Prognostication in young and elderly breast cancer patients

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Part I

PROGNOSTIC BIOMARKERS IN THE INTERACTIONS BETWEEN THE HOST'S IMMUNE SYSTEM AND BREAST CANCER

Chapter 2

The predictive value of hla class I tumor cell expression and presence of intratumoral tregs for chemotherapy in patients with early breast cancer

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ABSTRACT

Purpose We hypothesized that T cell immune interaction affects tumor development and thus clinical outcome. Therefore, we examined the clinical impact of human leukocyte antigen (HLA) class I tumor cell expression and regulatory T cell (Treg) infiltration in breast cancer.

Experimental Design Our study population (n=677) consisted of all early breast cancer patients primarily treated with surgery in our center between 1985 and 1994. Formalin-fixed paraffin-embedded tumor tissue was immunohistochemically stained using HCA2, HC10 and Foxp3 monoclonal antibodies.

Results HLA class I expression was evaluated by combining results from HCA2 and HC10 antibodies and classified into three groups: loss, downregulation and expression. Remarkably, only in patients who received chemotherapy, both presence of Treg ($p=0.013$) and higher HLA class I expression levels ($p=0.002$) resulted in less relapses, independently of other parameters. Treg and HLA class I were not of influence on clinical outcome in patients who did not receive chemotherapy.

Conclusions We showed that HLA class I and Treg both affect prognosis, exclusively in chemotherapy-treated patients and are therefore one of the few predictive factors for chemotherapy response in early breast cancer patients. Chemotherapy may selectively eliminate Treg, thus enabling Cytotoxic T-lymphocytes to kill tumor cells that have retained HLA class I expression. As a consequence, HLA class I and Treg can predict response to chemotherapy with high discriminative power. These markers could be applied in response prediction to chemotherapy in breast cancer patients.

INTRODUCTION

Breast cancer is the most common cancer in women: it affects one in nine women. Systemic treatment improves disease free survival (DFS) and overall survival (OS) in patients with early breast cancer.[1] Decisions regarding this systemic therapy are depending on prognostic and predictive factors, which divide patients into different risk-groups.[2] With the current classifications, however, prediction of outcome is still not optimal and additional prognostic and predictive factors are needed to improve tailored treatment.

It is widely accepted that the adaptive immune system plays an important role in controlling tumor growth and spread.[3] Cytotoxic T-lymphocytes (CTL) are capable to affect tumor development. However, due to their intrinsic genetic unstable nature, tumor cells may acquire properties to escape from CTL recognition. Among these properties are downregulation or complete loss of Human Leukocyte Antigen (HLA) class I expression. In addition, immunosuppressive regulatory T cells (Treg) may be induced.[4]

HLA class I molecules play a pivotal role in CTL-mediated immune responses and have been found to be a prognostic factor in various types of cancer.[5-7] Previous studies have demonstrated that HLA class I expression is frequently down-regulated in breast cancer.[8, 9] However, the reports on prognostic influence of HLA class I expression in breast cancer have contradictory results.[10-12] Some found no significant correlation between percentage of tumor cells expressing HLA class I and survival of breast cancer patients.[10, 12] In contrast, another study found that total loss of HLA class I was an independent indicator of good prognosis.[11]

Treg act as immunosuppressors and maintain immunological self-tolerance. Numbers of tumor-infiltrating Treg are known to be increased in several malignancies and a correlation was found with worse disease stage and prognosis in cancer. [13, 14]In breast cancer, the presence of Treg in the tumor environment has been found in several studies.[15-17] Moreover, these studies found a higher prevalence of Treg in tumor microenvironment and in peripheral blood of patients suffering breast cancer compared to healthy donors.[15-17] One study found higher numbers of Treg to be correlated with worse disease stage and shorter survival.[15] Interestingly, chemotherapy has been found to be involved in presence and prognostic influence of Treg.[16] The numbers of Treg in tumor tissue decreased after chemotherapy administration and there was an association between disappearance of Treg and pathologic complete response to preoperative chemotherapy.

The purpose of our study was to analyze the prognostic relevance of HLA class I expression and Treg infiltration in a large cohort of early breast cancer patients. In addition, we explored the predictive value of these markers for chemotherapy response.

MATERIALS 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 differentiation grade and morphology, TNM stage, local and systemic therapy, locoregional/distant tumor recurrence, secondary tumor, alive/death, estrogen receptor (ER), progesterone receptor (PgR),Ki67, and human epidermal growth factor receptor 2 (HER2). All tumors were graded according to current pathological standards, by one pathologist (VS).

Antibodies

The mouse monoclonal antibodies HCA2 and HC10 which recognize HLA class I heavy chains (kindly provided by Prof. Dr. J. Neefjes) were used. The reactivity spectrum of HCA2 comprises all HLA-A chains (except HLA-A24) as well as some HLA-B, HLA-C, HLA-E, HLA-F, HLA-G chains.[18, 19] HC10 reacts mostly with HLA-B and HLA-C heavy chains and some HLA-A (HLA-A10, -A28, -A29, -A30, -A31, -A32, -A33.[20, 21] Mouse antibodies against human Foxp3 (ab20034 clone 236A/E7: AbCam, UK) were used for Treg identification. The reactivity spectrum of Foxp3 comprises Treg and may include small numbers of CD8+ cells [22], but so far it is the best single marker of Treg.[23]

Immunohistochemistry

For HLA class I staining, slides of 4 μm were cut from a priory constructed tissue micro array (TMA). For staining of Treg sections of 4 μm were cut from the original formalin-fixed paraffin-embedded (FFPE) tumor blocks. Tissue sections were deparaffinised and rehydrated. Endogenous peroxidase was blocked for 20 minutes in hydrogen-peroxide methanol. For antigen retrieval, 0.01 M citrate buffer (pH6.0) was used for 10 minutes at maximum power in a microwave oven. Sections were incubated overnight with HCA2 or HC10 at room temperature using predetermined optimal concentrations. After incubation with secondary antibody envision anti-mouse (Dako Cytomation K4001), sections were visualised using DAB-solution (25ml DAB in 225 ml 0.05M Tris-HCl). Tissue section were counterstained with haematoxylin, dehydrated and mounted in malinol. All slides were stained simultaneously to avoid inter-assay variation. For each patient normal epithelium, stromal cells or lymphoid cells served as internal positive

	Total		HLA Class I						Treg			
			Loss		Downregulation		Expression		Absence		Presence	
	N	%	N	%	N	%	N	%	N	%	N	%
Age								P: 0.449				P: 0.902
<40	48	8.4	8	6.6	15	7.5	19	9.4	24	7.5	21	8.8
40-50	145	25.3	37	30.3	58	29.0	44	21.8	84	26.2	61	25.5
50-60	132	23.0	31	25.4	42	21.0	45	22.3	77	24.1	53	22.2
>60	249	43.4	46	37.7	85	42.5	94	46.5	135	42.2	104	43.5
Grade								P: <0.001				P: 0.047
I	80	14.2	28	23.7	26	13.1	16	8.0	52	16.5	27	11.4
II	282	49.9	55	46.6	114	57.6	87	43.3	163	51.7	113	47.7
III	203	35.9	35	29.7	58	29.3	98	48.8	100	31.7	97	40.9
Histological type								P: 0.135				P: 0.290
Ductal	513	89.4	102	86.4	178	89.9	190	94.5	286	90.8	215	90.3
Lobular	46	8.0	14	11.9	16	8.1	10	5.0	27	8.6	18	7.6
Other	7	1.2	2	1.7	4	2.0	1	0.5	2	0.6	5	2.1
Tumor stage								P: 0.760				P: 0.850
pT1	211	38.0	46	39.3	73	37.4	71	36.0	120	38.7	87	37.5
pT2	272	49.0	55	47.0	92	47.2	103	52.3	151	48.7	112	48.3
pT3/4	72	13.0	16	13.7	30	15.4	23	11.7	39	12.6	33	14.2
Nodal stage								P: 0.871				P: 0.831
pN0	307	55.1	64	53.3	107	55.4	111	56.3	170	54.5	128	55.4
pN+	250	43.6	56	46.7	86	44.6	86	43.7	142	45.5	103	44.6
Estrogen receptor								P: 0.004				P: 0.465
Negative	203	37.6	37	30.8	64	33.0	93	46.5	109	36.3	88	39.5
Positive	337	62.4	83	69.2	130	67.0	107	53.5	191	63.7	135	60.5
Progesterone receptor								P: <0.001				P: 0.140
Negative	223	41.6	44	37.6	64	32.8	105	52.5	116	38.8	100	45.2
Positive	313	58.4	73	62.4	131	67.2	95	47.5	183	61.2	121	54.8
Ki67 expression								P: 0.161				P: 0.001
Negative	458	85.4	103	88.0	169	87.1	161	81.3	270	90.0	176	80.0
Positive	78	14.6	14	12.0	25	12.9	37	18.7	30	10.0	44	20.0
HER2 overexpression								P: 0.147				P: 0.403
No overexpression	378	89.6	92	94.8	128	87.7	148	88.1	213	88.8	157	91.3
Overexpression	44	10.4	5	5.2	18	12.3	20	11.9	27	11.2	15	8.7
Local therapy								P: 0.051				P: 0.985
MST-radiotherapy	223	38.9	46	37.7	75	37.5	85	42.1	125	39.2	90	38.0
MST+radiotherapy	108	18.8	25	20.5	27	13.5	46	22.8	60	18.8	44	18.6
BCS-radiotherapy	5	0.9	2	1.6	2	1.0	0	0	3	0.9	2	0.8
BCS+radiotherapy	238	41.5	49	40.2	96	48.0	71	35.1	131	41.1	101	42.6
Systemic therapy								P: 0.426				P: 0.294
Chemotherapy	112	19.5	20	16.4	39	19.5	48	23.8	64	20.0	48	20.1
Endocrine therapy	75	13.1	12	9.8	25	12.5	28	13.9	49	15.3	24	10.0
Both	18	3.1	3	2.5	9	4.5	6	3.0	11	3.4	7	2.9
No systemic therapy	369	64.3	87	71.3	127	63.5	120	59.4	196	61.2	160	66.9

Table 1 Correlations between HLA class I expression and presence of Treg and well-established prognostic factors using chi-square test.

Abbreviations N number of patients; % percentage; HLA class I human leukocyte antigen class I; Treg regulatory T cell; HER2 human epidermal growth factor receptor 2; MST Mastectomy; BCS breast conservative surgery.

control for HLA class I antibody reactivity. Slides from human tonsil tissue served as positive control for Treg staining. For each staining, slides that did undergo the whole immunohistochemical staining procedure, but without primary antibodies served as negative controls.

Evaluation of immunostaining

Microscopic analysis of HCA2 and HC10 was assessed by two independent observers (EdK and QT) in a blinded manner. Percentage of tumor cells that showed membranous staining was assessed. HCA2 and HC10 staining were scored in 5 categories according to the defined standard method of the International HLA and Immunogenetics Workshop (IHIWS, score 1: 0-5 percent of tumor cells positively stained; score 2: 5-25; score 3: 25-50; score 4: 50-75; score 5: 75-100).[24] Quantification of Treg within the tumor was microscopically assessed in 10 high power fields (hpf) by two observers (EdK: 100%; AS: 30%) in a blinded manner. Treg was scored into two categories: absence and presence of Treg infiltration.

Statistical analysis

Statistical analyses were performed using the statistical package SPSS (version 15.0 for Windows, Spps Inc, Chicago, IL, USA). Cohen's kappa coefficient revealed a satisfactory agreement in classification (kappa=0.73). The χ^2 test was used to evaluate associations between various clinicopathological parameters and HLA class I expression and infiltration of Treg. Relapse-free period (RFP) was the time from date of surgery until a locoregional recurrence and/or distance recurrence, whichever came first. Clinical follow-up policy was equal for all patients in the study. Overall survival (OS) was defined from date of surgery until death. The Kaplan–Meier method was used for calculation of survival probabilities and the log-rank test for comparison of survival curves. RFP is reported as cumulative incidence function, after accounting for death as competing risk.[25] Cox regression was used for univariate and multivariate analysis for RFP and OS. Significant or close to significant variables ($p<0.1$) in univariate analysis were included in multivariate analysis. To analyze the predictive effect of HLA class I and Treg, analyses were performed in which was stratified for adjuvant chemotherapy administration.

RESULTS

Patient and tumor characteristics

Tumor material was available and incorporated in the TMA of 86% (574/677) of the patients. Clinicopathological and treatment characteristics are shown in table 1. Median age of patients was 57 years (range= 23-96 years). Median follow-up of patients alive

was 19 years (range=0-23 years). Chemotherapy treatment consisted of a combination of cytostatic drugs, always containing cyclophosphamide.

Expression of HLA class I and infiltration of Treg

Microscopical quantification was successful in 94% (538/574) of tumors for HC10 and in 96% (548/574) for HCA2 (figures 1 A, B). A total of 523/574 tumors (91%) could be quantified for both and were therefore available for total HLA class I expression evaluation. Three groups were defined for HLA class I expression: (1) HLA class I loss (both HCA2 and HC10 scored 0-5%), 23% of tumors, (2) HLA class I downregulation (either HCA2 or HC10 scored 0-5%), 38% of tumors and (3) HLA class I expression (both HCA2 and HC10 scored 5-100%), 39% of tumors (table1).

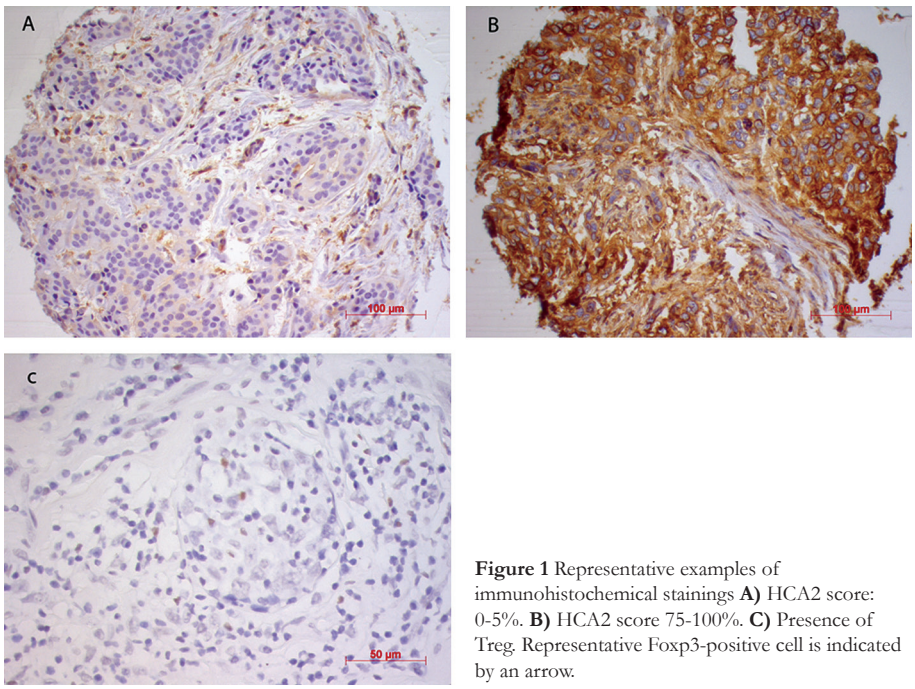

Figure 1 Representative examples of immunohistochemical stainings **A)** HCA2 score: 0-5%. **B)** HCA2 score 75-100%. **C)** Presence of Treg. Representative Foxp3-positive cell is indicated by an arrow.

A total of 556/574 (97%) tumors could be evaluated for Treg infiltration (figures 1 C,). Tumors with absence of Treg (0Treg/10hpf) and presence of Treg (≥1 Treg/10hpf) were seen in 57% and 43% of patients respectively (table 1).

Prognostic value of HLA class I and Treg

In order to analyze the prognostic effect of HLA class I and Treg, all patients who did not receive any systemic therapy were analyzed. There were no statistically significant differences in outcome for RFP (logrank: p=0.27) or OS (logrank: p=0.55) between different HLA class I expression levels (figures 2a, b). Treg showed no differences, neither in outcome for RFP (logrank: p=0.93) nor OS (logrank: p=0.14) between intra-tumoral

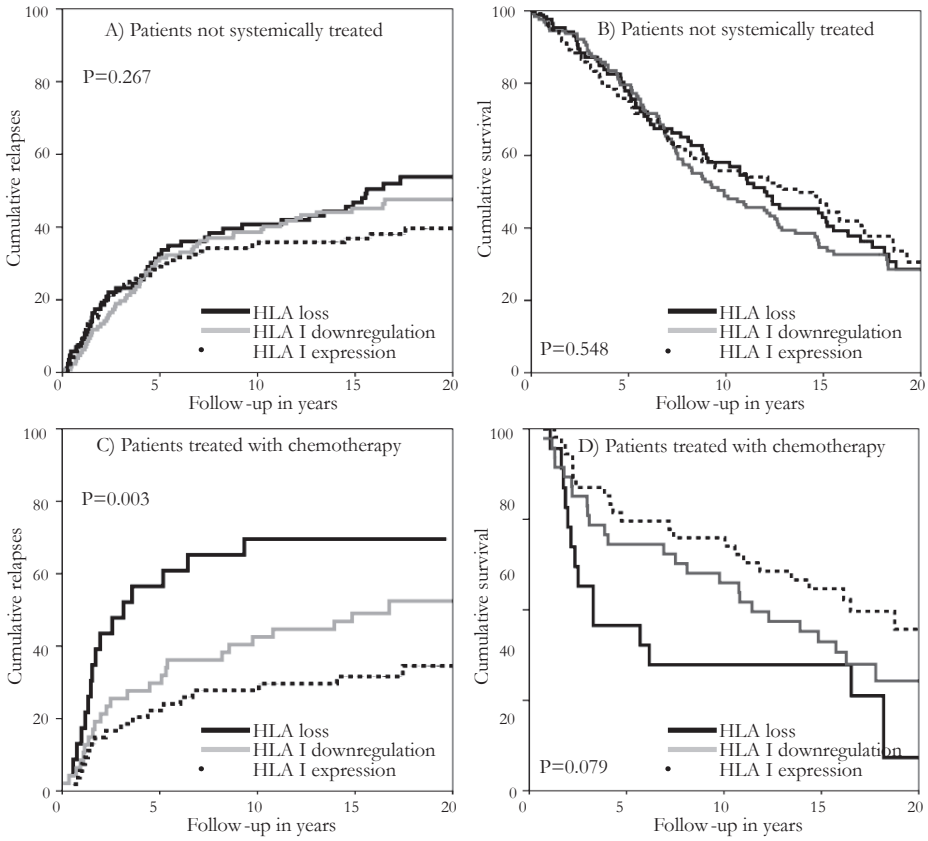

Figure 2 HLA class I tumor expression and clinical outcome. Relapses over time (A) and overall survival (B) of non-systemically treated patients. Relapses over time (C) and overall survival (D) of chemotherapy-treated patients.

absence and presence (figures 3a, b). In contrast with the data in patients who did not receive systemic treatment, analysis of HLA class I expression in chemotherapy-treated patients showed statistically significant differences for RFP between groups (logrank: p=0.003) (figure 2c). Of patients with expression of HLA class I, 68% were relapse free after 15 years, compared to 51% and 30% for downregulation and loss of HLA class I expression respectively. Infiltration of Treg showed, similarly to HLA class I, moderate differences in outcome between groups among chemotherapy-treated patients (logrank: p=0.06) (figure 3c). Patients with intra-tumoral infiltration of Treg had less relapses compared to patients with no infiltration of Treg. Cox proportional multivariate analysis was performed with data from chemotherapy-treated patients including the parameters that showed a trend of influence on outcome ($p<0.1$) in Cox proportional univariate analysis; lymph node status, HLA class I and Treg (table 2). This analysis revealed that lymph node status, HLA class I (p= 0.002; downregulation, HR 2.11, 95%CI 1.13-3.95;

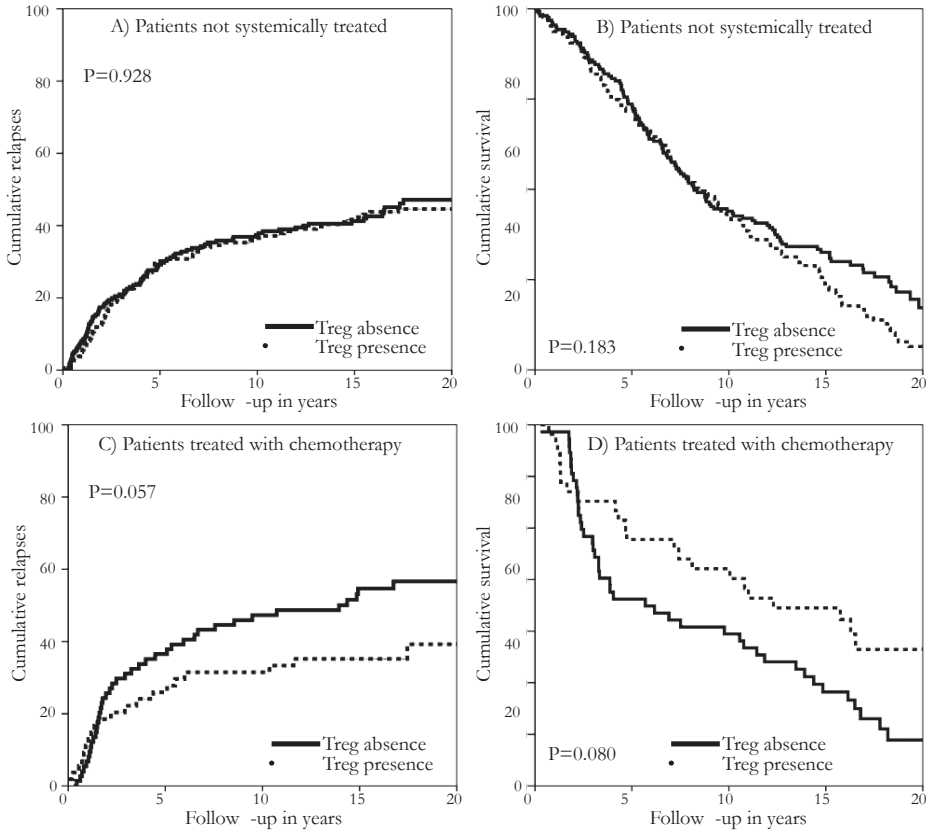

Figure 3 Treg tumor infiltration and clinical outcome. Relapses over time (A) and overall survival (B) of non-systemically treated patients. Relapses over time (C) and overall survival (D) of chemotherapy-treated patients.

loss, HR 3.34, 95%CI 1.67-6.67) and Treg (p=0.01, HR 2.04, 95%CI 1.16-3.57) were all independent prognostic factors for RFP among chemotherapy treated patients.

Predictive value of HLA class I and Treg

In order to prove that HLA class I and Treg were statistically significant cooperating with chemotherapy, an interaction term was introduced in Cox regression analysis. This analysis showed that both HLA class I (p< 0.001; Downregulation HR: 2.15, 95%CI: 1.17-3.96; Loss HR: 3.15, 95%CI: 1.92-5.15) and Treg (p<0.001; HR 2.47, 95%CI 1.54-3.95) significantly interacted with chemotherapy administration. These data indicated that both HLA class I expression and Treg tumor infiltration possess prognostic value specifically in breast cancer patients that are treated with chemotherapy.

		UNIVARIATE			MULTIVARIATE	
	N	HR	95% CI	p-value	HR	95% CI	p-value
Age							
<40	25	1.00		0.370			
40-50	57	1.01	0.537-1.888				
50-60	30	0.67	0.303-1.472				
>60	18	0.51	0.183-1.413				
Grade							
I	15	1.00		0.887			
II	57	1.12	0.490-2.558				
III	57	0.99	0.428-2.271				
Histological type							
Ductal	117	1.00		0.453			
Other	12	0.453	0.594-3.209				
Tumor stage							
pT1	35	1.00		0.416			
pT2	70	0.88	0.491-1.560				
pT3/4	20	1.39	0.657-2.950				
Nodal stage							
pN-	38	1.00		0.002	1.00		0.001
pN+	92	2.92	1.480-5.741		3.08	1.539-6.179	
Estrogen receptor							
Negative	58	1.00		0.207			
Positive	65	1.41	0.828-2.390				
Progesterone receptor							
Negative	57	1.00		0.377			
Positive	69	1.27	0.750-2.138				
Ki67 expression							
Negative	101	1.00		0.866			
Positive	23	0.68	0.438-1.712				
HER2 overexpression							
Negative	82	1.00		0.497			
Positive	17	1.29	0.622-2.663				
Local treatment							
MST-Radiotherapy	33	1.00		0.109			
MST +radiotherapy	35	1.78	0.889-3.548				
BCS +radiotherapy	62	1.01	0.524-1.955				
Endocrine therapy							
Negative	112	1.00		0.501			
Positive	18	0.76	0.347-1.678				
HLA							
Expression	54	1.00		0.005	1.00		0.002
Downregulation	47	1.71	0.929-3.159		2.11	1.127-3.947	
Loss	23	3.11	1.577-6.116		3.34	1.671-6.670	
Treg							
>0	54	1.00		0.060	1.00		0.013
0	75	1.67	0.979-2.857		2.04	1.164-3.568	

Table 2 Cox univariate and multivariate analysis for recurrence free period (RFP) of patients who did receive chemotherapy.

Abbreviations N number of patients; HR hazard ratio; 95%CI 95% Confidence Interval; HLA class I human leukocyte antigen class I; Treg regulatory T cell; HER2 human epidermal growth factor receptor 2; MST mastectomy; BCS breast conserving surgery.

DISCUSSION

Our study showed that HLA class I and Treg are independent prognostic markers for chemotherapy-treated patients with substantial discriminative power. Parameters that are able to determine which breast cancer patients may benefit from adjuvant chemotherapy are few.[26] Known factors which tend to indicate better chemotherapy response are negative ER, high tumor grade and high proliferative activity, but their predictive value is marginal.[27] In our study, independently of those factors, both high levels of HLA class I expression and presence of Treg resulted in statistically significant less relapses over time. Most importantly, these results can be explained by underlying biology and correspond with results of previous studies.

In concordance with previous studies, downregulation and loss of HLA class I was frequently seen in our study.[9-11] Prior studies indicate that breast cancer is immunogenic and induces tumor associated antigen (TAA)-specific CTL.[28] These findings may imply that breast cancer cells with downregulation or loss of HLA class I expression escaped from immune destruction and therefore selectively grew out.[29] This seems quite a common phenomenon in breast cancer considering the fact that we and others, found HLA class I downregulation or loss in more than half of the tumors. In addition, Treg were found in a significant number of tumors. Tumors may either attract these immune-suppressing cells in order to evade attack from effector T cells, or Treg may consider tumor cells as normal cells and thus prevent immune attack. Our data indicate that the immune system is closely involved in the development of breast cancer. At the time of a clinically manifest tumor, the balance between immune attack and tumor growth obviously is at the site of the tumor. [29]

We showed that expression levels of HLA class I had a specific prognostic effect, but only in chemotherapy-treated patients. Previous studies on HLA class I expression in breast cancer did not stratify for systemic therapy. A total of 3 studies have evaluated HLA class I expression and its effect on prognosis in breast cancer.[10-12] Two studies found that HLA class I expression levels had no influence on the prognosis of patients, which is in concordance with our findings in patients that were not systemically treated. [10, 12]Our study also showed that infiltration of Treg was a predictive marker for chemotherapy response in breast cancer patients. These finding are supported by Ladoire *et al.* who found that the number of Treg declined due to chemotherapy, showing that chemotherapy affects Treg and thus may counteract by restrained CTL. More importantly, complete absence of Treg after chemotherapy administration, resulted in a better response with higher rates of pathological complete response (pCR), further supporting our findings of a predictive role of Treg. [16] Other studies have shown that infiltration of Treg in breast tumors resulted in a worse prognosis in terms of relapses and survival.[15, 30] Our study could not statistically prove such a relation in patients who

did not receive systemic treatment. In order to unravel the complex tumor-immune system interactions during tumor development, further studies are needed.
The specific prognostic effects found for HLA class I and Treg among chemotherapy-treated patients can be explained by the following biological explanation. In our population, choice of chemotherapy comprised cyclophosphamide which positively influences host immune responses against cancer.[31-33] It is hypothesized that several mechanism are the basis for this phenomenon: enhanced homeostatic expansion of antigen-specific T cells by creation of a niche in the immune system, stimulation of dendritic cells (DC), induction of T cell growth factors such as type I interferons (IFN) and selective elimination of Treg. [31-33]Ceasing of Treg through cyclophosphamide effects, results amongst other things in enhanced expansion and function of responding of CTL.[32, 33] Ladoire *et al.* found that after preoperative chemotherapy absolute numbers of tumor-infiltrating Treg significantly declined, and numbers of effector T cells and CTL remained stable. In addition, a pathologic complete response was associated with a combination of absence of infiltration of Treg and presence of CTL after chemotherapy.[16] In our study this phenomenon was associated only in tumors that retained HLA expression, suggesting that upon counteraction of Treg, CTL are able to affect tumor metastases development.

In summary, HLA class I and Treg have an independent prognostic effect for chemotherapy-treated patients, which can be explained by underlying biology. Both factors resulted in a very high differentiation in sensitivity to chemotherapy. Predictive factors for chemotherapy response in breast cancer are highly necessitated. Therefore, we conclude that HLA class I and Treg are candidate markers for further investigation in randomized studies and may be applied for chemotherapy response prediction.

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