treated with neoadjuvant chemotherapy (n = 5) or adjuvant chemotherapy (n = 24), hormonal therapy (n = 17) and trastuzumab (n = 5). Twenty-six patients were in an early stage of the disease (stage 0-II) and 21 patients in advanced stages (stage III-IV). Twenty-seven patients had lymph node metastases (LNM). The histological classification of the disease mainly included ductal or lobular carcinomas. Overall, ten patients relapsed; two patients died during the follow-up for liver injury and cardiovascular diseases (Table 1).

Table 1
Clinical and biological features of the breast cancer (BC) patients.

| Variables | N (%) |
|---|---|
| *Number patients* (n = 47): | 1 (2.1) |
| - males | 46 (97.9) |
| - females | |
| *Median age*, years (range ) | 62 (45–87) |
| ≥ 50 | 30 (63.8) |
| ≤ 50 | 17 (36.2) |
| *Tumor Stage*: | 26 (55.3) |
| - early (0-II ) | 21 (44.7) |
| - advanced (III-IV) | |
| *Hormonal status* (n = 34): | 25 (73.5) |
| ER+ | 23 (67.6) |
| PR+ | 8 (23.5) |
| ER-/PR- | 22 (64.7) |
| ER + PR+ | 5 (14.7) |
| HER+ | 5 (14.7) |
| Triple negative | |
| *Ki67 index* (n = 30): | 17 (56.7) |
| > 20% | 13 (43.3) |
| < 20% | |
| *Chemotherapy and hormonal therapy* (n = 34): | 24 (70.6) |
| Adjuvant (anthracyclines /taxanes) | 5 (14.7) |
| Neoadjuvant | 17 (50.0) |
| Hormonal therapy | |
| *Anti-HER2* (n = 5): | 4 (11.8) |
| Trastuzumab | 1 (2.9) |
| Trastumab + pertuzumab | |
| *Metastasis site*: | 27 (57.4) |
| LNM+ | 20 (42.6) |
| LNM- | 6 (12.8) |
| Visceral | |
| *Tumor size*: | 34 (72.3) |
| T1-T2 | 8 (17.0) |
| T3-T4 | 5 (10.6) |
| NA (Tis etc) | |

| Variables | N (%) |
|---|---|
| *Relapse*: | 10 (33.3) |
| *Diagnosis* (n = 39): | 27 (69.2) |
| Ca ductal | 6 (15.4) |
| Ca lobular | 8 (20.9) |
| Other types (apocrine, mucinous etc.) | |

## 3.2 HLA-C allele associations in BC patients

First, we compared the distribution of HLA-C alleles and associated subsets (C1:C80N and C2:C80K) of BC patients at different tumor stages with that of healthy controls. The incidence of the C1 group, HLA-C*07:02:01 allele was higher in more advanced and LNM + tumors than in female (16.7% vs 4.0%, p = 0.040 OR = 4.867) or mixed controls (p = 0.020 OR = 4.867). Also the group C2, HLA-C*04:01:01 allele was positively associated with LN + metastasis (LNM+) compared to mixed controls (28.6% vs. 14.5%, p = 0.040 OR = 2.364). A univariate regression indicated that the presence of either HLA-C*04:01:01 or HLA-C*07:02:01 allele was significantly associated with advanced stage of tumor (p = 0.044 OR = 3.696) or LNM + status (p = 0.016 OR = 3.930, 95%CI: 1.292–11.950). On the contrary, in the whole population of BC patients, the HLA-C*05:01:01 was found to be significantly protective (0% vs 7.2%, p = 0.019 OR = 0.087) when compared to the mixed controls, Table 2. However, the significance of association of HLA-C alleles was not confirmed after the Bonferroni's correction, maybe because of limited number of BC patients and the high HLA-C allele variability.

Table 2
HLA-C alleles in breast cancer patients (BC, n = 35), female controls (FCTR, n = 38) and historical controls (n = 76)

| HLA-C* | BC tot (2n = 70) n AF(%) | BC stage 0-II (2n = 40) n AF(%) | BC stage III-IV (2n = 30) n AF(%) | LNM+ (2N = 42) n AF(%) | Female Ctrs (2n = 76) n AF(%) | P val | OR= | Mixed Ctrs (2n = 152) n AF(%) | P val | OR= |
|---|---|---|---|---|---|---|---|---|---|---|
| 01:02 | 1 1.4 | 1 2.5 | 0 0.0 | 0 0.0 | 4 5.3 | ns | | 4 2.6 | ns | |
| 02:02 | 4 5.3 | 2 5.0 | 2 6.7 | 2 4.8 | 7 9.2 | ns | | 6 4.0 | ns | |
| 03:03 | 1 1.4 | 1 2.5 | 0 0.0 | 0 0.0 | 1 1.3 | ns | | 3 2.0 | ns | |
| 03:04 | 1 1.4 | 0 0.0 | 1 3.3 | 1 2.4 | 2 2.6 | ns | | 4 2.6 | ns | |
| **04:01:01 (C2)** | 17 24.3 | 9 22.5 | 8 26.7 | 12 **28.6** | 16 21.1 | ns | | 22 **14.5** | **0.040** | **2.364** |
| **05:01:01 (C2)** | **0 0.00** | 0 0.0 | 0 0.0 | 0 0.0 | 3 4.0 | ns | | 11 **7.2** | **0.019** | **0.087** |
| 06:02:01 | 7 10.0 | 4 10.0 | 3 10.0 | 3 7.1 | 8 10.5 | ns | | 12 7.9 | ns | |
| 07:01:01 | 7 10.0 | 5 12.5 | 2 6.7 | 4 9.5 | 14 18.4 | ns | | 28 18.4 | ns | |
| **07:02:01 (C1)** | 7 10.0 | 2 5.0 | 5 **16.7** | 7 **16.7*** | 3 **4.0** | **0.04** | **4.867** | 6 **4.0** | **0.020** | **4.867** |
| 07:04:01 | 3 4.3 | 3 7.5 | 0 0.0 | 1 2.4 | 1 1.3 | ns | | 2 1.3 | ns | |
| 07:43 | 1 1.4 | 0 0.0 | 1 3.3 | 1 2.4 | 0 0.0 | ns | | 0 0.0 | ns | |
| 08:01 | 0 0.0 | 0 0.0 | 0 0.0 | 0 0.0 | 0 0.0 | ns | | 1 0.7 | ns | |
| 08:02:01 | 2 2.9 | 2 5.0 | 0 0.0 | 1 2.4 | 4 5.3 | ns | | 7 4.6 | ns | |
| 12:02 | 1 1.4 | 1 2.5 | 1 2.5 | 0 0.0 | 2 2.6 | ns | | 1 0.7 | ns | |
| 12:03:01 | 5 7.1 | 2 5.0 | 3 10.0 | 3 7.1 | 4 5.3 | ns | | 12 7.9 | ns | |
| 12:05 | 1 1.4 | 1 2.5 | 0 0.0 | 1 2.4 | 0 0.0 | ns | | 0 0.0 | ns | |
| 14:02 | 3 4.3 | 2 5.0 | 1 3.3 | 1 2.4 | 0 0.0 | ns | | 6 4.0 | ns | |
| 15:02:01 | 1 1.4 | 0 0.0 | 1 3.3 | 1 2.4 | 3 4.0 | ns | | 10 6.6 | ns | |
| 15:05 | 1 1.4 | 1 2.5 | 0 0.0 | 0 0.0 | 1 1.3 | ns | | 2 1.3 | ns | |
| 15:13 | 1 1.4 | 0 0.0 | 1 3.3 | 1 2.4 | 0 0.0 | ns | | 0 0.0 | ns | |
| 16:01 | 3 4.3 | 3 7.5 | 0 0.0 | 2 4.8 | 2 2.6 | ns | | 5 3.3 | ns | |
| 16:02:01 | 1 1.4 | 0 0.0 | 1 3.3 | 1 2.4 | 0 0.0 | ns | | 2 1.3 | ns | |
| 16:04 | 0 0.0 | 0 0.0 | 0 0.0 | 0 0.0 | 0 0.0 | ns | | 2 1.3 | ns | |
| 17:01,03 | 2 2.9 | 1 2.5 | 1 3.3 | 1 2.4 | 1 1.3 | ns | | 6 4.0 | ns | |
| **C1 subgr.** | 25 71.4 | 15 75.0 | 10 66.7 | 20 **47.6** | 28 73.7 | ns | | 48 **63.2** | 0.080 | |
| C2 subgr. | 25 71.4 | 13 65.0 | 12 80.0 | 22 52.4 | 28 73.7 | 0.14 | | 43 56.6 | ns | |

## 3.3 KIR/HLA genes distribution in the total of BC patients

The analysis of KIR genes distribution between BC patients and controls highlighted a significant reduction of the KIR2DS4ins in BC patients (25.5% vs 57.6%, p = 0.001 OR = 0.253), especially towards MCTR, also after Bonferroni's correction (p = 0.022), Table 3. The combined effect of KIR genes with their cognate HLA-C ligands evidenced that frequency of the KIR2DL2 gene, not licensed by HLA-C1 ligand (HLA-C2+), was significantly lower in BC patients than that of FCTR group (28.6% *vs.* 57.9%, p = 0.022 OR = 0.291). This negative correlation was also validated by univariable regression analysis (p = 0.013 OR = 0.291, 95%CI: 0.110–0.772). In presence of HLA-C2, the concurrent lack of KIR2DL2 and KIR2DS4ins was significantly more frequent in patients than in controls (FCTR: 37.1% *vs* 10.5%, p = 0.012 OR = 5.020; MCTR: 37.1% *vs* 12.7%, p = 0.010 OR = 3.886). This result suggests that this combination may increase the susceptibility to develop BC disease. Besides, the percentage of the KIR2DS5 gene (haplotype B), in association with the C2 allele group, was significantly lower in BC patients than in mixed controls (17.1% vs. 63.5%, p = 0.0001 OR = 0.119), Table 4.

Table 3
– Distribution of KIR allele frequencies in BC patients in comparison with two healthy controls groups.

| KIR GENES | BREAST CANCER | | | | | | HEALTHY CONTROLS | | | | COMPARISONS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TOTAL (N = 47) | | EARLY STAGE (N = 26) | | ADVANCED STAGE (N = 21) | | FEMALES (N = 39) | | MIXED * (N = 66) | | TOTAL VS. CONTROLS | | ADVANCED VS. CONTROLS | |
| | N | % | N | % | N | % | N | % | N | % | P-value | OR= | P-value | OR= |
| **2DL1** | 45 | 95.7 | 25 | 96.2 | 21 | 100.0 | 39 | 100.0 | 64 | 97.0 | NS | NS | NS | NS |
| **2DL2** | 24 | 51.1 | 15 | 57.7 | 9 | 42.9 | 26 | 66.7 | 40 | 60.6 | NS | NS | NS | NS |
| **2DL3** | 43 | 91.5 | 23 | 88.5 | 20 | 95.2 | 30 | 76.9 | 53 | 80.3 | NS | NS | NS | NS |
| **2DL4** | 47 | 100.0 | 28 | 100.0 | 19 | 100.0 | 39 | 100.0 | 66 | 100.0 | NS | NS | NS | NS |
| **2DL5A** | 19 | 40.4 | 7 | 26.9 | 12 | 57.1 | 18 | 46.2 | 26 | 39.4 | NS | NS | NS | NS |
| **2DL5B** | 22 | 46.8 | 11 | 42.3 | 11 | 52.4 | 25 | 64.1 | 41 | 62.1 | NS | NS | NS | NS |
| **2DS1 §** | 21 | 44.7 | 7 | **26.9** | 14 | **66.7** | 18 | 46.2 | 36 | 54.5 | 0.031 | 0.307 | NS | NS |
| **2DS2** | 22 | 46.8 | 14 | 53.8 | 8 | 38.1 | 21 | 53.9 | 35 | 53.0 | NS | NS | NS | NS |
| **2DS3** | 16 | 34.0 | 10 | 38.5 | 6 | 28.6 | 13 | 33.3 | 34 | 51.5 | NS | NS | NS | NS |
| **2DS4del*003** | 38 | 80.9 | 23 | 88.5 | 16 | 76.2 | 30 | 76.9 | 56 | 84.8 | NS | NS | NS | NS |
| **2DS4ins*001–002 *** | 12 | **25.5** | 8 | 30.8 | 3 | 14.3 | 14 | 35.9 | 38 | **57.6** | **0.022*** | **0.253** | 0.020 | 0.226 |
| **2DS5** | 16 | 34.0 | 7 | 26.9 | 10 | 47.6 | 15 | 38.5 | 22 | 33.3 | NS | NS | NS | NS |
| **3DL1** | 43 | 91.5 | 25 | 96.2 | 18 | 85.7 | 36 | 92.3 | 60 | 90.9 | NS | NS | NS | NS |
| **3DL2** | 47 | 100.00 | 28 | 100.0 | 19 | 100.0 | 39 | 100.0 | 66 | 100.0 | NS | NS | NS | NS |
| **3DL3** | 47 | 100.00 | 28 | 100.0 | 19 | 100.0 | 39 | 100.0 | 66 | 100.0 | NS | NS | NS | NS |
| **3DS1** | 19 | 40.43 | 7 | 26.9 | 11 | 52.4 | 18 | 46.2 | 29 | 43.9 | NS | NS | NS | NS |

§ KIR2DS1: III-IV grade vs. 0-II grade tumors, **p = 0.015**. * Total BC pts vs mixed healthy controls (p = 0.0014), with Bonferroni correction. (in bold) .

Table 4
– KIR/ KIR-ligand (HLA-C) interaction frequencies in the study groups with comparisons.

| KIR / HLA-C combinations | BREAST CANCER | | | | | | CONTROLS | | | | COMPARISONS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TOTAL (N = 35) | | EARLY STAGE (N = 20) | | ADVANCED STAGE (N = 15) | | FEMALES (N = 38) | | MIXED (N = 63) | | TOTAL VS. CONTROLS | | ADVANCED VS. CONTROLS | |
| | N | % | N | % | N | % | N | % | N | % | P-value | OR= | P-value | OR= |
| 2DL1 HLA-C2+ | 24 | 68.6 | 12 | 60.0 | 12 | 80.0 | 28 | 73.7 | 43 | 68.3 | NS | NS | NS | NS |
| 2DL1 HLA-C1+ | 24 | 68.6 | 14 | 70.0 | 10 | 66.7 | 28 | 73.7 | 46 | 73.0 | NS | NS | NS | NS |
| 2DL2 HLA-C1+ | 14 | 40.0 | 10 | 50.0 | 4 | 26.7 | 17 | 44.7 | 28 | 44.4 | NS | NS | NS | NS |
| **2DL2 HLA-C2+** | 10 | **28.6** | 7 | 35.0 | 3 | **20.0** | 22 | **57.9** | 31 | 49.2 | **0.022** | **0.291** | **0.016** | **0.182** |
| 2DL2 HLA-C1C1 | 7 | 20.0 | 4 | 20.0 | 3 | 20.0 | 4 | 10.5 | 7 | 11.1 | NS | NS | NS | NS |
| **2DL2 HLA-C2C2 §** | 3 | 8.6 | 2 | 10.0 | 1 | **6.7** | 8 | **21.1** | 11 | 17.5 | NS | NS | NS | NS |
| **2DL2 HLA-C1C2 §** | 7 | 20.0 | 5 | 25.0 | 2 | **13.3** | 13 | **34.2** | 20 | 31.7 | NS | NS | NS | NS |
| 2DL3 HLA-C2 | 23 | 65.7 | 11 | 55.0 | 12 | 80.0 | 20 | 52.6 | 43 | 68.3 | NS | NS | NS | NS |
| **2DS1 HLA-C2*** | 8 | 22.9 | **2** | **10.0*** | 6 | **40.0*** | 14 | 36.8 | 22 | 34.9 | NS | NS | NS | NS |
| 2DS1 HLA-C1 | 12 | 34.3 | 4 | 20.0 | 8 | 53.3 | 12 | 31.6 | 21 | 33.3 | NS | NS | NS | NS |
| **2DS4ins HLA-C2** | 4 | 11.4 | 4 | 20.0 | 0 | **0.00** | 11 | **28.9** | 15 | 23.8 | 0.084 | NS | **0.023** | **0.077** |
| 2DS4ins HLA-C1 | 8 | 22.9 | 6 | 30.0 | 2 | 13.3 | 10 | 26.3 | 15 | 23.8 | NS | NS | NS | NS |
| **2DS5 HLA-C2** | 6 | **17.1** | 3 | 15.0 | 3 | **20.0** | 12 | 31.6 | 40 | **63.5** | **0.0001** | **0.119** | **0.003** | **0.144** |
| **$2DL2^{-}$ $2DS4INS^{-}C2+$** | 13 | **37.1** | 4 | 20.0 | 8 | **53.3** | 4 | **10.5** | 8 | 12.7 | **0.012** | **5.020** | **0.002** | **9.71** |

*KIR2DS1/HLA-C2: early tumors vs. advanced tumors p = 0.05. §The comparison of KIR2DL2 HLA-C2/C2 plus KIR2DL2 HLA-C1C2 frequencies between BC patients with advanced tumors and FCTR was significant (p = 0.0313).

## 3.4 Involvement of KIR genes and HLA-C allele subsets in tumor progression.

To distinguish the role of different biomarkers in breast malignancy, the influence of variables such as the invasion type, clinical tumor stage and the presence of immunohistochemistry markers, including ER, PR and HER2, was also considered.

The incidence of the KIR2DS1 gene in patients with advanced stages of the disease was significantly higher than in patients at early stages (66.7% vs. 26.9%, p = 0.015, Table 3) and interestingly, an increased coexistence of KIR2DL1 and KIR2DS1 genes was evidenced in these advanced BC patients (66.7% vs. 19.2% p = 0.002). On the contrary, the presence of KIR2DL1 without KIR2DS1 was instead higher in patients at early stages (0-II grade:18/26 = 69.2% *vs* III:IV grade: 6/21 = 28.6%, p = 0.013). The frequency of the KIR2DS4ins gene was lower in patients with advanced cancer than MCTRs (14.3% vs 57.6%, p = 0.020 OR = 0.226). In the context of KIR/HLA-C combinations, we confirmed the negative association of $KIR2DL2/HLA\text{-}C2^{+}$ (20% vs 57.9% FCTR, p = 0.016 OR = 0.182) and KIR2DS5 gene/HLA-C2+ (20.0% vs. 63.5% MCTR, p = 0.003 OR = 0.144) with advanced disease (Table 4). The influence of HLA-C2 *licensing* for KIR2DL2 + NK cells is shown by the significant difference of the KIR2DL2 HLA-C2/C2 and/or HLA-C1/C2 combinations between patients with advanced tumors and FCTR (20.0% vs. 55.3%, p = 0.031). The same relationship was found in BC patients with LN + metastasis where the AF of KIR2DL2 genes in combination with the homozygous HLA-C1 (C1/C1) was significantly higher than in both controls (vs. FCTR p = 0.042 OR = 4.250; *vs.* MCTR p = 0.042 OR = 4.000). Always in this type of

patients LNM+, the absence of KIR2DL2 and KIR2DS4ins genes in the presence of HLA-C2 was higher (42.9% vs. FCTR:10.5%, p = 0.008 OR = 6.375), Supplemental Table 1.

## 3.5 Evaluation of KIR polymorphisms in BC patients depending on steroid hormone receptors.

Taking into account the presence of specific steroid hormone receptors, $ER^+$ BC patients (n = 25) had a lower frequency of KIR2DS4ins gene compared to controls (20.0% vs MCTR 57.6%, p = 0.002 OR = 0.174). All $ER^+$, $LNM^+$ BC patients (n = 16) carried the KIR2DL3 gene (100.0% *vs* FCTR 76.9%, p = 0.046 OR = 10.28). Similar results were obtained in $PR^+$ and $LNM^+$ BC patients group (KIR2DL3: 100% *vs.* FCTR 76.9%, p = 0.049; KIR2DS4ins: 26.7% *vs* MCTR 57.6%, p = 0.045). Interestingly, the frequency of KIR2DS4ins in ER/PR double-positive patients (n = 22) was significantly lower than that of mixed controls (4/22 18.2% vs 38/66 57.6%, p = 0.001 OR = 0.164) and they showed a lower incidence of B haplotype in homozygosity, compared to female controls (1/22 4.5% *vs* 10/39 25.6%, p = 0.045 OR = 0.138). Noteworthy, KIR2DS1 gene was more frequent in $HER2^+$ patients than in $HER2^{--}$patients (5/5 100% vs. 12/28 42.9%, p = 0.04 OR = 14.52).

## 3.6 FCGR3A polymorphisms in BC onset and clinical outcome

Although no significant difference for FCGR3A 48 and 158 amino acid polymorphisms was found in terms of alleles, we observed the prevalent presence of a valine (G) in position 158 and a leucine (T) in position 48 both in BC patients and controls (Supplemental Table 2). We noted a reduced incidence of the 158TT (FF) homozygous genotype in the whole group of BC patients (2.2% vs. 11.1% FCTR), including those with more aggressive tumors (0% vs. 11.1%, p = 0.13). A significant negative relationship between 48G/T (LR) genotype and the proliferative index Ki-67 expression was observed, as indicated by Spearman's correlation (p = 0.020 rs= -0.439). Besides, the FCGR3A 48–158 haplotype-based analysis showed a different distribution between patients and controls: 48T-158G was the most frequent haplotype in BC patients (HF = 44.2%), while 48T-158T was more commonly identified in FCTR (HF = 44.4%), Suppl. Table 3.

## 3.7 Correlations between FCGR3A haplotypes and KIR genes in BC patients.

When FCGR3A 48–158 haplotypes were combined with KIR genes by using Spearman's ranks method (Table 5), we made some interesting observations: 1. The FCGR3A 48A-158G haplotype was found negatively linked with the KIR2DL2 gene (p = 0.034 rs= -0.434) in LNM + BC patients, while it was negatively associated with KIR2DS4del (p = 0.029 rs = -0.365) and positively with KIR2DS5 gene (p = 0.043 r = 0.339) in the female control group; 2. The 48T-158T haplotype was positively correlated either with KIR2DL2 (p = 0.006 rs = 0.542) or with the KIR2DS2 gene (p = 0.026 rs = 0.454 in LNM + BC patients), while it was positively associated with KIR2DS4ins (p = 0.038 rs = 0.348) and negatively with KIR2DS5 gene (p = 0.041 rs= -0.343) in the controls. It must be noted that there was a significant correlation between the 48T-158T haplotype and KIR2DL2 (p = 0.035 rs = 0.500) in advanced stages of the disease and with KIR2DL3 (p = 0.032 rs = 0.431) in patients with early tumors. It is interesting to note that KIR2DL2 and KIR2DL3 genes are alleles of the same locus, which segregates in different haplotypes (B and A, respectively). In contrast, in relapsed BC patients, a positive relationship with the KIR2DL1gene (p = 0.016 rs = 0.674) (sited on the haplotype A) was also observed. 3. The 48T-158G haplotype was the third haplotype detected in the studied patient population. While it was negatively correlated with KIR2DL1 gene (p = 0.046 rs=-0.475) in more advanced tumors, it was positively associated with KIR2DL5B (p = 0.009, rs = 0.512) and KIR2DS3 (p = 0.012, rs = 0.493) genes (loci near in haplotype B) in early-stage tumors and in FCTRs to KIR2DS4ins gene.

Table 5
Spearman's correlation analysis between FCGR3A 48–158 haplotypes and KIR genes in BC patients compared to female healthy controls.

| **FCGR3A** | **Grade 0-II BC patients (n = 25)** | **P=** | **rs** | **FCTR (n = 36)** | **P=** | **rs** |
|---|---|---|---|---|---|---|
| 48–158 haplotype | KIR gene | | | KIR gene | | |
| **48G-158G** | 2DL5B | 0.046 | -0.411 | — | | |
| **48T-158T** | 2DL3 | 0.032 | 0.431 | 2DS4ins | 0.038 | 0.348 |
| | | | | 2DS5 | 0.041 | -0.343 |
| **48T-158G** | 2DL5B | 0.009 | 0.512 | 2DS4ins | 0.000 | -0.565 |
| | 2DS3 | 0.012 | 0.493 | | | |
| **FCGR3A** | **Grade III-IV BC patients (n = 18)** | **P=** | **rs** | **FCTR (n = 36)** | **P=** | **rs** |
| **48T-158T** | 2DL2 | 0.035 | 0.500 | 2DS4ins | 0.038 | 0.348 |
| | | | | 2DS5 | 0.041 | -0.343 |
| **48T-158G** | 2DL1 | 0.046 | − 0.475 | 2DS4ins | 0.000 | -0.565 |
| **FCGR3A** | **LNM* BC patients (n = 24)** | **P=** | **rs** | **FCTR (n = 36)** | **P=** | **rs** |
| **48A-158G** | 2DL2 | 0.034 | − 0.434 | 2DS4del | 0.029 | -0.365 |
| | | | | 2DS5 | 0.043 | 0.339 |
| **48T-158T** | 2DL2 | 0.006 | 0.542 | 2DS4ins | 0.038 | 0.348 |
| | 2DS2 | 0.026 | 0.454 | 2DS5 | 0.041 | -0.343 |
| | 2DL2/HLA-C1 | 0.032 | 0.480 | | | |
| **48T-158G** | – | | | 2DS4ins | 0.000 | -0.565 |

# 4 Discussion

Breast cancer is characterized by a microenvironment often infiltrated by immune cells, including tumor-infiltrating T cells (TILs) and to a lesser extent, NK cells (25). Metastatic dissemination of cancer cells consists of an interplay between cancer cell-intrinsic factors (genetic and epigenetic diversification) and micro environmental immunosuppressive determinants, such as metabolic, stromal and immunological factors. Interindividual variability in the NK receptors repertoire is also influenced by KIR genes, HLA class I alleles and FCGR3A (CD16A) genetic polymorphisms. The presence of steroid hormone receptors, including ER (estradiol E2), mediates immunostimulation or immunosuppression, particularly on NK cells in a time-dependent manner (26–28).

To date, the analysis of gene polymorphisms at the nucleotide level of the HLA-C locus, in combination with those of FCGR3A in breast cancer, hasn't been rated yet. For this purpose, we carried out a case-control study between BC patients and healthy controls in order to evaluate the impact of the KIR/HLA-C and FCGR3A genes on the pathogenesis and progression of breast cancer in the Italian population and correlate them with proliferation and some key clinical features of BC, including tumor stage and recurrence.

Based on our observations, first of all the absence of HLA-C*05:01:01 allele could be considered protective toward BC, while the presence of C*07:02:01 could play a role in BC progression. We can speculate that a differential expression of these alleles might influence the efficacy of the immune response toward cancer cells given that, as suggested by previous studies (29), different expression of HLA-C7 and HLA-C5 antigens were found. Low levels of HLA-C*07 allele cell surface expression are due to a more restrictive peptide-binding pocket than the HLA-C*05 allele, that has a flatter cleft that allows binding of a larger range of peptides.

This situation can stabilize the HLA-C molecule affecting its expression level on the cell surface. As a consequence, the HLA-C7 and HLA-C5 antigens may negatively and positively regulate T cell immunosurveillance, respectively. Also the predisposing effect of the HLA-C*04:01:01 allele to lymph node metastasis could be due to its low cell surface expression (30). These features might cause NK hyporesponsiveness predisposing to tumor invasiveness.

Defective NK cell cytotoxicity has been described in a variety of solid tumors, including breast cancer (25, 31) which was associated with increased frequency of CD56*bright* NK cells in peripheral blood (32). Thus, while the interaction between *inhibitory* KIRs and their HLA class I ligands, by the process of "licensing", allows NK cells to acquire full effector functions, a mismatch makes NK cells hypo-responsive. Also the *activating* KIR-HLA class I licensing may influence NK unresponsiveness to cancer cells. NK cells expressing activating KIR2DS1 in presence of self-HLA-C2 ligands are poorly responsive toward cancer (33). Our study indicates that in BC patients KIR2DS4ins and KIR2DL2 genes, not licensed by their HLA-C1 ligands, seem to be protective from the neoplasm onset. Conversely, the absence of both KIR2DS4 and KIR2L2 genes may increase the risk of BC occurrence. The protective role of KIR2DS4ins could be due to its inhibitory action on NK response towards bacterial or viral infections, predisposing to a particular tumor microenvironment. Indeed, it's known that KIR2DS4ins receptor recognizes recA peptides derived by pathogens, mainly in the context of HLA-C5 antigen (34). Also, the protective role on BC pathogenesis of the inhibitory KIR2DL2 receptor, unlicensed by C1 ligands, maybe due to the NK cell unresponsiveness to cancer cells. Our study evidenced that a simultaneous absence of the inhibiting KIR2DL2, not licensed by its cognate ligand, and the KIR2DS4ins gene increases the risk of tumor progression nine-fold.

Furthermore, we can also speculate that the activating KIR2DS1 might play a role in breast cancer aggressiveness, maybe supporting a chronic over-stimulation of NK cells. The association of this gene with increased BC risk had already been highlighted in another analysis on Turkish patients with advanced BC compared to controls (35). This correlation might depend by the influence in patients with advanced BC from the simultaneous presence of its equivalent inhibitory, KIR2DL1 gene. On the contrary, the presence of KIR2DL1 in the absence of the KIR2DS1 counterpart, in advanced tumors, suggests a putative "antitumor role" of the KIR2DL1 receptor. This information is also supported by analysis of Ashoury E (36). In addition, interestingly, BC patients with lymph node metastasis have shown a significantly higher frequency of the KIR2DL2 gene in presence of HLA-C1/C1 (OR = 4.25) than healthy controls, that by the effect of the process of "licensing" could favor full pro-inflammatory effector functions.

We could speculate that the activation of inflammation mediated by KIR2DS1 gene could be turned off by KIR2DL2/KIR2DS4, but only when they are not licensed by the HLA-C1 ligand. Such results suggest that the susceptibility to BC transformation and progression might depend on the type of genotype combinations. Indeed, the involvement of *activating* KIRs in cancer pathogenesis was also observed by other Authors (37, 38) in chronic myeloid leukemia (CML) and nasopharyngeal carcinoma, respectively. Increases in the levels of innate immune response stimulation may contribute to an increased risk of some virus-associated cancers, maybe through an amplified inflammatory response triggered by NK cells (or other effector cells) expressing activating KIRs.

Examining the influence of KIR genes on lymph node status, in the context of hormone markers, we did not evidence any difference in the progression of disease between ER or PR positive patients, even though in these patients a prevalent frequency of the KIR2DL3 gene and a reduction of KIR2DS4ins gene were specifically detected. It is possible to hypothesize that breast cancer hormones may influence in different way some immune responses depending on genetic background (i.e. every HER-$2^+$ BC patients in our study carried the KIR2DS1 gene and showed signs of metastasis).

Fcγ receptors are essential for the ADCC pathway and FCGR3A gene functional polymorphisms may affect the killing function of immune effector cells. FCGR3A-158 G/T gene polymorphism is the most studied biomarker for ADCC and several reports have already demonstrated its correlation with the efficacy of monoclonal antibodies treatment in solid tumors (39, 15, 40). One of the goals of this work was to define the impact of FCGR3A gene polymorphisms together with KIR/HLA-C interaction on BC pathogenesis and malignancy. While FCGR3A gene polymorphisms alone did not show a prominent role in the development of BC, the 48–158 haplotypes in the context of particular KIR genes had different correlations in BC patients compared to female controls. Our results suggested the possible existence of a cooperation between these genetic factors involved in ADCC response and KIR/HLA functional repertoire.

## Conclusion

Although in a relatively small sample size, our study examined first the influence of the genetic basis of KIR receptors, together with their HLA-C ligands and FCGR3A genes for NK antitumor activity using high-resolution techniques. Our findings suggest that in the development of breast cancer exists a disorder of immune regulation and that NK cells might represent a promising target for the development of immunotherapeutic strategies for metastatic disease. The KIR-HLA and FCGR3A associations evidenced in this analysis constitute a hypothetical indication of genetic risk scores for tailored screening of BC patients. Further studies in BC patients with advanced tumors are needed to verify the specific *in vivo* activation of NK cells. These results could potentially guide immunotherapy against breast cancer by specifically targeting NK cell clones with particular KIR-HLA-CD16A patterns favoring antitumor activity.

# Declarations


### Acknowledgments

This work was supported by the IG10555 grant from the Italian Association for Cancer Research (AIRC) and partly by CNR funds.


### Author's contributions

A.C. and A.A. designed the study, analyzed and interpreted the data. A.C. performed KIR and HLA-C genotyping and analyzed results. T.D.B. performed FCGR3A SBT and A.A. analyzed data. AC, A.A. and G.S. wrote the manuscript. G.N., O.B., P.R., A.V. and F.P. critically revised the manuscript. G.S. provided fundings. All the authors approved the final version.


**Corresponding author**: Angelica Canossi, C.N.R. Institute of Translational Pharmacology via Carducci 32C, 67100 L'Aquila, Italy. E-mail: angelica.canossi@cnr.it. Phone +39.0862.318843.

**Co-corresponding author**: Giuseppe Sconocchia, Laboratory of Tumor Immunology and Immunotherapy, C.N.R. Institute of Translational Pharmacology (IFT), Via Fosso Del Cavaliere 100 – 00133, Roma, Italy. E-mail: giuseppe.sconocchia@ift.cnr.it.


### Competing Interests

The authors report there are no competing interests to declare.

# References


1. DeSantis CE, Ma J, Gaudet MM, Newman LA, Miller KD, Goding Sauer A et al. Breast cancer statistics. CA Cancer J Clin. 2019, 69(6):438–451.
2. Segovia-Mendoza M, Morales-Montor J. Immune Tumor Microenvironment in Breast Cancer and the Participation of Estrogen and Its Receptors in Cancer Physiopathology. Front Immunol. 2019; 10: 348.
3. Coppola A, Arriga R, Lauro D, Del Principe MI, Buccisano F, Maurillo L et al. NK Cell Inflammation in the Clinical Outcome of Colorectal Carcinoma. Front Med (Lausanne) 2015; 2:33.
4. Wende H, Colonna M, Ziegler A, Volz A. Organization of the leukocyte receptor cluster (LRC) on human chromosome 19q13.4. Mamm Genome 1999; 10(2):154–160.
5. Hollenbach JA, Meenagh A, Sleator C, Alaez C, Bengoche M, Canossi A et al. Report from the killer immunoglobulin-like receptor (KIR) anthropology component of the 15th International Histocompatibility Workshop: worldwide variation in the KIR loci and further evidence for the co-evolution of KIR and HLA. Tissue Antigens 2010; 76(1):9–17.
6. Biassoni R, Falco M, Cambiaggi A, Costa P, Verdiani S, Pende D et al. Amino acid substitutions can influence the natural killer (NK)-mediated recognition of HLA-C molecules. Role of serine-77 and lysine-80 in the target cell protection from lysis mediated by "group 2" or "group 1" NK clones. J Exp Med. 1995; 182(2):605–9.
7. Parham P. MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol. 2005; 5(3):201–214.
8. Al Omar S, Middleton D, Marshall E, Porter D, Xinarianos G, Raji O et al. Associations between genes for killer immunoglobulin-like receptors and their ligands in patients with solid tumors. Hum Immunol. 2010; 71(10):976–81.
9. Middleton D, Vilchez JR, Cabrera T, Meenagh A, Williams F, Halfpenny I et al. Analysis of KIR gene frequencies in HLA class I characterised bladder, colorectal and laryngeal tumours. Tissue Antigens 2007; 69(3):220–226.

10. De Re V, Caggiari L, De Zorzi M, Talamini R, Racanelli V, D' Andrea M et al. Genetic diversity of the KIR/HLA system and outcome of patients with metastatic colorectal cancer treated with chemotherapy. PLoS One. 2014; 9(1):e84940.
11. Berghella AM, Aureli A, Canossi A, Beato TD, Colanardi A, Pellegrini P. Redox, immune and genetic biomarker system for personalized treatments in colorectal cancer. World J Gastrointest Oncol. 2019; 11(2):117–138.
12. Canossi A, Aureli A, Del Beato T, Rossi P, Franceschilli L, De Sanctis F et al. Role of KIR and CD16A genotypes in colorectal carcinoma genetic risk and clinical stage. J Transl Med. 2016; 14(1):239.
13. Bhat R, Watzl C. Serial killing of tumor cells by human natural killer cells-enhancement by therapeutic antibodies. PLoS One 2007; 2(3):e326.
14. Arriga R, Caratelli S, Lanzilli G, Ottaviani A, Cenciarelli C, Sconocchia T et al. CD16-158-valine chimeric receptor T cells overcome the resistance of KRAS-mutated colorectal carcinoma cells to cetuximab. Int J Cancer 2020: 146(9):2531–2538.
15. D'Aloia MM, Caratelli S, Palumbo C, Battella S, Arriga R, Lauro D et al. T lymphocytes engineered to express a CD16-chimeric antigen receptor redirect T-cell immune responses against immunoglobulin G-opsonized target cells. Cytotherapy 2016; 18(2):278–290.
16. Terszowski G, Klein C, Stern M. KIR/HLA interactions negatively affect rituximab- but not GA101 (obinutuzumab)-induced antibody-dependent cellular cytotoxicity. J Immunol. 2014: 192(12):5618–5624.
17. Caratelli S, Sconocchia T, Arriga R, Coppola A, Lanzilli G, Lauro D, Venditti A, Del Principe MI, Buccisano F, Maurillo L, Ferrone S, Sconocchia G (2017) FCgamma Chimeric Receptor-Engineered T Cells: Methodology, Advantages, Limitations, and Clinical Relevance. Front Immunol 8:457.
18. Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de Haas M Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. Blood 1997; 90(3):1109–1114.
19. de Haas M, Koene HR, Kleijer M, de Vries E, Simsek S, van Tol MJ et al. A triallelic Fc gamma receptor type IIIA polymorphism influences the binding of human IgG by NK cell Fc gamma RIIIa. J Immunol. 1996: 156(8):2948–2955.
20. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987; 235(4785):177–82.
21. Hortobagyi GN. Trastuzumab in the treatment of breast cancer. N Engl J Med. 2005; 353(16):1734–1736.
22. Luen S, Virassamy B, Savas P, Salgado R, Loi S. The genomic landscape of breast cancer and its interaction with host immunity. Breast 2016; 29:241–250.
23. Savas P, Salgado R, Denkert C, Sotiriou C, Darcy PK, Smyth MJ et al. Clinical relevance of host immunity in breast cancer: from TILs to the clinic. Nat Rev Clin Oncol. 2016; 13(4):228–241.
24. Campbell KS, Purdy AK. Structure/function of human killer cell immunoglobulin-like receptors: lessons from polymorphisms, evolution, crystal structures and mutations. Immunology 2011; 132(3):315–325.
25. Sconocchia G, Arriga R, Tornillo L, Terracciano L, Ferrone S, Spagnoli GC Melanoma cells inhibit NK cell functions. Cancer Res. 2012; 72(20):5428–5429; author reply 5430.
26. Screpanti I, Santoni A, Gulino A, Herberman RB, Frati L. Estrogen and antiestrogen modulation of the levels of mouse natural killer activity and large granular lymphocytes. Cell Immunol. 1987; 106(2):191–202.
27. Manukyan G, Martirosyan A, Slavik L, Ulehlova J, Dihel M, Papajik T et al. 17beta-Estradiol Promotes Proinflammatory and Procoagulatory Phenotype of Innate Immune Cells in the Presence of Antiphospholipid Antibodies. Biomedicines 2020; 8:8(6):162.
28. Nilsson N, Carlsten H. Estrogen induces suppression of natural killer cell cytotoxicity and augmentation of polyclonal B cell activation. Cell Immunol. 1994; 158(1):131–139.
29. Kaur G, Gras S, Mobbs JI, Vivian JP, Cortes A, Barber T et al. Structural and regulatory diversity shape HLA-C protein expression levels. Nat Commun. 2017; 8:15924.
30. Kulkarni S, Savan R, Qi Y, Gao X, Yuki Y, Bass SE et al. Differential microRNA regulation of HLA-C expression and its association with HIV control. Nature 2011; 472 (7344):495–498.
31. Sconocchia G, Spagnoli GC, Del Principe D, Ferrone S, Anselmi M, Wongsena W et al. Defective infiltration of natural killer cells in MICA/B-positive renal cell carcinoma involves beta(2)-integrin-mediated interaction. Neoplasia 2009; 11(7):662–671.

32. Verma C, Kaewkangsadan V, Eremin JM, Cowley GP, Ilyas M, El-Sheemy MA et al. Natural killer (NK) cell profiles in blood and tumour in women with large and locally advanced breast cancer (LLABC) and their contribution to a pathological complete response (PCR) in the tumour following neoadjuvant chemotherapy (NAC): differential restoration of blood profiles by NAC and surgery. J Transl Med. 2015; 13:180.
33. Venstrom JM, Pittari G, Gooley TA, Chewning JH, Spellman S, Haagenson M et al. HLA-C-dependent prevention of leukemia relapse by donor activating KIR2DS1. N Engl J Med. 2012; 367(9):805–816.
34. Sim MJW, Rajagopalan S, Altmann DM, Boyton RJ, Sun PD, Long EO. Human NK cell receptor KIR2DS4 detects a conserved bacterial epitope presented by HLA-C. Proc Natl Acad Sci U S A 2019; 116 (26):12964–12973.
35. Ozturk OG, Gun FD, Polat G. Killer cell immunoglobulin-like receptor genes in patients with breast cancer. Med Oncol. 2012, 29(2):511–515.
36. Ashouri E, Rajalingam K, Barani S, Farjadian S, Ghaderi A, Rajalingam R Coexistence of inhibitory and activating killer-cell immunoglobulin-like receptors to the same cognate HLA-C2 and Bw4 ligands confer breast cancer risk. Sci Rep. 2021; 11(1):7932.
37. Zhang Y, Wang B, Ye S, Liu S, Liu M, Shen C et al. Killer cell immunoglobulin-like receptor gene polymorphisms in patients with leukemia: possible association with susceptibility to the disease. Leuk Res. 2010; 34(1):55–58.
38. Butsch Kovacic M, Martin M, Gao X, Fuksenko T, Chen CJ, Cheng YJ et al. Variation of the killer cell immunoglobulin-like receptors and HLA-C genes in nasopharyngeal carcinoma. Cancer Epidemiol Biomarkers Prev. 2005; 14(1 Pt1):2673–2677.
39. Caratelli S, Arriga R, Sconocchia T, Ottaviani A, Lanzilli G, Pastore D et al. In vitro elimination of epidermal growth factor receptor-overexpressing cancer cells by CD32A-chimeric receptor T cells in combination with cetuximab or panitumumab. Int J Cancer 2020; 146(1):236–247.
40. Gavin PG, Song N, Kim SR, Lipchik C, Johnson NL, Bandos H et al. Association of Polymorphisms in FCGR2A and FCGR3A With Degree of Trastuzumab Benefit in the Adjuvant Treatment of ERBB2/HER2-Positive Breast Cancer: Analysis of the NSABP B-31 Trial. JAMA Oncol. 2017; 3(3):335–341.

## Figures

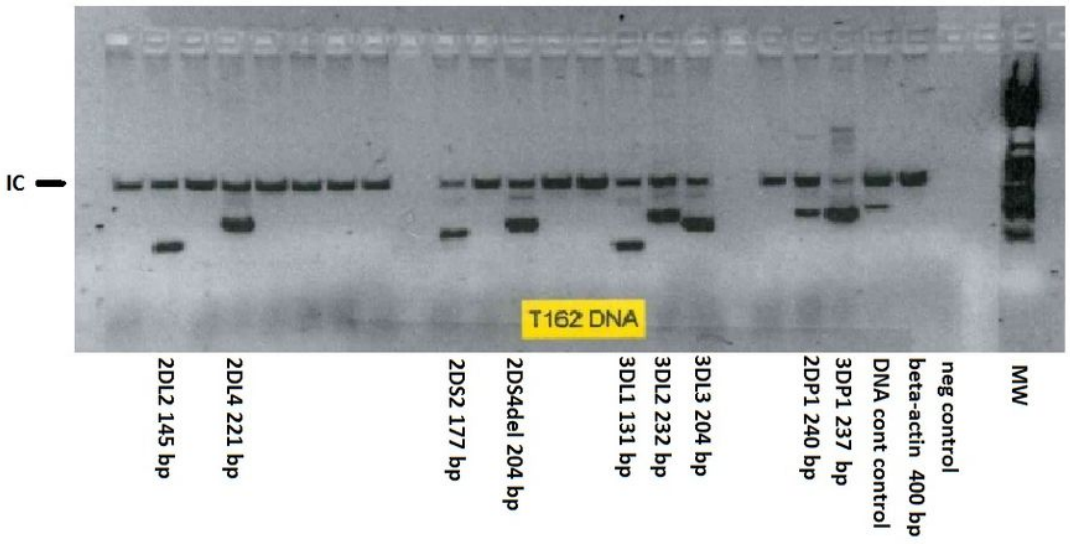


Figure 1

An example of a gel image of KIR genotyping. Every amplified KIR gene corresponding to the lanes is indicated below the gel image. All the PCR products contains an internal control and their molecular weight is estimable by comparison with a molecular weight.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TabS1LNM.docx
- TableS2FCGR3A.docx
- TABLES3FCGR3AHAPLO.docx