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ORIGINAL RESEARCH

Human Leukocyte Antigen Class I Expression and Natural Killer Cell Infiltration and Its Correlation with Prognostic Features in Luminal Breast Cancers

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Purpose: The aim of this study was to determine whether low HLA-I expression and NK cells infiltration are related to prognostic features in breast cancer, as observed in cancers in other locations and non-hormone dependent breast cancers. Particularly, we explored their relation to infiltrated axillary lymph nodes (ALNs), with the aim of finding new predictors helping to decide the extent of axillary surgery.

Patients and Methods: We conducted a retrospective correlational analysis of 35 breast cancers from 35 breast cancer patients showing axillary infiltration at diagnosis and with upfront surgery. HLA-I H-score and the number of NK cells x 50 high power fields (HPF) in the biopsy specimen were correlated with pathological variables of the surgical specimen: number of infiltrated ALNs, tumor size, histological type, the presence of ductal carcinoma in situ, focality, histological grade, necrosis, lymphovascular and perineural invasion, Her2Neu status, and the percentages of tumor-infiltrating lymphocytes (TILs), estrogen receptor, progesterone receptor, ki67, and p53.

Results: All tumors showed hormone receptor expression and three of them Her2Neu positivity. A positive correlation (p=0.001**) was found between HLA-I H-score and TILs and Ki67 expression. HLA H-score increased with histological grade and was higher in unifocal than in multifocal disease (p=0.044 and p=0.011, respectively). No other correlations were found.

Conclusion: High HLA-I H-score values correlated with features of poor prognosis in this cohort of luminal breast tumors, but not with infiltrated ALNs. This finding highlights the differences between luminal breast cancer, and cancers in other locations and non-hormone dependent breast cancers, in which low HLA-I expression tends to be associated with poor prognostic features.

Keywords: luminal breast cancer, HLA-I, natural killer cells, prognostic features, axillary lymph nodes

Introduction

Breast cancer is the most common cancer in women worldwide.[1] Its prognosis depends on tumour size, lymph node involvement, tumour grade, hormone receptor status, Her2 status, proliferation rate, genetic mutations and patients age.[2] Currently, surgical treatment is the standard approach for all new non-metastatic breast cancer cases, resulting in a high number of breast cancer survivors dealing with the secondary effects of this treatment. Several studies have contributed to making breast cancer surgery in the 21st century progressively less radical to minimize these secondary effects, especially in the management of the axilla. In this context, recent trials have assessed the possibility of limiting surgery not only in patients with no evidence of axillary involvement but also in those with a low axillary tumor burden.[3]

Therefore, quantifying the extent of axillary tumor burden is crucial to tailor surgical interventions. To do this, imaging tests are essential,[4] but some authors have postulated that the pathological and genetic characteristics of the tumor,[5] as well as the interaction between tumor cells and patient immunity,[6] may also be useful in predicting axillary tumor burden before treatment.

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Studies aiming to predict axillary tumor burden by investigating the interaction between tumors and the immune system have reported contradictory results. Some authors have reported that downregulation of human leukocyte class I antigen (HLA-I) is an immune evasion mechanism that occurs in 32.5% to 54% of breast cancers and is associated with a higher axillary tumor burden.[7] Other authors, however, claim that higher HLA-I expression is associated with positive axillary lymph nodes.[8]

Equally, the possible role of natural killer (NK) cells in breast cancer is also unclear, as some authors suggest these cells constitute a very small percentage of tumor-infiltrating lymphocytes (TILs), casting doubt on their role in tumor containment.[9] However, Rezaeifard et al reported that infiltration by activated NK cells was higher in tumors from patients with negative lymph nodes than in those with neoplastic lymph nodes.[6]

The main aim of this study was to determine whether HLA-I expression and NK cell tumor infiltration could be associated with prognostic features in luminal breast cancer, particularly axillary tumor burden. This information could be relevant for deciding the extent of axillary surgery, as most luminal breast cancers are treated upfront with surgery.

Materials and Methods

Study Design

We planned a retrospective correlation study to correlate HLA-I expression and NK cells infiltration in breast cancer tissue samples with clinical and pathology features, including axillary tumor burden.

Ethical Aspects

Patients included in this study were informed of the possibility of storing their tissue samples in the Tissue Bank of the Parc de Salut Mar (MARBiobanc) for research purposes. Their voluntarily had agreed for their clinical and pathology data, from both the diagnostic biopsy and the surgical specimen, to be held by the MARBiobanc. All patients signed the informed consent form for this purpose. This study was approved by the Ethics Committee of the Hospital del Mar Medical Research Institute (IMIM, number 2018/8361/I, MARBiobanc 2022S009) and complied with the Declaration of Helsinki statements.

Patient Cohort

Based on studies conducted by our group on HLA-I expression in other tissues, we hypothesized that breast cancers with a low axillary burden would exhibit HLA-I expression in 50% of the cells, whereas breast cancers with a high axillary burden would exhibit HLA-I expression in 90% of the cells. Using a unilateral test with a 95% confidence interval and 80% statistical power, 15 patients were needed in each group for the study. Assuming a 10% loss in recruitment, 17 patients were needed in each group to conduct this pilot study.

We identified 35 diagnostic breast biopsy samples from the MarBiobanc, taken from 35 breast cancer patients with axillary lymph node involvement who had undergone initial surgical treatment including lymphadenectomy in our institution in the previous 3 years. These samples were processed for immunohistochemical analysis of HLA-I expression, NK cell infiltration, and p53 expression. We decided to include p53 expression in the study as it is a marker of tumors with poor prognosis[10] that is not routinely examined by the Breast Pathology Unit of our hospital when analyzing breast cancer samples.

We created a new database for the study using our prospective database from Breast Diseases Unit and employing MS Excel. This new database included clinical information (age and body mass index), data from the diagnostic biopsy (TILs, HLA-I and p53 expression, and NK cell quantification) and data from the surgical biopsy following the American College of Pathologists protocol[11] (histological type and grade, tumor size, tumor focality; presence/absence of ductal carcinoma in situ, tumor necrosis, lymphovascular invasion, perineural invasion, axillary lymph node status including the total number of infiltrated lymph nodes, the number of lymph nodes with macrometastases, micrometastases and isolated tumor cells, and the size of the largest metastatic nodal deposit). Estrogen receptor (ER), progesterone receptor (PR), and Her2 expression were collected in surgical biopsies and assessed following clinical routine guidelines together with Ki67 expression, that was reported as proliferation index.

Sample Processing

We used standard methods for tissue fixation (10% buffered formalin) and processing. TILs were evaluated on hematoxylin-eosin stained sections of the diagnostic biopsy following the scoring guidelines of the International Immuno-Oncology Biomarker Working Group on breast cancer.[12] TILs scores were defined as the percentage of tumor stromal area occupied by mononuclear inflammatory cells. We excluded areas of crush artifacts, necrosis, regressive hyalinization, immune cell infiltrates related to areas of carcinoma in-situ or adjacent to normal breast cancer lobules.

Immunohistochemical (IHC) studies to assess the surrogate molecular profile were performed on 3µm, formalin-fixed paraffin-embedded whole tissue sections, in both diagnostic and resection biopsies, although only the studies in the resection biopsies were included in this study. The antibody panel included ER (clone SP1; pre-diluted; Roche Diagnostics; Basel; Switzerland), PR (clone 1E2; pre-diluted; Roche Diagnostics), HER2 (clone 4B5; pre-diluted; Roche Diagnostics) and Ki67 (clone 30–9; pre-diluted; Roche Diagnostics) and was performed on an automated immunostainer "BenchMark XT IHQ Roche". ER, PR and HER2 expression were assessed following routine clinical guidelines.[11] ER and PR positivity were defined considering a cut-off value of ≥ 1% positively stained nuclei. HER2 expression was analyzed according to current guidelines: negative cases included: "0", meaning there was no immunostaining or weak incomplete membranous staining in ≤10% of the tumor cells; "1+", if there was weak incomplete membranous staining in >10% of the tumor cells; positive cases were those with an intense complete membranous staining in >10% of the tumor cells (positive 3+). Equivocal cases or "2+" included those with a weak or moderate complete membranous staining in >10% of tumor cells. Equivocal cases were further evaluated for HER2 gene amplification by in situ hybridization (ISH), with fluorescence or silver, and were classified as positive or negative accordingly.

HLA-I and p53 expression together with NK cells infiltration were evaluated on the diagnostic biopsies with IHC studies. The reason for that was that diagnostic biopsies end to be better preserved than resection biopsies. To assess HLA-I expression, we used clone EMR8-5, dilution 1/2000 (Abcam, Cambridge, UK). To quantify NK cell infiltration, the positivity for CD56 was identified with clone MRQ-42, pre-diluted (Cell Marque, Rocklin, California, USA). To assess p53 expression, we used clone DO7, pre-diluted (Roche Diagnostics). These slides were digitally scanned at 20X magnification on the "Aperio CS2 ScanScope Leica Biosystems". HLA-I immunostaining in tumor cells was scored as an H-score by two expert pathologists (LC, IV) on a multiheaded microscope.

Slides to evaluate p53 and CD56 expression were analyzed with the Quantitative Pathology & Bioimage Analysis Software (QuPath, version 0.2.3). For p53, we used this software to establish the absolute number and percentage of positive cells, together with the intensity of the expression: low (+), moderate (++) or high intensity (+++). To evaluate NK cell infiltration, we counted cells with a morphology corresponding to NK cells and positive for CD56 in the tumor areas of the slide per mm^2. Then, we estimated the number of NK cells in 50 high power fields (HPF, magnification x40).

Correlation Between HLA-I Expression and NK Cells Tumor Infiltration with Other Variables

The HLA-I H-score and the number of NK cells x 50 high-power field (HPF) were presented as median and range when appropriate and were correlated with other variables in our database.

The continuous variables studied were age in years, body mass index, tumor size in mm, number of infiltrated lymph nodes, and percentages of TILs, ER, PR, Ki67 and p53.

The categorical variables analyzed were histological type (non-special type (NST) carcinoma vs other], ductal carcinoma in situ (DCIS) present in the biopsy (yes vs no), focality (unifocal vs multifocal/multicentric disease), histological grade (I vs II vs III), necrosis (yes vs no), lympho-vascular invasion (yes vs no), perineural invasion (yes vs no), and Her2Neu (negative vs positive).

Statistical Analysis

To correlate the HLA-I H score and the number of NK cells in 50 hPF with continuous variables, we performed the non-parametric Spearman correlation test. To correlate HLA-I score and the number of NK cells in 50 hPF with categorical variables, we performed the Mann–Whitney test, comparing the median for both in each category.

All statistical analyses were two-sided and P values <0.05 were considered significant. Statistical analyses were performed using PASW version 18 (IBM SPSS software, USA).

Results

The general characteristics of the cohort are summarized in Table 1. Because the included patients had lymph node involvement at diagnosis and initial surgical treatment, most of them had the Her2-negative luminal immunophenotype.

On correlating HLA-I H-score and the number of NK cells in 50 hPF with continuous variables, we found no correlation with the number of infiltrated lymph nodes. We did, however, observe a positive correlation between HLA-I

Table 1 Description of the Breast Cancer Patients Included in the Study

Feature	Measure	Value
Age, years	Mean (SD)	58.30 (12.65)
Menopause		
No	Number (%)	13 (43.3)
Yes	Number (%)	17 (56.7)
Body mass index, kg/m^2	Mean (SD)	25.34 (5.60)
Tumor size, mm	Median (range)	18.27 (17.71–41.62)
Infiltrated lymph nodes, number	Median (range)	2 (1–13)
Pathology type		
Carcinoma NST	Number (%)	20 (66.7)
Lobular carcinoma	Number (%)	9 (30)
Other	Number (%)	1 (3.3)
DCIS in the biopsy		
No	Number (%)	15 (50%)
Yes	Number (%)	15 (50%)
Focality		
Unifocal	Number (%)	24 (80)
Multifocal or multicentric	Number (%)	6 (20)
Histological grade		
I	Number (%)	4 (13.33)
II	Number (%)	19 (63.33)
III	Number (%)	7 (23.34)
Necrosis		
No	Number (%)	27 (90)
Yes	Number (%)	3 (10)
Lymphovascular infiltration		
No	Number (%)	14 (46.66)
Yes	Number (%)	16 (53.34)
Perineural infiltration		
No	Number (%)	24 (80)
Yes	Number (%)	6 (20)
TILs %	Median (range)	5 (1–30)
ER %	Median (range)	98.50 (0–99)
PR %	Median (range)	90 (0–99)
Her2Neu		
Negative	Number (%)	27 (90)
Positive	Number (%)	3 (10)
Ki67 %	Median (range)	20 (5–40)
p53 %	Median (range)	6.82 (0–60.36)
NKs x50 HPF	Median (range)	2.85 (0–61.31)
HLA-I H score	Median (range)	60.0 (0–285.0)

Abbreviations: NST, non-specified type; DCIS, ductal carcinoma in situ; tils, tumor infiltrating lymphocytes; ER, estrogen receptor; PR, progesterone receptor; NKs, natural killer cells; HPF, high power fields.

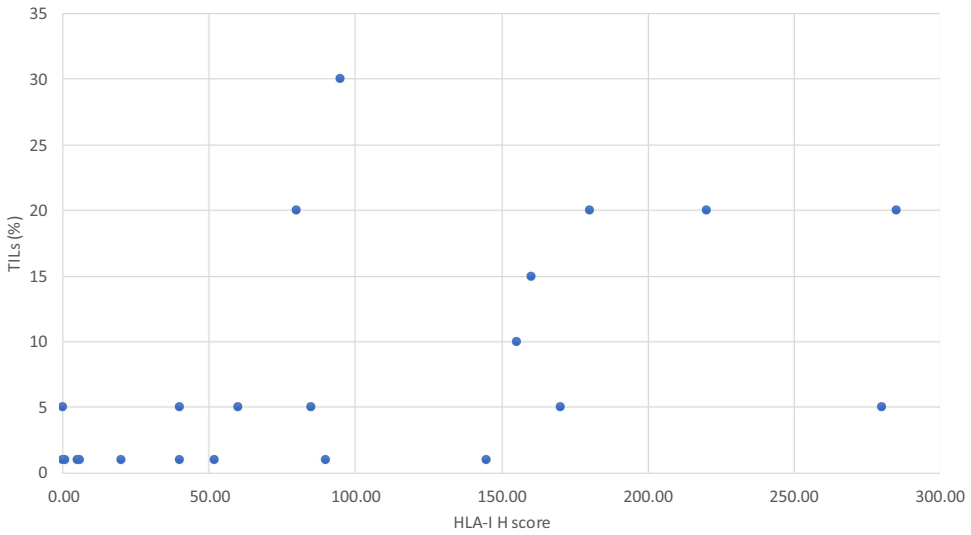

Figure 1 Correlation between HLA-I H score and TIL infiltration in the cohort of evaluated tumors.

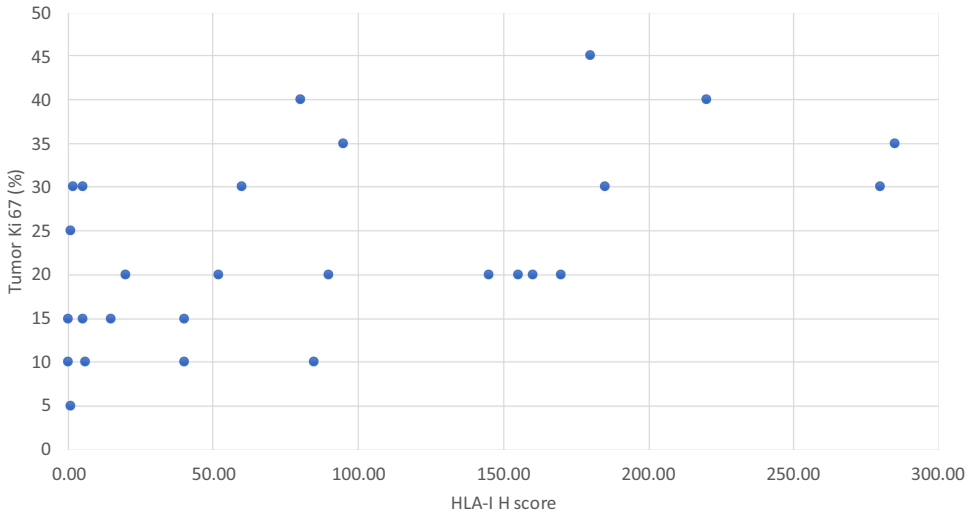

Figure 2 Correlation between the Ki67 of the tumor (%) and HLA-I H score.

score and the percentage of TILs (Figure 1) as well as with the percentage of cells expressing Ki67 (Figure 2). No correlation was found between NK cells and any of the continuous variables. These results are summarized in Table 2.

Correlating HLA-I score and NK cells with categorical variables revealed that the higher the HLA-I score, the higher the histological grade (p=0.044, Figure 3). HLA-I score was also higher in unifocal than in multifocal/multicentric disease (p=0.011). No association was found between the percentage of NK cells and categorical variables. These results are summarized in Table 3.

Discussion

In this study, high HLA-I expression was not significantly associated with axillary tumor burden but was associated with higher histological grade and Ki67 index, greater TIL infiltration, and unifocal tumors.

To our knowledge, this is the first study to associate HLA-I expression with tumor focality. Other authors have previously reported differences in the biology of unifocal tumors compared with multifocal/multicentric breast cancers.

Table 2 Correlation Between HLA-I H Score and the Number of NK Cells x 50 HPF with Continuous Clinical and Histopathological Variables

	NK x50 HPF			HLA I score		
	n	Pearson correlation	p	n	Pearson correlation	p
Age, years	30	0.16	0.4	29	0.165	0.39
Bodymass index, kg/m^2	29	−0.167	0.39	28	0.204	0.3
Tumor size, mm	30	0.219	0.25	29	0.241	0.21
Infiltrated lymph nodes, number	30	0.281	0.13	29	0.015	0.94
TILs %	26	0.002	0.99	25	0.668	0.00
ER %	30	−0.143	0.45	29	0.073	0.71
PR %	30	−0.249	0.19	29	0.188	0.33
Ki67 %	30	−0.084	0.66	29	0.53	0.00
p53 %	29	0.287	0.13	27	0.088	0.66
NKs x 50 HPF				26	0.053	0.79

Abbreviations: ER, estrogen receptor; HPF, high power field; NK, natural killer; PR, progesterone receptor; TIL, tumor infiltrating lymphocytes; HPF, high power fields.

Weissenbacher et al found down-regulated expression of E-cadherin in multifocal/multicentric breast cancer compared with unifocal disease.[13] Zehni et al concluded that hormone receptor expression was a prognostic factor in unifocal but not in multifocal or multicentric breast cancer[14] Akbulut et al confirmed that the miRNA expression profile differed between unifocal and multifocal/multicentric breast tumors.[15] Our results are consistent with those of these authors, also showing differences in the biology of multifocal/multicentric and unifocal breast tumors. Further research should clarify the significance of the lower HLA-I expression in multifocal/multicentric breast tumors compared with unifocal disease.

Our results are also consistent with those of other authors who have linked higher HLA-I expression with markers of poor tumor prognosis,[16] but contrast with those of other studies that have related downregulation of HLA-I expression in breast cancer to poor prognosis.[17,18] These conflicting results have been attributed to the different antibodies used and the various cutoffs employed to assess staining. Moreover, these differences may be also due to the different breast cancer subtypes evaluated.[16]

HLA-I downregulation is an immune evasion mechanism observed in multiple tumors, including some breast tumors[7,17] but not all. Low HLA-I expression is more common in HER2-positive subtypes[16,19] and triple-negative subtypes[16] than in luminal subtypes, in which high expression is associated with markers of poor prognosis. Likewise,

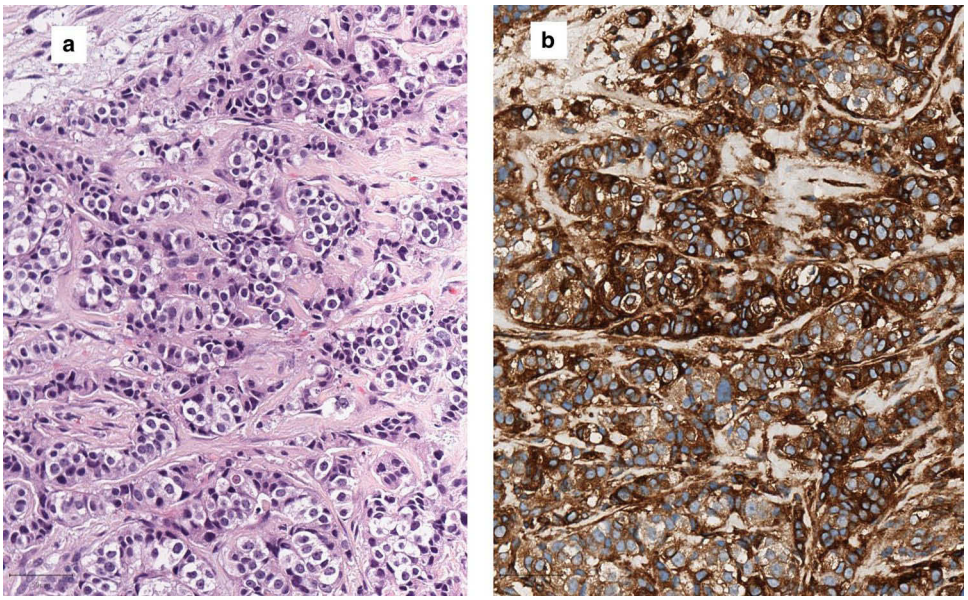

Figure 3 A breast tumor area showing high histological grade in the Hematoxylin-Eosin staining (**a**) and a high HLA-I H-score in the immunohistochemical study (**b**).

Table 3 Correlation Between HLA-I H Score and the Number of NK Cells x 50 HPF with Categorical Histopathological Variables

Features		NKs x50 HPF			HLA I score		
		n	Median (range)	p	n	Median (range)	p
Pathological type	Carcinoma NST	20	1.43 (0–61.31)		19	90 (0–285)	
	Lobular carcinoma	9	5.33 (0–21.95)	0.365	9	40 (0–160)	0.205
DCIS in the biopsy	No	15	3.68 (0–26.7)		13	90 (0–285)	
	Yes	15	2.16 (0–61.31)	0.87	16	50 (0–280)	0.174
Focality	Unifocal	24	4.50 (0–26.7)		24	87.5 (0–285)	
	Multifocal or multicentric	6	0.30 (0–61.31)	0.174	5	5 (1–15)	0.011
Histological grade	I	4	4.19 (0–61.31)		4	10 (1–20)	
	II	19	2.16 (0–26.65)		19	60 (0–285)	
	III	7	5.63 (0–26.7)	0.601	6	162.5 (5–280)	0.044
Necrosis	No	27	2.16 (0–61.31)		26	46 (0–285)	
	Yes	3	5.63 (2.26–26.70)	0.283	3	180 (95–220)	0.067
Lymphovascular infiltration	No	14	1.29 (0–61.31)		14	30 (1–285)	
	Yes	16	4.5 (0–26.70)	0.334	15	95 (0–280)	0.217
Perineural infiltration	No	24	4.50 (0–61.31)		22	46 (0–285)	
	Yes	6	1.27 (0–36.65)	0.402	7	80 (1–170)	0.94
Her2Neu	Negative	27	3.68 (0–61.31)		28	70 (0–285)	
	Positive	3	0.6 (0–90)	0.2	1	6 (6–6)	0.552

Abbreviations: NK, natural killer; NST, non-specified type; DCIS, ductal carcinoma in situ.

high HLA-I expression has been associated with lower disease-free survival in HER2-negative luminal tumors but not in HER2-positive or triple-negative tumors.[8] This information strongly suggests that, in breast cancers with strong hormonal dependence for their growth and spread, HLA-I expression could be positively related to their poor clinical outcomes, while less hormone-dependent breast tumors could behave like non-hormone-dependent tumors in other locations.

This association between high HLA-I expression and markers of poor prognosis in tumors with high hormonal dependence is unsurprising, as the interaction between HLA-I and NK cells has a major role in the physiology and pathology of hormone-dependent organs. For instance, NK physiological cell infiltration in the endometrium is well established, as is their role in endometrial changes during the menstrual cycle[20] and their interaction with HLA-I molecules in the process of implantation and tolerance to paternal antigens, activating NK cells inhibitory receptors.[21] NK cell dysfunction, partly induced by HLA-I overexpression in the endometrium,[22] has been associated with the pathogenesis of diseases such as endometriosis.[23,24]

Epithelial cells in the breast undergo continuous proliferation and regression during the menstrual cycle, a feature they share with endometrial cells. The presence of NK cells in healthy mammary gland and in breast tumors has already been confirmed.[25] Therefore, it is important to investigate whether the role of NK cells in the breast has a certain overlap with their role in the endometrium, and whether HLA-I overexpression could contribute to neoplastic degeneration and progression. Linked to this idea, it has been proved that, at one hand, progesterone increases HLA-I expression,[26] and, at the other hand, progesterone exposure increases the risk of developing breast cancer.[27] It would be sensible investigating if the increased breast cancer risk due to progesterone exposure could be linked to NK cells inhibition secondary to progesterone-induced HLA-I overexpression.

A possible explanation for the association between high HLA-I expression and tumors with poor prognosis at the molecular level is that aggressive tumors accumulate more mutations and, therefore, more neoantigens. These neoantigens may attract immune cells that could produce cytokines and interferon gamma, which in turn could increase HLA-I expression in tumor cells.[28]

In this study, we found no association between NK cells with either axillary tumor burden or with other tumor characteristics. Nevertheless, we believe that NK cells should be a focus of attention in breast cancer immunity research.

NK infiltration in luminal tumors is limited[29] and is clearly lower than that observed in triple-negative tumors.[30] Even so, breast cancer hormone receptor-positive cell lines are more susceptible to lysis by NK cells stimulated with interleukin 2 than Her2-positive or triple-negative breast cancer cell lines.[31] It has also been reported that node-negative tumors have a greater number of activated NK cells than those with infiltrated nodes, and that larger tumors have a higher percentage of regulatory NK cells.[6] Future studies should be conducted to establish the role of NK cell immunosurveillance in normal glandular tissue and breast cancer.

Our study has some limitations. The patient cohort is small and with highly specific characteristics, as all of them had axillary lymph node infiltration, and most were Her2-negative luminal tumors. The reason for the small number of patients was that, at that stage of the project, we aimed to carry out a pilot study, limited in time and resources, to better guide the following steps for our research group. In this regard, because this was a highly generic study of both HLA-I and NK cells, we may not have detected differences that might have been detected in a more in-depth study of these parameters.

Despite these limitations, we believe that these results could be useful, as they may help other researchers in the design of future studies, given that significant differences were detected by analyzing only a few cases. The present study, together with those of other authors, suggest that immune evasion mechanisms in hormone-dependent tumors could differ markedly from those of hormone-independent tumors. This notion could be key in the research of the pathophysiology of breast cancer and in the search for therapeutic targets.

Conclusion

In this study we observed that, in a cohort with predominantly Her2-negative luminal breast cancer, high HLA-I expression was not associated with axillary tumor burden but was associated with unifocal tumors, high histological grade, a high ki67 index, and greater TIL infiltration than other luminal tumors. Further research should establish the importance of the HLA-I score as a prognostic and predictive tool in breast cancer.

Data Sharing Statement

The data supporting the results of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Disclosure

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