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ORIGINAL ARTICLE

HLA class I alterations in breast carcinoma are associated with a high frequency of the loss of heterozygosity at chromosomes 6 and 15

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Abstract

HLA class I (HLA-I) molecules play a crucial role in the presentation of tumor antigenic peptides to CD8+ T cells. Tumor HLA-I loss provides a route of immune escape from T cell-mediated killing. We analyzed HLA-I expression in 98 cryopreserved breast cancer tissues using a broad panel of anti-HLA-I antibodies. Genomic HLA-I typing was performed using DNA obtained from autologous normal breast tissue. Analysis of the loss of heterozygosity (LOH) in the HLA-I region of chromosome 6 (LOH-6) and in the β2-microglobulin (B2M) region of chromosome 15 (LOH-15) was done by microsatellite amplification of DNA isolated from microdissected tumor areas. B2M gene sequencing was done using this DNA form HLA-I-negative tumors. Immunohistological analysis revealed various types of HLA-I alterations in 79 tumors (81%), including total HLA-I loss in 53 cases (54%) and partial loss in 16 samples (14%). In 19 cases (19%), HLA-I expression was positive. Using microsatellite analysis, we detected LOH in 36 cases out of 92 evaluated (39%), including 15 samples with only LOH-6, 14 with LOH-15, and seven tumors with LOH-6 and LOH-15 at the same time. Remarkably, we detected LOH-6 in eight tumors with positive HLA-I immunolabeling. We did not find any B2M mutations in HLA-I-negative breast tumors. In conclusion, LOH at chromosomes 6 and 15 has a high incidence in breast cancer and occurs in tumors with different HLA-I immunophenotypes. This common molecular mechanism of HLA-I alterations may reduce the ability of cytotoxic T lymphocytes to kill tumor cells and negatively influence the clinical success of cancer immunotherapy.

Keywords HLA class I · Breast cancer · Loss of heterozygosity · Cancer immune escape

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Abbreviations

HLA	Human leukocite antigens
MHC	Major histocompatibility antigens
LOH	Loss of heterozygosity
B2M	Beta-2-microglobilin
FFPE	Formalin fixed paraffin embedded
IDC	Infiltrating ductal carcinoma
ILC	Infiltrating lobular carcinoma
TNM	Tumor-node-metastasis
ER	Estrogen receptor
PgR	Progesterone receptor
SSO	Sequence-specific oligonucleotide analysis
STR	Short tandem repeat

Introduction

It is widely accepted that one of the principal mechanisms of anti-tumor immunity is T cell-mediated cytotoxicity, which

relies on the recognition of tumor antigenic peptides presented by HLA class I (HLA-I) molecules expressed on tumor cell surface (Boesen et al. 2000; Coulie et al. 2014; Wang et al. 2008; Ryschich et al. 2005). The absence of HLA-I expression is a common finding in different tumor tissues (Lopez-Nevot et al. 1989; Garrido et al. 1993; Marincola et al. 2000; Seliger et al. 2002) and is a major mechanism used by tumor cells to escape from T cell-mediated immune surveillance (Romero and Coulie 2014; Boesen et al. 2000; Garrido et al. 1997a, 2017a). In addition, there is a growing evidence demonstrating that HLA-I expression in different types of cancer has a prognostic value and is associated with disease progression, invasiveness, metastatic potential, and resistance to therapy (del Campo et al. 2014; Perea et al. 2017; Sade-Feldman et al. 2017). It has been recently reported that adoptive transfer of tumor-infiltrating lymphocytes specific for neo-antigens detected in the tumor in conjunction with interleukin-2 and checkpoint blockade induced a complete durable regression of metastatic breast cancer (Zacharakis et al. 2018). This neoantigen-specific T cell therapy requires normal expression of HLA-I molecules on tumor cell surface. Therefore, the status of tumor HLA-I expression is important for the success of T cell and peptide-mediated cancer immunotherapy.

It is also well established that the molecular mechanisms responsible for the HLA-I loss plays a crucial role in the ability to recover the HLA expression in different types of cancer immunotherapy. These mechanisms can be subdivided into two major groups: reversible/"soft" or irreversible/"hard" (Garrido et al. 2010a, 2010b). Reversible alterations are associated with transcriptional downregulation of HLA-I and antigen-presenting machinery (APM) genes and can be recovered by different cytokines. The "hard" alterations are caused by mutations/deletions in HLA-I, B2M, and/or IFN-gamma genes and cannot be corrected by cytokines or by immunotherapy. Hence, it is essential to investigate molecular mechanisms responsible for HLA-I alterations in cancer to understand the mechanisms of tumor escape and predict the response to therapy.

There have been several reports describing the frequency of HLA-I alterations in breast cancer, ranging from as high as 90% (Cabrera et al. 1996; Perez et al. 1986) to 30% (Kaneko et al. 2011). This discrepancy could be due to the different methods and anti-HLA antibodies used in various studies. For example, in formalin fixed paraffin embedded (FFPE) tissues, HLA heavy chain/B2M complex on the cell surface loses conformational epitopes and cannot be detected by the commonly used w6/32 antibody. In addition, there is little information about the molecular mechanisms involved in HLA-I altered expression in breast cancer (Madjd et al. 2005; Concha et al. 1991a, b; Pedersen et al. 2017).

In this manuscript, we present the results of immunohistological and molecular analysis of HLA-I and II (HLA-II) expression performed on 98 cryopreserved breast cancer samples. We also evaluated 53 HLA-I-negative samples for the presence of mutation/deletions in B2M gene and investigated the frequency of the loss of heterozygosity at chromosomes 6 (LOH-6, HLA heavy chain genes) and 15 (LOH-15, B2M gene) in 92 samples.

Material and methods

Patients and samples

Ninety-eight patients with breast carcinoma were included in this study. All patients were female and their mean age was 59 (ranging from age 26 to 81). Patient samples (tumor specimens and autologous normal breast tissue samples) were obtained from Virgen de las Nieves University Hospital (Granada, Spain). Demographic, clinical, and histological characteristics of the studied subjects/tumors are summarized in Table 1. Before the study, all medical records and tumor sections were reviewed by an oncologist and a surgical pathologist. Signed informed consent approved by the Ethics Committee of our institution was obtained from all the patients. The specimens included 67 infiltrating ductal carcinoma (IDC), 17 infiltrating lobular carcinoma (ILC), and 14 tumors of various histological type (see Table 1) based on WHO criteria of histopathological classification. Tumors were classified as stages I ($n = 24$), II ($n = 27$), III ($n = 46$), and IV ($n = 1$) based on the American Joint Committee on Cancer Guidelines, tumor-node-metastasis (TNM) (Sobin et al. 2009). Expression of the estrogen receptor (ER), progesterone receptor (PgR), and HER2 were examined by immunohistochemical staining.

HLA typing

HLA typing of the patients using DNA isolated from the autologous normal breast tissue was performed in our laboratory using low-resolution genomic sequence-specific oligonucleotide analysis (SSO) from Dynal RELI HLA-A, B, C, DR kits (Dynal Biotech Ltd., Wittal, UK).

Immunohistological analysis of HLA-I and HLA-II expression in breast cancer specimens

Tumor samples from primary breast tumors and autologous normal breast tissue samples were obtained by surgical excision and immediately stored at − 80 °C. Four to eight-micrometer-thick cryopreserved tumor tissue sections were allowed to dry at room temperature for 4–18 h, fixed in acetone at 4 °C for 10 min, and stored at − 40 °C until immunohistological analysis using Biotin-Streptavidin System (NovolinkTM Polymer Detection System). Table 2 summarizes all mouse monoclonal antibodies (mAbs) used to analyze HLA-I and HLA-II expression. Total HLA-I loss was considered when less than 25% tumor cells were stained.

Table 1 Association between clinicopathologic parameters and HLA class I expression in 98 breast cancer patients

		Negative (n = 79)	Positive (n = 19)	p value
Medium age	59.88 ± 13.30			
Tumor size	T1	26 (33%)	11 (57.9%)	
	T2	48 (60.7%)	6 (31.5%)	
	T3	2 (2.5%)	1 (5.3%)	0.044*
	T4	3 (3.8%)	1 (5.3%)	
Nodal invasion				
	Negative	39 (49%)	12 (63%)	0.280
	Positive	40 (51%)	7 (37%)	
Estrogen receptor				
	Negative	18 (23%)	6 (32%)	0.553
	Positive	61 (77%)	13 (68%)	
Progesterone receptor				
	Negative	24 (30%)	9 (47%)	0.159
	Positive	55 (70%)	10 (53%)	
Metastasis				
	Negative	65 (82%)	17 (90%)	0.730
	Positive	14 (18%)	2 (10%)	
TNM				
	I	9 (24%)	5 (26.3%)	
	II	22 (28%)	5 (26.3%)	
	III	38 (48%)	8 (42.1%)	
	IV	0 (0%)	1 (5.3%)	
Histology				
	IDC	54 (68%)	13 (68%)	
	ILC	12 (15%)	5 (27%)	
	Other	13 (17%)	1 (5%)	
HER2/neu receptor				
	Negative	67 (85%)	15 (79%)	0.506
	Positive	12 (15%)	4 (21%)	

*Statistically significant correlation between tumor size and HLA-I expression was found ($p = 0.044$) when tumors were divided into two groups: T1 versus T2+T3+T4

When between 25 and 75% of tumor cells were labeled positively, tumor was considered to have a heterogeneous pattern. Finally, immunolabeling was considered to be positive when more than 75% of tumor cells labeled with W6/32 and GRH-1 mAbs according to the criteria established by the HLA and cancer component of the 1996 International Histocompatibility Workshop (Garrido et al. 1997b; Cabrera et al. 2003). In negative controls, the primary antibody was replaced with PBS.

Tumor microdissection and DNA isolation

Four to eight-micrometer-thick cryopreserved tumor tissue sections were fixed in 70% ethanol, stained with a 0.05% *w/v* solution of toluidine blue and microdissected using a laser micromanipulator (PALM Micro Laser Systems, ZEISS). Microdissected tumor fragments were collected in PALM Adhesive Caps and used to isolate DNA with Qiagen DNA isolation kit (QIAamp Tissue Kit, the Netherlands). This DNA was used for microsatellite analysis (LOH studies) and for B2M sequencing (in HLA-I-negative tumors).

Microsatellite analysis to detect LOH at chromosomes 6 and 15

This analysis was done on 92 of the studied tumors. Eight short tandem repeat (STRs) markers (7 in 6p21 y 1 in 6q21) were used for the LOH study at chromosome 6 (HLA heavy chain genes) (D6S291, D6S273, D6S265, D6S105, D6S276, C.1.2.C, C.1.2.5, and D6S311 respectively). Five markers spanning B2M genes were used for the analysis of LOH-15 (D15S126, D15S146, D15S1028, D15S153 in the 15q21 adjacent to the B2M gene and a telomeric marker D15S209) (Maleno et al. 2001; Maleno et al. 2006; Ramal et al. 2000). The amplification reaction was done in 15 μl volume using

Table 2 Panel of monoclonal antibodies

Monoclonal antibody	Specificity	Laboratory	
W6/32	HLA-ABC/ß$_2$m	Dr. W. Bodmer	(Barnstable et al. 1978)
GRH1	ß$_2$m	Dr. F. Garrido	(López-Nevot et al. 1986)
A131	HLA-A	Dr. J. Kornbluth	(Spear et al. 1985)
YTH-76	HLA-B	Dr. C. Milstein	(Burrone et al. 1985)
HC-10	HLA-BC free heavy chain	Dr. H. Ploegh	(Neefjes and Ploegh 1988)
Kre-501	HLA-A2,28	Dr. M. Kreisler	(Madrid, España)
160-30	HLA-A3	Dr. K. Gelsthorpe	(Sheffield, Inglaterra)
41-HA	HLA-A23,24	One Lambda	
LT-129.11	HLA-A30,31	Dr. A.G. Palma-Carlos (Lisboa, Portugal)	
BB7.1	HLA-B7	Dr. W. Bodmer	(Oxford, Inglaterra)
KS4	HLA-B7 CREG	Dr. S. Ferrone	
MRE-4	HLA-B8	Dr. R. Fauchet	(Rennes, Francia)
66-HA	HLA-B12	One Lambda	
116-5-28	HLA-Bw4	Dr. K. Gelsthorpe	
126.39	HLA-Bw6	Dr. K. Gelsthorpe	
GRB-1	HLA-DR	Dr. F. Garrido	(Cabrera et al. 1986)

1.5 μl of DNA (0.50 μg/μl) and 1 μl of the primer mixture (5 μl of each). The products of the amplification were analyzed by 5% acrylamide gel electrophoresis and sequenced using an automatic sequencer ABI PRISM 377 ADN (PE Applied Biosystems). Data analysis was performed using the software Genotyper program (PE Applied Biosystem). As a reference control, we used DNA obtained from normal autologous breast tissue. LOH was calculated as height of the signal of the tumor allele two/height of area of tumor allele one divided by the height of the signal of normal allele two/height of area of normal allele one. LOH was assigned when more than 25% of signal reduction of one allele was observed in the tumor simples as compared to the normal tissue. Haplotype loss was considered to exist when a tumor exhibited an allelic reduction in three or more STRs markers in chromosome 6. LOH-15 was assigned to the sample when signal was reduced in two or more STRs in chromosome 15 (Ramal et al. 2000).

B2M gene sequencing in HLA-I-negative tumors

The amplification of B2M gene from tumor fragments microdissected from HLA-I-negative tumors was performed using genomic DNA and Illustra PuRe-Taq Ready-To-GoTM PCR Beads (GE Healthcare Europe, Barcelona, Spain) with the following forward primers: 5′-CG ATATTCCTCAGGTACTC C-3′ and 5′-GGTG AATTCAGTG TAGTACAAG-3′, and one reverse primer: 5′-ACACAACTTTCAGCAGCTTAC-3′. The predicted PCR product sizes were 311 and 114 bp, respectively. Sequencing was performed with the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Warrington, UK) using Centri-Sep Columns (Applied Biosystems) and ABI 3130× = Genetic Analyzer and Sequencing Analysis v5.2 software (Applied Biosystems).

Statistical analysis

All statistical analyses were performed using the Statistical Package for the IBM-SPSS Statistics Ver.21. Variables with normal distribution are expressed as means with standard deviation, minimum, maximum, and range. The categorical variables such as tumor size, nodal invasion, estrogen receptor, progesterone receptor, TNM, histological tumor characteristics, HER2 receptor, and HLA-I expression were coded in two groups and analyzed using the chi-square (χ^2) or Fisher exact test in case when the validity criteria were not reached. Differences were considered statistical significant for $p < 0.05$.

Results

Immunohistological analysis of HLA class I expression in breast cancer tissue samples

The expression of HLA-I antigens was evaluated in 98 breast cancer tissue using immunohistochemical staining with monoclonal antibodies directed against B2M and against monomorphic, HLA-A and HLA-B locus-specific and HLA-I allelic determinants. We detected various types of HLA-I alterations in 79 out of 98 studied samples (81%) (Tables 3 and 4). Total HLA-I loss, with negative immunolabeling of both B2M (GRH-1 antibody) and HLA-I/B2M complex (W6/32 antibody), was found in

Table 3 HLA class expression in breast cancer

Sample	HLA typing Normal tissue	Tumor size	HLA-A-B-C-B2M complex	B2M	HLA-ABC free heavy chain	HLA-A	HLA-B	HLA-DR
HLA class I negative (n = 53)								
BC-1	A*32,*34; B*52,*53; C*04,*12	T2	−	−	−			NT
BC-2	A*36,*68; B*14,*53; C*05,*08	T1	−	−	−			NT
BC-3	A*02,*24; B*07,*44; C*02,*07	T2	−	−	−			NT
BC-4	A*01,*02; B*08,*49; C*07,-	T2	−	−	−			NT
BC-15	A*18,-; B*24,-; C*07,-	T2	−	−	−			NT
BC-10	A*02,-; B*44,*57; C*05,*07	T1	−	−	−			NT
BC-13	A*02,*30; B*35,*42; C*04,*17	T2	−	−	−			NT
BC-14	A*01,*25; B*08,*18; C*07,*12	T2	−	−	−			NT
BC-19	A*02,*32; B*41,*42; C*07,*17	T2	−	−	−			NT
BC-30	NT	T1	−	−	−			NT
BC-31	A*01,*02; B*18,*49; C*05,*07	T1	−	−	−			NT
BC-35	A*02,*03; B*08,*49; C*05,*07	T2	−	−	NT			−
BC-36	A*11,*30; B*18,*35; C*04,*05	T2	−	−	−			NT
BC-37	A*01,*02; B*8,*51; C*07,*15	T1	−	−	−			NT
BC-38	A*26,*30; B*15,*18; C*05,*07	T1	−	−	−			NT
BC-39	A*02,*24; B*18,*51; C*04,*15	T1	−	−	−			−
BC-40	A*01,*02; B*35,*51; C*04,*15	T1	−	−	−			−
BC-42	A*02,*11; B*40,*57; C*03,*04	T2	−	−	−			−
BC-43	A*30,*32; B*18,*45; C*06,*12	T2	H	−	−			NT
BC-45	A*02,*03; B*14,*41; C*15,*17	T2	−	−	H			H
BC-48	A*30,*32; B*18,*44; C*04,*05	T1	−	−	−			−
BC-49	A*29,*31; B*44,*45; C*06,*16	T2	−	−	−			−
BC-50	A*24,*33; B*14,*44; C*05,*08	T1	−	−	+			−
BC-51	A*01,*32; B*08,*38; C*07,*12	T1	−	−	−			−
BC-52	A*2,*24; B*51,-; C*02,*14	T2	−	−	+			+
BC-55	A*2,*68; B*14,*38; C*08,*12	T2	−	−	+			−
BC-56	A*02,*11; B*44,*51; C*05,*16	T2	−	−	−			−
BC-63	A*02,*68; B*15,*51; C*07,*16	T4	−	−	+			−
BC-64	A*24,*29; B*39,*44; C*04,*12	T2	−	−	H			NT
BC-65	NT	T1	−	−	−			NT
BC-68	A*11,*26; B*37,*52; C*06,*12	T2	−	−	NT			NT
BC-70	A*23,*24; B*39,*78; C*07,*16	T2	−	−	−			−
BC-73	A*24,*74; B*35,*37; C*04,*06	T4	−	−	+			+
BC-74	A*03,*24; B*14,* 15; C*03,*08	T2	−	H	H			−
BC-75	A*02,*24; B*35,*35; C*04,*04	T1	−	−	H			−
BC-77	A*11,*33; B*14,*35; C*04,*08	T2	−	−	+			−
BC-79	A*24,*25; B*07,*44; C*05,*07	T2	−	−	−			−
BC-82	A*02,*29; B*44,*45; C*05,*06	T1	H	H	+			+
BC-83	A*03,*24; B*35,*35; C*04,*06	T1	NT	−	−			NT
BC-84	A*11,*24; B*35,*44; C*04,*05	T1	−	H	+			NT
BC-85	A*03,*11; B*35,*51; C*24,*15	T1	−	−	−			−
BC-86	A*02,*24; B*35,*45; C*04,*16	T1	−	−	−			−
BC-87	A*02,*24; B*35,*45; C*04,*06	T4	−	−	+			−
BC-88	A*02,*66; B*44,*49; C*04,*07	T1	−	−	−			−
BC-91	A*24,*30; B*40,*50; C*04,*50	T3	−	−	−			−
BC-92	A*02,*29; B*40,*57; C*02,*06	T2	−	−	−			−
BC-95	A*23,*11; B*35,*35; C*02,*04	T2	−	−	+			−

Table 3 (continued)

Sample	HLA typing Normal tissue	Tumor size	HLA-A-B-C-B2M complex	B2M	HLA-ABC free heavy chain	HLA-A	HLA-B	HLA-DR
BC-96	A*03,*23; B*07,*44; C*07,*07	T1	−	−	+			−
BC-97	A*26,*68; B*14,*49; C*07,*49	T2	−	−	+			−
BC-27	A*01,*24; B*14,*44; C*02,*07	T2	H	H	H			−
BC-41	A*01,*02; B*07,*18; C*05,*07	T2	−	H	H			−
BC-80	A*02,*23; B*44,*49; C*05,*07	T2	H	+	H			−
BC-93	A*26,*32; B*49,*50; C*07,*50	T2	H	H	+			+
HLA locus (A or B) negative (n = 9)								
BC-21	A*11,*26; B*50,*53; C*04,*07	T2	+	+	+	H	+	−
BC-22	A*02,*24; B*07,*51; C*07,*02	T2	+	+	+	H	+	−
BC-26	A*02,-; B*_07_,*44; C*05,*07	T2	+	+	NT	+	−	−
BC-32	A*02,*11; B*35,*57; C*04,*06	T2	+	+	+	H	H	+
BC-47	A*01,*02; B*15,*53; C*04,-	T2	NT	+	+	+	H	−
BC-59	A*24,*26; B*14,*40; C*02,*08	T2	−	+	+	−	+	−
BC-62	A*02,*11; B*15,*50; C*03,*06	T1	−	+	−	H	+	−
BC-24	A*02,*68; B*18,*40; C*05,*12	T1	+	NT	+	NT	−	+
BC-81	A*02,*24; B*15,*38; C*04,*12	T2	+	+	+	−	+	H
HLA haplotype loss (n = 8)								
BC-7	A*11,*30; B*18,*51; C*05,*15	T1	+	+	+	+	+	−
BC-11	A*03,*25; B*07,*15; C*03,*07	T2	+	+	+	+	+	−
BC-20	A*02,*29; B*14,*_44_; C*05,*08	T2	+	+	+	+	+	NT
BC-29	A*02,*68; B*14,*35; C*04,*08	T2	+	+	+	+	+	−
BC-53	A*02,*25; B*15,*18; C*03,*05	T1	+	+	+	+	+	−
BC-71	A*_02_,*24; B*_07_,*15; C*03,*07	T1	+	+	+	+	+	+
BC-78	A*30,*33; B*14,*18; C*05,*18	T2	+	+	+	+	+	−
BC-98	A*03,*_25_; B*35,*41; C*03,*07	T2	+	+	+	+	+	NT
HLS allele loss (n = 5)								
BC-16	A*_02_,*03; B*07,*57; C*06,*07	T2	+	+	+	+	+	NT
BC-23	A*02,*30; B*_15_,*51; C*03,*14	T2	+	+	NT	+	+	+
BC-28	A*02,*29; B*_07_,*44; C*07,*16	T2	+	+	+	+	+	+
BC-54	A*11,*29; B*40,*_44_; C*03,*05	T2	+	+	+	+	+	H
BC-46	A*_02_,*11; B*35,*52; C*04,*12	T2	+	+	+	+	NT	+
Combined HLA losses (n = 4)								
BC-33	A*03,*11; B*07,*51; C*04,*07	T1	+	+	H	−	−	−
BC-67	A*24,*32; B*35,*44; C*04,*07	T2	+	+	+	H	+	−
BC-34	A*01,*_02_; B*07,*18; C*05,*07	T2	+	+	+	+	−	+
BC-90	A*03,*03; B*07,*52; C*07,*12	T3	−	+	+	+	H	H
No alterations detected (n = 19)								
BC-5	B*18,*51; C*05,*15	T2	+	+	+	+	+	NT
BC-6	A*01,*02; B*27,*37; C*01,*06	T4	+	+	+	+	+	−
BC-8	A*01,*31; B*18,*38; C*05,*12	T1	+	+	+	+	+	−
BC-9	A*02,*23; B*14,*51; C*02,*08	T2	+	+	+	+	+	+
BC-12	A*01,*11; B*08,*35; C*04,*07	T3	+	+	+	+	+	−
BC-17	A*02,*23; B*18,*49; C*07,-	T2	+	+	+	+	+	NT
BC-18	A*02,-; B*07,*41; C*01,*04	T1	+	+	+	+	+	NT
BC-25	A*23,*69; B*40,*44; C*03,*04	T1	+	+	NT	+	+	+
BC-44	A*02,*03; B*35,*51; C*01,*04	T1	+	+	+	+	+	+
BC-57	A*11,*23; B*07,*58; C*07,*07	T1	+	+	+	NT	NT	
BC-58	A*29,*30; B*15,*18; C*03,*05	T1	+	+	+	+	+	−

Table 3 (continued)

Sample	HLA typing Normal tissue	Tumor size	HLA-A-B-C- B2M complex	B2M	HLA-ABC free heavy chain	HLA-A	HLA-B	HLA-DR
BC-60	A*01,*02; B*08,*51; C*07,*15	T1	−	+	+	+		−
BC-61	A*03,*30; B*07,*08; C*05,*07	T1	+	+	+	+	NT	+
BC-66	A*31,*36; B*38,*50; C*12,*16	T1	+	+	+	+	+	−
BC-69	A*02,*11; B*35,*40; C*03,*04	T2	+	+	+	+	+	+
BC-72	A*26,*26; B*38,*51; C*14,*16	T1	+	+	H	NT	+	H
BC-76	A*02,*26; B*41,*51; C*12,*17	T2	+	+	+	+	NT	−
BC-94	A*02,*32; B*18,*44	T2	+	+	+	+	+	H
BC-89	A*24,*30; B*44,*50; C*04,*07	T1	+	+	H	+	+	+

HLA-I alleles presented in italics are lost in the corresponding tumor samples

H heterogeneous immunolabeling pattern, *NT* not tested

54% (53 out of 98) of the samples. We also included in this group 7 cases with heterogeneous HLA-I staining. Interestingly, in 13 cases out of these 53 HLA-I-negative cases, we observed positive intracellular labeling of free heavy chains (HC-10 antibody). Sequencing of exons 1 and 2 of B2M gene in all breast HLA-I-negative tumors ($n = 53$) did not reveal any mutations/deletions. In 9 cases out of 98 (9%), we detected a selective loss of HLA-A or HLA-B locus expression and in 5 cases (5%), we observed single HLA-I allelic losses. In 4 tumors, we found a combination of different HLA-I alterations. Only 19 out of 98 (19%) tumors showed “normal” expression of HLA-I antigens. We also studied HLA-II expression in 71 tumors using monoclonal antibody against HLA-DR (GRB-1), 54 of which (76%) were negative and 17 (24%) were positive (Table 3). Figure 1 depicts representative images of HLA-ABC-positive and HLA-ABC-negative tumors (W6/32 antibody). Table 1 summarizes a correlation between different clinicopathologic parameters (age, tumor size, nodal invasion, estrogen and progesterone receptor, metastasis, TNM, histology, and HER2 receptor) and tumor HLA expression. The only statistically significant correlation ($p < 0, 05$) was found between HLA-I expression and tumor size demonstrating that tumor with larger size have less HLA-I expression.

Genomic HLA typing of normal breast tissue

Table 3 shows the results of HLA-A, B, and C locus genomic typing performed on normal autologous tissue of 98 breast cancer patients. Only patient BC15 was homozygous for HLA-I. Loss of single HLA-I alleles was determined by lack of immunostaining of tumor tissue with specific antibodies and confirmed by its presence in patient’s HLA haplotype. In Table 4, HLA-I alleles lost in tumors are in italics.

Molecular mechanisms involved in the alterations of HLA class I expression: analysis of LOH and B2M sequencing

One of the objectives of our work was to investigate the molecular mechanisms involved in HLA-I alterations in breast cancer. Of the 92 cases studied for LOH-6 and LOH-15 (Table 4), 36 cases (39%) showed LOH at one or both chromosomes. Using microsatellite analysis, loss of heterozygosity was detected in 36 cases (39%). Among them, 15 cases had only LOH-6, 14 tumors only LOH-15, and in 7 samples, we detected LOH at chromosomes 6 and 15 (Table 4). Interestingly, some tumors positive for HLA-I expression by immunohistochemistry (w6/32 MoAbs) had LOH in chromosomes 6 or 15 (Table 4). Figure 2 illustrates a localization of STR markers at chromosome 6 and demonstrates representative results of the LOH-6 analysis in some breast cancer samples, including samples with haplotype loss.

Discussion

Tumor HLA-I expression is one of the fundamental factors responsible for the efficacy of adaptive anti-tumor immune response, while the high frequency of HLA-I loss suggests that this is a key route of cancer immune escape from T cell-mediated lysis (Garrido et al. 1997b; Garrido and Algarra 2001; Aptsiauri et al. 2007).

In this work, using immunohistochemistry with a large panel of monoclonal antibodies against monomorphic, locus and allelic specific HLA-I and HLA-II determinants, we found that loss and downregulation of HLA-I expression is a frequent event in breast cancer. Around 81% of the studied tumors demonstrated various types of HLA-I alterations. Molecular analysis of the microdissected tumor samples revealed that 39% of the tumor samples have loss of heterozygosity (LOH) at chromosomes 6 and/or 15, which harbor the

Table 4 LOH at chromosomes 6 and 15 affecting HLA class I heavy chain and B2M genes

Tumor sample	LOH chromosome 6	LOH chromosome 15	HLA class I expression
LOH-6 (n = 15)			
BC-40	+	−	HLA-I negative
BC-48	+	−	HLA-I negative
BC-49	+	−	HLA-I negative
BC-83	+	−	HLA-I negative
BC-96	+	−	HLA-I negative
BC-97	+	−	HLA-I negative
BC-1	+	−	HLA-I negative
BC-33	+	−	HLA-A and HLA-B negative
BC-20	+	−	HLA-I positive**
BC-71	+	−	HLA-I positive**
BC-78	+	−	HLA-I positive**
BC-7	+	−	HLA-I positive**
BC-11	+	−	HLA-I positive**
BC-29	+	−	HLA-I positive**
BC-53	+	−	HLA-I positive**
LOH-6 and LOH-15 (n = 7)			
BC-50	+	+	HLA-I negative
BC-38	+	+	HLA-I negative
BC-79	+	+	HLA-I negative
BC-67	+	+	HLA-A negative
BC-90	+	+	HLA-B negative
BC-34	+	+	HLA-A2 negative
BC-98	+	+	HLA-I positive**
LOH-15 (n = 14)			
BC-35	−	+	HLA-I negative
BC-37	−	+	HLA-I negative
BC-45	−	+	HLA-I negative
BC-65	−	+	HLA-I negative
BC-77	−	+	HLA-I negative
BC-87	−	+	HLA-I negative
BC-70	NI	+	HLA-I negative
BC-21	−	+	HLA-A negative
BC-47	−	+	HLA-B negative
BC-62	−	+	HLA-A negative
BC-46	−	+	HLA-A2 negative
BC-18	−	+	HLA-I positive
BC-57	−	+	HLA-I positive
BC-76	−	+	HLA-I positive

Ninety-two breast tumor tissues were analyzed for LOH at chromosomes 6 and 15. This table includes 39 samples with LOH and corresponding HLA-I immunophenotype

NI not informative

**These tumors were classified as "positive" based on immunohistology with anti-HLA-I antibodies directed against HLA-I monomorphic determinants (w6/32). Nevertheless, LOH-6 was detected in these tumors, which is usually associated with HLA-I haplotype loss. We could not specify which HLA-I alleles were missing in each case

HLA-I region (6p21) and B2M gene (6p15), respectively. Previous studies demonstrated that HLA-I expression is often downregulated in different types of malignancy, including breast cancer (de Kruijf et al. 2010; Aptsiauri et al. 2007). There have been several reports showing different results of the immunohistological analysis of HLA expression in breast

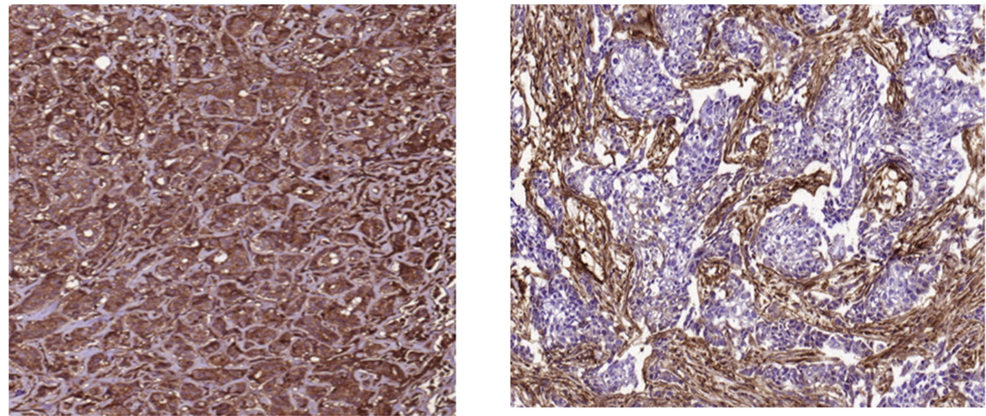
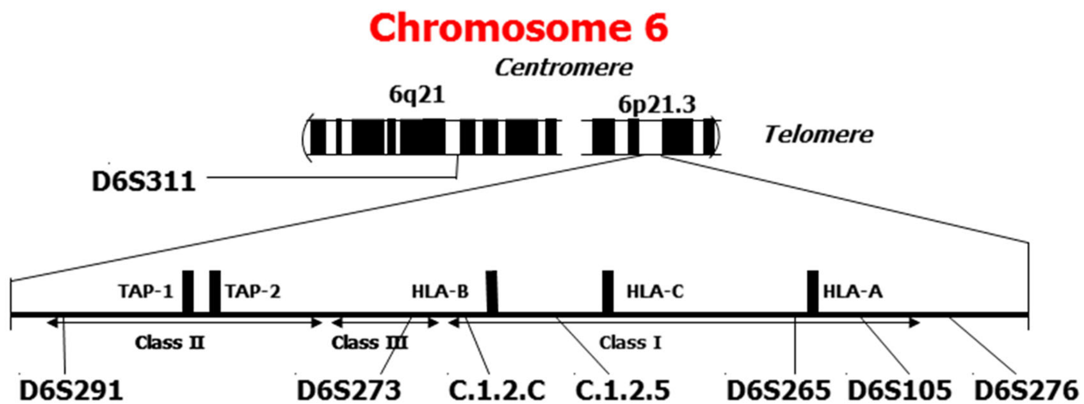


Fig. 1 Representative images of HLA-ABC positive (left) and negative (right) breast carcinoma samples. Immunohistological analysis was done with w6/32 monoclonal antibody. **a** HLA-ABC positive. **b** HLA-ABC negative



Tumor	Tumor HLA class I tumor phenotype by immunohistology	6q	6p						
		311	291	273	C.1.2c	C.1.2.5	265	105	276
BC-7	HLA-positive	L	L	N	N	N	L	L	NI
BC-11	HLA-positive	NI	NI	L	NI	N	N	L	L
BC-20	HLA-positive	NI	L	L	L	L	NI	N	NI
BC-29	HLA-positive	N	L	L	N	L	N	N	NI
BC-53	HLA-positive	NI	NI	NI	L	NI	NI	L	L
BC-71	HLA-positive	L	L	L	L	L	NI	NI	L
BC-78	HLA-positive	NI	NI	NI	L	L	L	L	NI
BC-98	HLA-positive	L	NI	NI	NI	L	L	L	L

L in grey box= LOH N= Normal, absence of alterations NI= Not informative

Fig. 2 Localization of STR markers on chromosome 6 and representative results of the LOH analysis at chromosome 6 in breast cancer samples. D6S291 is centromeric, and D6S105 and D6S276 are telomeric to the HLA region. L, LOH; N, normal, absence of alterations; NI, not informative

cancer in correlation with clinical parameters. The discrepancy could be associated with the type of tissue and antibodies used.

On the other hand, there are only few publications on molecular analysis of genetic defects in HLA-I genes responsible for HLA alterations in breast cancer. These alterations could be due to structural defects in HLA-I genes ("hard" lesions) or regulatory aberrations ("soft" lesions) reversible by pro-inflammatory cytokines in the tumor microenvironment (Garrido et al. 2010b; Garrido et al. 2016; Aptsiauri et al. 2014). HLA-I expression in tumors can potentially predict the response to immunotherapy in cancer patients, and it depends on the nature of the alteration. It is believed that if the defect is genetic, it is unlikely that immunostimulation of T cells in tumor microenvironment induced by immunotherapy can upregulate normal HLA-I expression and antigen presentation. In this case, the escape mechanisms could prevail and lead to the generation of dangerous HLA-I-negative tumor escape variants providing the basis for tumor heterogeneity. Therefore, the success or failure of immunotherapy depends on the nature of preexisting HLA-I alterations (Garrido et al. 2016).

Among the structural ("hard") HLA-I alterations, LOH-6 is an important mechanism that generates HLA-I haplotype loss in various human tumors with high incidence (Maleno et al. 2004; Maleno et al. 2006). Mutations in B2M gene together with the loss of another gene copy caused by LOH-15 are also responsible for the irreversible total HLA-I and have been described in various types of malignancy, both in cell lines and in tumor tissues, including a proportion of HLA-I-deficient melanoma and MSI-H colorectal cancers (Bernal et al. 2012). In the present study, we did not find mutations/deletions in B2M gene. LOH-15 may be unnoticed in tumor cells with "normal" HLA-I immunolabeling pattern and could represent one of the early events in malignant transformation driving pre-committed tumors to become HLA escape variants. Most tumors derive from HLA-I-positive normal epithelia and constantly acquire new HLA-I alterations during cancer progression and dissemination. It creates variability within primary tumors as well as between the primary carcinoma and metastases and may negatively impact survival and treatment efficacy.

In our study, we found a significant correlation between tumor size and HLA-I expression (when tumors were divided into two groups: T1 versus T2+T3+T4), demonstrating that tumors with larger size have less HLA-I expression. It can be explained by a gradual elimination of HLA-positive tumor cells by CTLs and escape of HLA-I-negative cells during tumor growth.

Intratumor heterogeneity among cancer cells is promoted by reversible or irreversible genetic alterations, by different microenvironmental factors and also by immunotherapy. In patients with bladder carcinoma, recurrent tumors after BCG therapy have a higher percentage of LOH-6 and LOH-15 and increased incidence of other HLA-I alterations (Carretero et al. 2011). Deletion of HLA genes may enable the clonal expansion of HLA-negative tumor cells and this selective pressure could explain the increased frequency of LOH within the HLA genes after immunotherapy. In melanoma, we have previously observed that LOH-6 and LOH-15 were the earliest HLA-I alterations occurring in a primary tumor, followed by an emergence of a B2M mutation and complete loss of HLA-I in successive metastatic lesions (del Campo et al. 2014). The role of LOH in HLA genes during cancer evolution has been also recently described by us in a lung cancer study (Perea et al. 2017). In this context, another group reported that LOH affecting HLA-I genes in lung cancer is one of the immune escape mechanisms that is subject to strong immune selection pressure during tumor evolution (McGranaham et al. 2017). Selective allelic HLA-I losses caused by LOH-6 (HLA haplotype loss) could also potentially compromise T cell cytotoxicity directed against tumor antigenic peptide presented by a particular HLA-I allele.

Loss of HLA class I expression should render tumor tissues prone to destruction by NK cells. However, as it has been reported in different types of solid tumors, NK cells are rarely detected in the tumor infiltrate or even in the tumor margin (del Campo et al. 2014; Garrido et al. 2017b). In addition, tumor area in lung cancer has been reported to be enriched with the NK cell subpopulation with non-ctytotoxic $CD56^{bright}$ $CD16^{-}$ phenotype (Del Mar Valenzuela-Membrives et al. 2016). It is probably induced by the tumor microenvironment, which could locally impair NK homing and differentiation rendering these cells less cytotoxic and favoring the immune escape of HLA-I-negative tumor cells.

The results reported in this work suggest that a combination of multiple molecular mechanisms is responsible for HLA-I loss in breast cancer. It has been previously reported that HER2/neu oncogene expression in breast cancer has a correlation with HLA-I downregulation (Vertuani et al. 2009; Herrmann et al. 2004; Seliger and Kiessling 2013). However, in this study, we did not find any association between HLA-I and HER2/neu receptor expression (see Table 1).

Overall, the findings presented in this article indicate that LOH has a high frequency in breast cancer, even in tumor samples with positive HLA-I expression. These tumors were classified as positive with the anti-HLA-I antibody directed against HLA-I monomorphic determinants (w6/32). Nevertheless, these tumors harbor molecular HLA-I alterations, such as LOH-6 causing loss of HLA-I haplotype. However, we could not define the missing HLA-I alleles in each case. LOH, as a mechanism of HLA-I alteration, is definitely underestimated and may negatively influence T cell-mediated tumor rejection and clinical success of cancer eradiation by immunotherapy. We believe that the high frequency of LOH-6 and/or LOH-15 in breast cancer

(approximately in 39% of the samples) and especially the coincidence of the LOH at chromosomes 6 and 15 might have a strong impact on tumor immunogenicity and on the efficacy of cancer immunotherapy, since the loss of any given HLA-I locus or of a single allele could result in the lack of CD8+T cell stimulation by a potentially important tumor-associated neo-antigen.

It is becoming increasingly evident that immune checkpoint blocking therapies are associated with recurrent metastatic tumor lesions harboring mutations in B2M and IFN genes (Zaretsky et al. 2016), which are interfering with tumor antigen presentation to T lymphocytes (Sade-Feldman et al. 2017). However, the massive sequencing techniques used in these modern studies do not take into account loss of genetic material in chromosomes 6 and 15, which represent structural/ "hard" HLA-I lesions described by us and other groups in different types of cancer.

With the recent development of the field of cancer immunotherapy, the focus has shifted to the investigation of how tumors acquire resistance to treatment and to the discovery of novel predictive markers of the efficacy of therapy. Although anti-"immune checkpoint" immunotherapies have produced dramatic results in a subset of some malignancies, the percentage of non-responders, mixed responders, and post-treatment recurrences is rather high. Based on the existing scientific evidence, it could be, at least partially, explained by the loss of tumor HLA-I expression caused by structural genetic lesions in HLA-I/B2M genes. Therefore, investigation of genetic aberrations underlying altered tumor HLA-I expression is necessary for developing effective therapies.

Conclusions

Based on immunohistological and molecular analysis of breast tumor tissues, we discovered that partial and total HLA-I loss is a frequent finding in breast carcinoma. Here, we demonstrated that the leading molecular mechanism responsible for HLA-I altered expression is a loss of heterozygosity in the HLA-I region of chromosome 6 and in the B2M region of chromosome 15, which was detected in about 39% of studied tumors. It can be potentially overlooked and underestimated in tumors analyzed only by immunohistochemistry, when apparently HLA-I-positive tumors (labeled with antibodies against HLA monomorphic determinants) harbor potentially dangerous structural/irreversible genetic aberrations. These alterations may reduce the ability of tumor cells to present antigens to T lymphocytes in the context of HLA-I molecules and, consequently, could lead to immune escape and resistance to immunotherapy. Nevertheless, mechanisms responsible for total HLA-I loss (observed in 54% of the studied breast tumors) still remain to be defined.

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Compliance with ethical standards

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

The study protocol was approved by the ethical committee of the Virgen de las Nieves University Hospital and Instituto de Investigación Biosanitaria "ibs. Granada" (Comité de Ética de la Investigación de Centro de Granada (CEI Granada), number 2014-22/12). Signed informed consent approved by the Ethics Committee of our institution was obtained from all the patients.

Competing interest The authors declare that they have no competing interests.

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