Chapter 3

HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients

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ABSTRACT

Non-classical human leukocyte antigens (HLA), HLA-E and HLA-G, are known to affect clinical outcome in various tumor types. We examined the clinical impact of HLA-E and HLA-G expression in early breast cancer patients, and related the results to tumor expression of classical HLA class I.

Our study population (n=677) consisted of all early breast cancer patients primarily treated with surgery in our center between 1985 and 1995. Tissue micro array (TMA) sections of arrayed tumor and normal control material were immunohistochemically stained for HLA-E and HLA-G. For evaluation of HLA-E and HLA-G and the combined variable, HLA-EG, a binary score was used. Expression of classical HLA class I molecules was previously determined.

HLA-E, HLA-G and HLA-EG on breast tumors were classified as expression in 50%, 60% and 23% of patients respectively. Remarkably, only in patients with loss of classical HLA class I tumor expression, expression of HLA-E ($p=0.027$), HLA-G ($p=0.035$) or HLA-EG ($p=0.001$) resulted in a worse relapse free period. An interaction was found between classical and non-classical HLA class I expression ($p=0.002$), suggestive for a biological connection.

We have demonstrated that, next to expression of classical HLA class I, expression of HLA-E and HLA-G is an important factor in the prediction of outcome of breast cancer patients. These results provide further evidence that breast cancer is immunogenic, but also capable of evading tumor eradication by the host's immune system, by up- or down regulation of HLA class Ia and class Ib loci.

INTRODUCTION

There has been strong evidence that tumor progression is controlled by the host's immune system 1. However, due to their intrinsic genetic unstable nature, tumor cells may acquire properties to escape from immune recognition 2. These poorly immunogenic clones frequently have lost expression of classical human leukocyte antigen (HLA) class I (HLA-A, HLA-B, HLA-C) which enables them to escape cytotoxic T lymphocyte (CTL) attack. However, in that case they may be vulnerable to natural killer (NK) cell elimination. Expression of non-classical HLA class I molecules (HLA-E, HLA-G), which play a pivotal role in immune surveillance by NK-cells, may therefore also determine outcome of tumor immune interaction 3. Under normal circumstances, expression of the HLA-E molecule is found in most tissues that express HLA-A, -B, -C or -G molecules and is thought to provide an important "self-signal" to the immune system by accommodating and presenting peptide fragments from leader sequences of these molecules [3, 4]. HLA-G expression, on the other hand, has very restricted tissue expression and has been mostly found in extravillous trophoblastic cells, where it mediates semi-allograft immunotolerance during pregnancy 5. Expression of HLA-E and HLA-G on the cell surface can respectively bind with the inhibitory receptors CD94/NKG2A and KIR2DL4/p49 of NK cells, and thereby cause inhibition of their proliferation and cytotoxic effector functions [6, 7]. HLA-E also binds activating CD94/NKG2C receptors, present on T and NK cells, however with a 6-fold lower affinity 8.

Tumors may acquire or upregulate expression of HLA-E and HLA-G as protective property against immune recognition and elimination of tumors 3. HLA-E is regularly expressed in various healthy tissues and correlates with expression of classical HLA class I molecules. This physiological correlation with classical HLA class I molecules has been found to be disturbed in tumors, suggesting that malignant cells which escape T cell immune recognition by downregulation of classical HLA class I expression, may further escape immune recognition by upregulation of HLA-E 9. In addition, expression of HLA-G protects against "missing self" recognition of NK. Expression of this molecule, which is rarely found in healthy tissues, is frequently observed in pathological conditions such as in tumors [10, 11]. Previous studies showed that both HLA-E and HLA-G had increased expression in different types of tumor 12-15. Studies on the prognostic value of HLA-E expression in colorectal and cervix cancer showed that expression of this molecule correlated to tumor progression and had a trend towards a worse clinical outcome. The prognostic value of HLA-G expression has been investigated in colorectal, gastric, esophageal squamous cell carcinoma and non-small cell lung **cancer and** revealed it to be an independent prognostic factor for poor clinical outcome 16-19. **In addition,** expression of HLA-G has also been found in breast cancer, however no statistically significant associations were found with outcome of patients 20-22.

The prognostic effect of HLA-E and HLA-G expression in breast cancer is unknown. The purpose of this study was to analyze the prognostic relevance of expression of HLA-E and HLA-G in a large cohort of early breast cancer patients. Previously, we determined classical HLA class I expression in the same patient cohort. Therefore, we were able to stratify patients based on classical HLA class I expression of tumors and to analyze the impact of HLA-E and HLA-G expression on clinical outcome of early breast cancer patients.

PATIENTS AND METHODS

Patients and tumors

The patient population comprised all non-metastasized breast cancer patients primarily treated with surgery in the Leiden University Medical Center between 1985 and 1994 (n=677). Patients with bilateral tumors or a prior history of cancer (other than basal cell carcinoma or cervical carcinoma in situ) were excluded. The following data were known: age, tumor grade, histological type, TNM stage, local and systemic therapy, locoregional/distant tumor recurrence, secondary tumor, survival, and expression of estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2) 23. All tumors were graded according to current pathological standards, by a pathologist (VS). In addition, for about half the cohort of patients (n=266) a TMA of paired histologically normal breast tissue was available. Normal breast tissue originated from the cancer-affected breast, but localized more distal from the tumor tissue.

Immunohistochemistry

MEM-E/02 (AbCam, UK) and 4H84 (Nuclilab, NL) antibodies were used to recognize HLA-E and HLA-G respectively. MEM-E/02 reacts specifically with the denatured heavy chain of human HLA-E24. The 4H84 antibody recognizes denatured HLA-G molecules and has been described to react with classical HLA class I molecules 25-27. Tissue section of 4 μm were cut from a previously constructed tissue micro array (TMA) of formalin-fixed paraffin-embedded tumors 23. Tissue sections were deparaffinized and rehydrated. For antigen retrieval, 0.01 M Trizma EDTA (TE) buffer (pH6.0) was used for 10 minutes at maximum power in a microwave oven. Endogenous peroxidase was blocked for 20 minutes in 0.3% hydrogen-peroxide methanol. Sections were incubated overnight with primary monoclonal antibodies using predetermined optimal concentrations. After 30 minutes incubation with secondary antibody Envision anti-mouse (Dako Cytomation K4001), sections were visualised using DAB-solution. Tissue section were counterstained with haematoxylin, and then dehydrated and finally mounted in malinol. For each primary antibody, all slides were stained simultaneously to avoid inter-assay variation. For each staining, placenta tissue slides served as positive

control. Negative controls were placenta tissue slides that did undergo the whole immunohistochemical staining without primary antibodies. Sections of paired normal tissue TMA were stained with MEM-E/02 and 4H84 in order to assess frequency of staining in normal breast tissue samples.
Tumor staining for classical HLA class I using the mouse monoclonal antibodies HCA2 and HC10 (anti-HLA-A and anti-HLAB/C respectively) was previously described 28.

Evaluation of immunostaining

Microscopic analysis of HLA-E and HLA-G was assessed by two independent observers (AS and EdK) in a blinded manner. Both markers were scored in a binary manner, considering any specific staining of tumor cells as positive expression and no staining as no expression. A combined variable of HLA-E and HLA-G scores was created: HLA-EG. HLA-EG expression was considered positive when both HLA-E and HLA-G were expressed and negative when either HLA-E or HLA-G was not expressed.

Statistical analysis

Statistical analyses were performed using the statistical package SPSS (version 16.0 for Windows, Spps Inc, Chicago, IL, USA). Cohen's kappa coefficient was used to asses inter-observer agreement in quantification. This revealed a substantial agreement in classification for HLA-E (kappa=0.72) and a very good agreement in classification for HLA-G (kappa=0.90). The χ^2 test was used to evaluate associations between various clinicopathological parameters and HLA-E and HLA-G expression. Relapse free period (RFP) was the time from date of surgery until an event (locoregional recurrence and/or a distance recurrence, whichever came first). Overall survival (OS) was defined as date of surgery until death. The Kaplan–Meier method was used for survival plotting and log-rank test for comparison of survival curves. RFP is reported as cumulative incidence function, after accounting for death as competing risk 29. Cox regression was used for univariate and multivariate analysis for RFP and OS. Significant variables ($p<0.1$) in univariate analysis were included in multivariate analysis. To analyze the independent prognostic effect of HLA-E and HLA-G on clinical outcome, tumors were stratified based on a previously determined expression characteristics of classical HLA class I molecules.
We finally analyzed whether the specificity of the anti-HLA-G antibody would interfere with the results of our survival analyses by separately analyzing the set of patients in which those who stained positive for this antibody on normal breast tissue were excluded.

RESULTS

Patient and tumor characteristics

Tumor material was available and incorporated in the TMA of 86% (574/677) of the patients. Paired normal breast tissue was available on TMA in 46% (266/574) of the patients. Median age of patients was 57 years (range= 23-96 years). Median follow-up of patients alive was 19 years (range=14-23 years). Clinicopathological and treatment characteristics are shown in table I.

Expression of HLA-E and HLA-G

Microscopical quantification was successful in 86% (493/574) of tumors for HLA-E and in 87% (501/574) for HLA-G. Respectively 14% and 13% of tumors were damaged or lost on the TMA slides, a problem associated with preparation, staining and mounting of TMA slides. Two groups, expression versus no expression, were defined for HLA-E and HLA-G (figure 1 A-D). Expression was found in 50% (247/493) and in 60% (299/501) of tumors for HLA-E and HLA-G respectively (table I). Expression of HLA-EG was found in 23 % (100/428) of tumors. HLA-G stained positive in 1% (3/266) of normal tissue samples (figure 1E, F), while HLA-E showed positive staining in all normal tissue samples (figure 1 G).

HLA-E, HLA-G and HLA-EG and prognostic associations with outcome

In the whole cohort of patients HLA-E, HLA-G and HLA-EG showed no statistically significant difference in outcome between expression versus no expression for RFP (log rank p-values respectively: 0.52, 0.95, 0.72) or OS (log rank p-values respectively: 0.86, 0.74, 0.27) (figures 2 A, D, G and 3 A, D, G).

Next, we stratified patients based on classical HLA class I tumor expression, classified as expression versus loss. Among the subgroup of classical HLA class I expression results were similar as in the whole cohort of patients: neither for HLA-E, HLA-G, nor HLA-EG a statistically significant difference was found for different expression levels in outcome for RFP (log rank p-values respectively: 0.73, 0.69, 0.51) or OS (log rank p-values respectively: 0.64, 0.74, 0.22) (figures 2 B, E, H and 3 B, E, H). Interestingly, among the subgroup of patients with loss of tumor expression of classical HLA class I, HLA-E and HLA-G expression showed significant differences for RFP (log rank p-values respectively: 0.03, 0.04) and OS (log rank p-values respectively: 0.03, 0.12) between both expression groups (figures 2 C, F and 3 C, F). Of the patients with no tumor expression of HLA-E or HLA-G, respectively 60% and 56% of patients were relapse free after 10 years, whereas of the patients with tumor expression of HLA-E or HLA-G, respectively 35% and 39% of patients were relapse free after 10 years. The combination variable HLA-EG showed, similarly to HLA-E and HLA-G separately, differences in outcome between expression and no expression among the subgroup of classical HLA class I loss, but at a much higher level of significance than each separately

	Total		HLA-E					HLA-G				
			No expression		Expression		p-value	No expression		Expression		p-value
	N	%	N	%	N	%		N	%	N	%	
Age							0.378					0.221
<40	48	8,4	17	6,9	28	11,4		22	7,4	16	7,9	
40-50	145	25,3	64	25,9	59	24		74	24,7	59	29,2	
50-60	132	23	57	23,1	56	22,8		61	20,4	50	24,8	
>=60	249	43,4	109	44,1	103	41,9		142	47,5	77	38,1	
Grade							<0.001					0.242
I	80	14,2	44	18,1	29	12		40	13,4	23	11,8	
II	282	49,9	132	54,3	105	43,6		158	53	92	47,2	
III	203	35,9	67	27,6	107	44,4		100	33,6	80	41	
Histological type							0.094					0.465
Ductal	513	90,6	214	87,7	225	93,4		266	89	180	92,3	
Lobular	53	9,4	30	12,3	16	6,6		33	10,1	15	7,7	
Tumor stage							0.094					0.616
pT1	211	38	96	40,2	87	36,6		112	38,8	67	34,4	
pT2	272	49	108	45,2	128	53,8		142	49,1	103	52,8	
pT3/4	72	13	35	14,6	23	9,7		35	12,1	25	12,8	
Nodal stage							0.332					0.151
pN0	307	55,1	138	57,7	129	53,5		159	54,3	112	57,7	
pN1-3	250	44,9	101	42,3	112	46,5		134	45,7	82	42,3	
Estrogen receptor							0.004					0.095
Negative	203	37,6	72	31,4	106	44,7		100	35,3	82	42,9	
Positive	337	62,4	157	68,6	131	55,3		183	64,7	109	57,1	
Progesterone receptor							0.021					0.499
Negative	223	41,6	81	35,1	106	45,9		115	41,1	84	44,2	
Positive	313	58,4	150	64,9	125	54,1		165	58,9	106	55,8	
Her2 overexpression							0.008					0.014
No overexpression	435	80,9	200	87,7	186	78,5		236	84,6	145	75,5	
Overexpression	103	19,1	28	12,3	51	21,5		43	15,4	47	24,5	
Classical HLA I							0.003					<0.001
Negative	112	21,3	68	30,1	40	17,9		78	28,4	28	14,6	
Positive	401	69,9	158	69,9	183	82,1		197	71,6	164	85,4	
Local Therapy							0.407					0.661
MAST-RT	223	38,9	109	44,1	92	37,4		116	38,8	78	38,6	
MAST+RT	108	18,8	41	16,6	50	20,3		52	17,4	43	21,3	
BCS-RT	5	0,9	2	0,8	1	0,4		2	0,7	2	1	
BCS+RT	238	41,5	95	38,5	103	41,9		129	43,1	79	39,1	
Systemic therapy							0.076					0.004
Chemotherapy	112	19,5	37	15	57	23,2		43	14,4	52	25,7	
Endocrine therapy	75	13,1	42	17	32	13		52	17,4	20	9,9	
Both	18	3,1	7	2,8	10	4,1		12	4	6	3	
None	369	64,3	161	65,2	147	59,8		192	64,2	124	61,4	
Total	574	100	247	100	246	100		299	100	202	100	

TABLE 1. Correlations between HLA-E and HLA-G expression and well-established prognostic factors using chi-squared test. *Abbreviations* N number of patients; % percentage; HLA-E human leukocyte antigen E; HLA-G human leukocyte antigen G; HER2 human epidermal growth factors receptor 2; MAST Mastectomy; BCS breast conservative surgery.

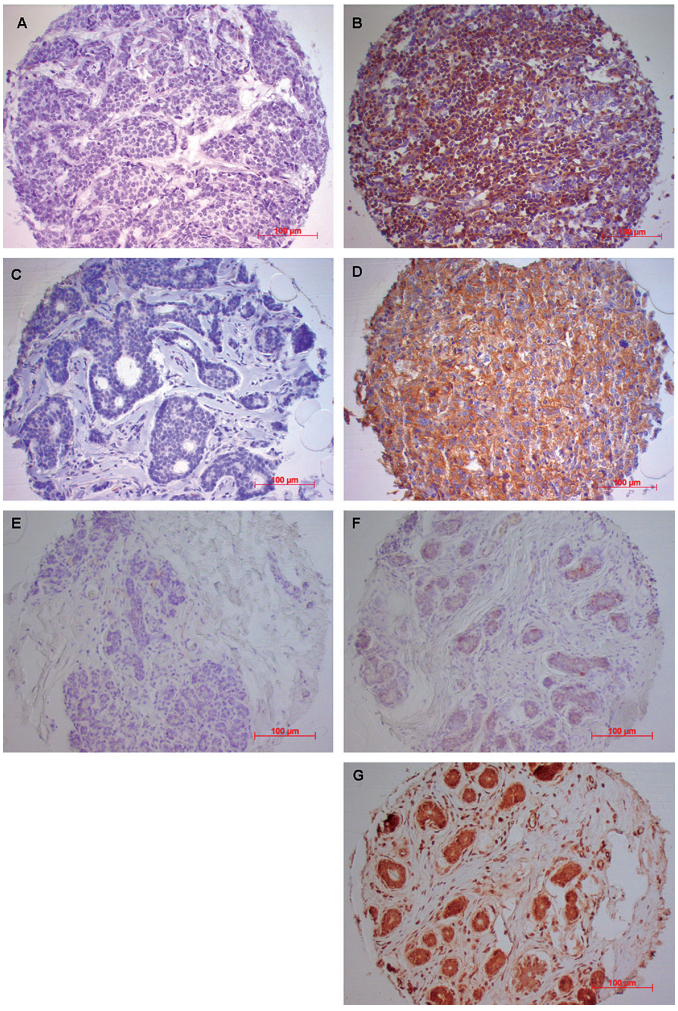

Figure 1 Representative examples of immunohistochemical stainings with MEM-E/02 and 4H84 antibodies on mammary tissues, performed according to standard protocols (details in Materials and Methods) **A)** HLA-E negative tumor **B)** HLA-E positive tumor **C)** HLA-G negative tumor **D)** HLA-G positive tumor **E)** HLA-G negative normal tissue **F)** HLA-G positive normal tissue **G)** HLA-E positive normal tissue.

(log rank p-values: RFP:0.001; OS: 0.007) (figures 2 I and 3 I). Among the patients with no expression of HLA-EG, 55% were relapse free after 10 years, compared to 17% for expression of HLA-EG. Cox proportional multivariate analysis was performed for relapses over time including the following factors: tumor stage, lymph node status, ER-status, HER2 expression, local therapy, endocrine therapy and HLA-EG. This analysis revealed that lymph node status and HLA-EG (p= 0.011, Hazard Ratio (HR): 2.87, 95% Confidence interval (CI): 1.28-6.43) were independent factors for RFP among the subgroup of classical HLA class I loss patients (table II). These data showed that HLA-EG possesses a specific prognostic effect, but only among classical HLA class I loss patients. In order to prove that classical HLA class I and HLA-EG were significantly cooperating variables, an interaction term was introduced in Cox regression analysis. This analysis showed a statistically significant interaction (p= 0.002) between the two

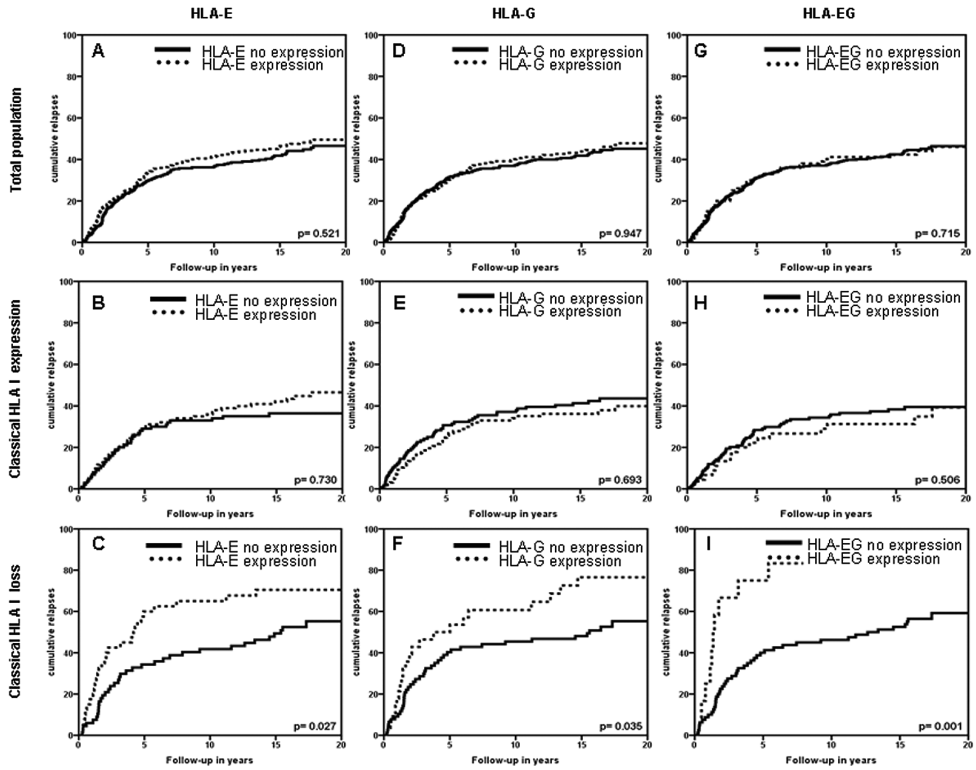

Figure 2 Relapses over time related with HLA-E **(A,B,C)**, HLA-G **(D,E,F)** and HLA-EG **(G,H,I)** tumor expression, among the total population **(A,D,G)**, patients with classical HLA class I tumor expression **(B,E,H)**, and patients with loss of classical HLA class I tumor expression **(C,F,I)**. Remarkably, only in patients with loss of classical HLA class I expression, HLA-E, HLA-G and HLA-EG affect relapses over time. Log-rank p-values are shown in each graph.

markers, suggesting that there is a biological connection between classical HLA class I and HLA-EG.

Correction for specificity of antibodies

The 4H84 antibody has been described to occasionally cross-react with classical HLA class I molecules 27. Therefore, we performed additional immunohistochemical analyses to examine whether this cross-reaction would interfere with our survival results.

Expression on paired normal breast tissue of half the cohort was found in 1% (3/266) for HLA-G. These 3 patients who showed weakly positive staining for HLA-G on normal breast tissue, also stained positive for classical HLA class I on normal and tumor tissue, indicating that the 4H48 antibody possibly occasionally cross-reacted with these classical HLA class I molecules. It should be noted however that the staining on normal tissue was only modest when compared to tumor staining with the 4H84 antibody (compare fig 1F with 1D). In order to examine whether the occasional cross-reaction of the 4H84 antibody would interfere with our results, we performed a sub-analysis

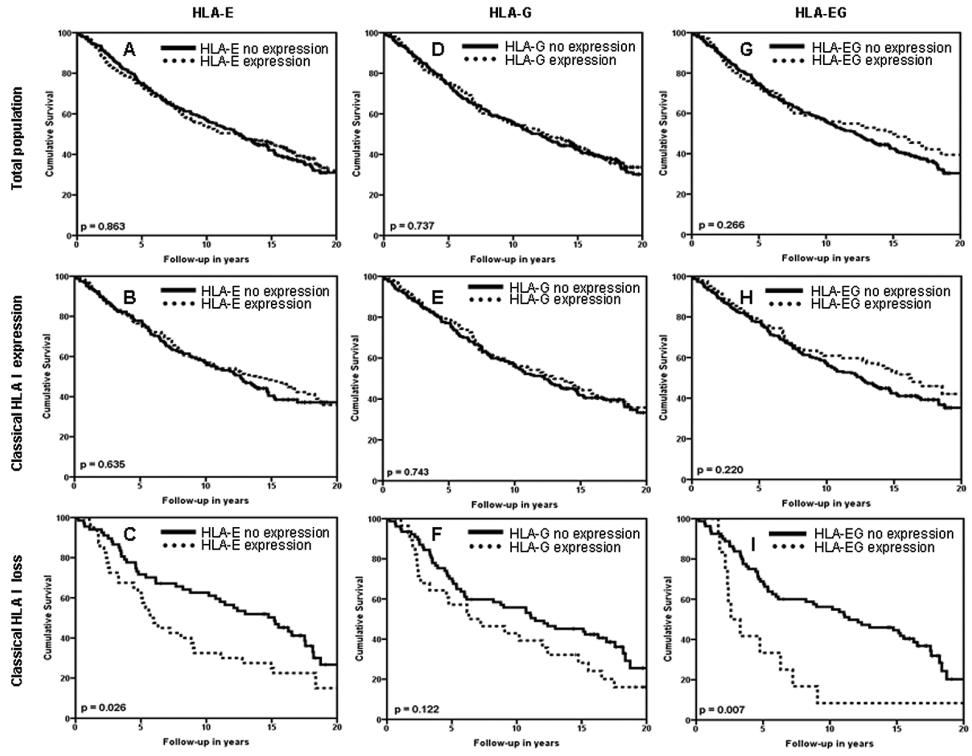

Figure 3 Kaplan Meier analysis of overall survival related with HLA-E **(A,B,C)**, HLA-G **(D,E,F)** and HLA-EG **(G,H,I)** tumor expression, among the total population **(A,D,G)**, patients with classical HLA class I tumor expression **(B,E,H)**, and patients with loss of classical HLA class I tumor expression **(C,F,I)**. Remarkably, only in patients with loss of classical HLA class I expression, HLA-E, HLA-G and HLA-EG affect overall survival. Log-rank p-values are shown in each graph.

by selecting only the tumors of the 266 patients of whom paired normal tissue was available. In this analysis, we excluded the 3 cases which showed positive staining for HLA-G on normal breast tissue (the presumed cases which showed cross-reaction for the 4H84 antibody) and examined whether survival analyses would reveal similar results as to when these cases would not be excluded. When excluding these 3 cases, no survival analyses reached statistical significance (log rank p-values ≥ 0.426) in neither the total population of patients nor the patient population with expression of classical HLA class I. This was concordant with the results found without exclusion of these cases (log-rank p-values ≥ 0.693). Importantly, no expression was seen of HLA-G in normal breast tissue of patients whose tumor showed no classical HLA class I expression, but resulted positive for HLA-G expression. Together, these results suggests that the occasional cross-reaction of 4H84 with classical HLA class I molecules did not interfere with our results.

		Univariate			Multivariate	
	N	HR	95% CI	p-value	HR	95% CI	p-value
Age							
<40	8	1.00		0.580			
40-50	37	1.23	0.426-3.544				
50-60	31	1.54	0.526-4.508				
>60	46	1.02	0.349-2.949				
Grade							
I	28	1.00		0.068			
II	55	1.29	0.661-2.507				
III	35	2.09	1.048-4.172				
Histological type							
Ductal	102	1.00		0.884			
Other	16	0.95	0.470-1.917				
Tumor stage							
pT1	46	1.00		0.006	1.00		0.679
pT2	55	1.71	0.994-2.953		1.25	0.590-2.644	
pT3/4	16	2.99	1.526-5.870		0.88	0.260-2.964	
Nodal stage							
pN-	64	1.00		<0.001	1.00		<0.001
pN+	56	4.10	2.482-6.783		3.60	1.812-7.165	
ER-status							
Negative	37	1.00		0.057	1.00		0.237
Positive	83	0.62	0.376-1.014		0.70	0.385-1.266	
PgR-status							
Negative	44	1.00		0.202			
Positive	73	0.73	0.445-1.186				
HER2							
No overexpression	102	1.00		0.075	1.00		0.069
Overexpression	10	1.73	0.947-3.176		2.21	0.939-5.217	
Ki67							
Ki67-	91	1.00		0.841			
Ki67+	26	0.94	0.523-1.695				
Local therapy							
MAST-RT	46	1.00		<0.001	1.00		0.320
MAST+RT	25	2.97	1.631-5.422		2.15	0.796-5.813	
BCS	51	0.96	0.542-1.703		1.23	0.572-2.663	
Endocrine therapy							
ET+	15	1.00		0.048	1.00		0.471
ET-	107	0.52	0.273-0.994		0.74	0.318-1.698	
Chemotherapy							
CT+	23	1.00		0.130			
CT-	99	0.65	0.371-1.136				
HLA-EG							
No expression	81	1.00		0.002	1.00		0.011
Expression	12	3.08	1.512-6.251		2.87	1.278-6.430	

TABLE 2. Cox univariate and multivariate analysis for relapses free period (RFP).
Abbreviations N number of patients; HR hazard ratio; 95%CI 95% confidence interval; HER2 human epidermal growth factor receptor 2; MAST Mastectomy; BCS breast conservative surgery.

DISCUSSION

Tumor-immune interaction may be of great importance for clinical outcome 2. In this study we showed that in tumors devoid of classical HLA class I expression, HLA-E and HLA-G expression were of statistically significant influence on outcome of breast cancer patients independently of known clinicopathological parameters, with an almost 3 times higher risk of relapse over time for patients with expression of HLA-EG compared to patients with no expression of HLA-EG. This is the first study providing evidence for a prognostic value of non-classical HLA class I molecule expression in a large cohort of breast cancer patients. In addition, to our knowledge we are the first to report that such an effect on outcome of patients interplays with expression of classical HLA class I molecules. Importantly, these results can be explained by underlying biology and support and add to previous studies on tumor-immune interaction in breast cancer [3, 12-19].

Previous studies have found elevated expression levels of the non-classical HLA class I molecules, HLA-E and HLA-G, in tumor tissues [3, 12-19]. Normally, HLA-G is not expressed on non-malignant cells. Corresponding to this fact, we found in our study that 4H84 HLA-G antibody did stain in a considerable number of tumor tissues, but in a negligible number of normal mammary tissues. Under normal circumstances HLA-E surface expression is dependent on the availability of HLA class I signal sequence-derived peptides. Therefore, HLA-E surface expression is usually found to be co-expressed with classical HLA class I, which comes to expression in almost all healthy tissues [3, 4]. Corresponding to this fact, we did not find any normal mammary tissue that did not express HLA-E molecules. In some tumor tissue however, HLA-E expression seems to be independent of the availability of classical HLA class I sequence-derived peptides and can be expressed in cells that lack classical HLA class I expression [9, 30]. Indeed, we found cytoplasmic expression of HLA-E in classical HLA class I negative tumors in our study. The disturbed balances of expression of classical HLA class I, HLA-E and HLA-G, as found in our study, suggests a cooperation between these molecules in evading immune recognition. According to the immunoediting hypothesis, tumors may become shaped through interaction with the immune system, leading to the selective outgrowth of highly tumorigenic clones that escape from immune recognition and elimination 31. Downregulation of classical HLA class I expression in tumors, with simultaneous loss of cell surface expression of HLA-E due to lack of peptide fragments which it can bind, is believed to reflect CTL immune escape 3. However, these tumor cells become highly vulnerable to NK cells, which recognize these "missing self" cells 14. Through a variety of factors, such as epigenetic control, hypoxia, stress and cytokines, expression of HLA-G and HLA-E may be upregulated and counteract this susceptibility to NK cells [3, 10, 32, 33]. Supportive for a specific NK cell inhibition of the non-classical HLA class I molecules, for both HLA-E and HLA-G an inverse

correlation was found with NK cell infiltrate in a colorectal cancer and gastric cancer study respectively [34, 35]. In addition, in various studies using colon cancer and melanoma cell lines it was demonstrated that overexpression of HLA-E and HLA-G respectively directly inhibited NK-mediated cell lysis 35-39. The statistical interaction between HLA-E and HLA-G with classical HLA class I molecules, as found in our study, adds to this evidence, suggesting that specifically in tumors devoid of classical HLA class I expression, upregulation of HLA-E and HLA-G expression counteracts the resulting NK cell susceptibility, leading to immune escape of tumor cells. Our study supports and adds to previous findings, suggesting that HLA-E and HLA-G contribute to tumor immune escape, specifically NK cells, a phenomenon that is likely to have impact on clinical outcome of patients.

Prognostic associations of HLA-E and HLA-G have been studied in various types of tumors [16-19, 30, 35]. In cervical cancer HLA-E expression increased with the progression of the lesion. One study analyzed the prognostic effect of HLA-E expression in colorectal cancer. A statistically significant association with outcome was noticed where high expression of HLA-E resulted in a worse disease free survival of patients 35. HLA-G expression showed a positive correlation with higher histological grade and clinical stage in colorectal cancer, gastric cancer, epithelial squamous cell carcinomas (ESCC) and cutaneaous T cell lymphoma. In addition, expression of HLA-G was an independent prognostic factor for a worse outcome of patients in colorectal cancer, ESCC and non small cell lung cancers [16, 18, 19]. We described that tumor expression of HLA-E and HLA-G has an independent prognostic influence in breast cancer patients, resulting in a worse patient outcome. Previously, similar results for disease free survival were found for breast cancer, albeit that these results did not reach statistical significance 20. This study was similar to ours in terms of patients selection criteria and immunohistochemical staining methods, but was probably limited by the small number of breast cancer patients studied (n=43). The results of our study demonstrate for the first time a statistically significant association of HLA-E and HLA-G expression with clinical outcome in a large cohort of breast cancer patients, which is particularly revealed in patients with tumors lacking expression of classical HLA class I molecules. Moreover, patients with tumors with simultaneous expression of HLA-E and HLA-G had an increased risk of relapses compared to patients with tumors expressing either HLA-E or HLA-G, a phenomenon that has been previously described as well 13. In addition we were able to demonstrate a statistical interaction in outcome analyses, indicating that the effect on outcome of HLA-E and HLA-G expression and the effect on outcome of HLA class I expression do not only operate simultaneously, but that the combined effect on outcome of these molecules is more than additive. These data correspond to the hypothesis that tumor expression of the non-classical HLA class I molecules E and G may indeed serve to protect tumor cells from NK-cell attack, but

this is mostly relevant in a situation that NK cells are activated, i.e. in case classical HLA class I molecule expression is downregulated 10.

Together, these results provide new insights in breast cancer tumorigenesis and provide further evidence that the immune system is able to recognize and eliminate breast cancer cells. However, it is also evident that breast cancer cells are capable of escaping immune attack. A better understanding of the various phases of tumor immune interactions in breast cancer, i.e. elimination, equilibrium and finally escape, may lead to a better prediction of clinical outcome of patients. Furthermore, this knowledge may be used for the development of tailored immunotherapeutic treatment modalities.

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