Chapter 5

Tumor immune subtypes distinguish tumor subclasses with clinical implications in breast cancer patients

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ABSTRACT

Introduction There is strong evidence that the host's cellular immune response is linked to tumor progression, however its impact on patient outcome in breast cancer is poorly understood. The purpose of this study is to define tumor immune subtypes, focusing on cellular immune responses and investigate their prognostic effect in breast cancer patients.

Methods Our training (n=440) and validation cohort (n=382) consisted of all early breast cancer patients primarily treated with surgery in our center between 1985 and 1996. Tumor tissue sections were immunohistochemically stained for CD8 (CTL) and PEN5 (NK cells). Tumor expression of classical and non-classical HLA class I, and tumor-infiltrating Tregs were previously determined. Tumor immune subtypes were constructed based on quantification of these markers and biological rationale.

Results High, intermediate and low immune susceptible tumor immune subtypes were found in respectively 16%, 63% and 20% of patients in the training cohort and 16%, 71% and 13% in the validation cohort. The subtypes showed to be statistically significant prognostic in multivariate analyses for relapse free period (RFP) ($p<0.0001$, intermediate versus high: hazard ratio (HR) 1.95; low versus high HR 2.98) and relative survival (RS) ($p=0.006$, intermediate versus high HR 3.84; low versus high: HR 4.26). Validation of these outcome analyses confirmed the independent prognostic associations: RFP ($p=0.025$) and RS ($p=0.040$).

Conclusion The tumor immune subtypes that we present represent a prognostic profile with solid underlying biological rationale and with high discriminative power confirmed in an independent validation cohort. Our results emphasize the importance of tumor immune surveillance in the control of tumor development and, therefore, in determining patient prognosis. Tumor immune subtype profiling is promising for prognosis prediction and the achievement of tailored treatment for breast cancer patients.

INTRODUCTION

Breast cancer is the most commonly diagnosed female cancer and is the leading cause of death from cancer in women in the western world[1]. Decisions regarding use of systemic therapy in primarily non-metastasized breast cancer patients are mainly based on prognostic and predictive factors like lymph node status, tumor size, grade, hormone receptor and human epidermal growth factor receptor 2 (HER2) expression[2]. However, currently these do not provide optimal risk-stratification. Therefore, additional prognostic and predictive information is sought in order to improve tailored treatment for patients with breast cancer.

There is strong evidence that a host's cellular immune response is able to control tumor progression[3]. However, due to their intrinsic genetic unstable nature, tumor cells may acquire properties to escape from such immune recognition[4]. Various interactions underlie the balance between immune control and tumor escape (Figure 1). Cytotoxic

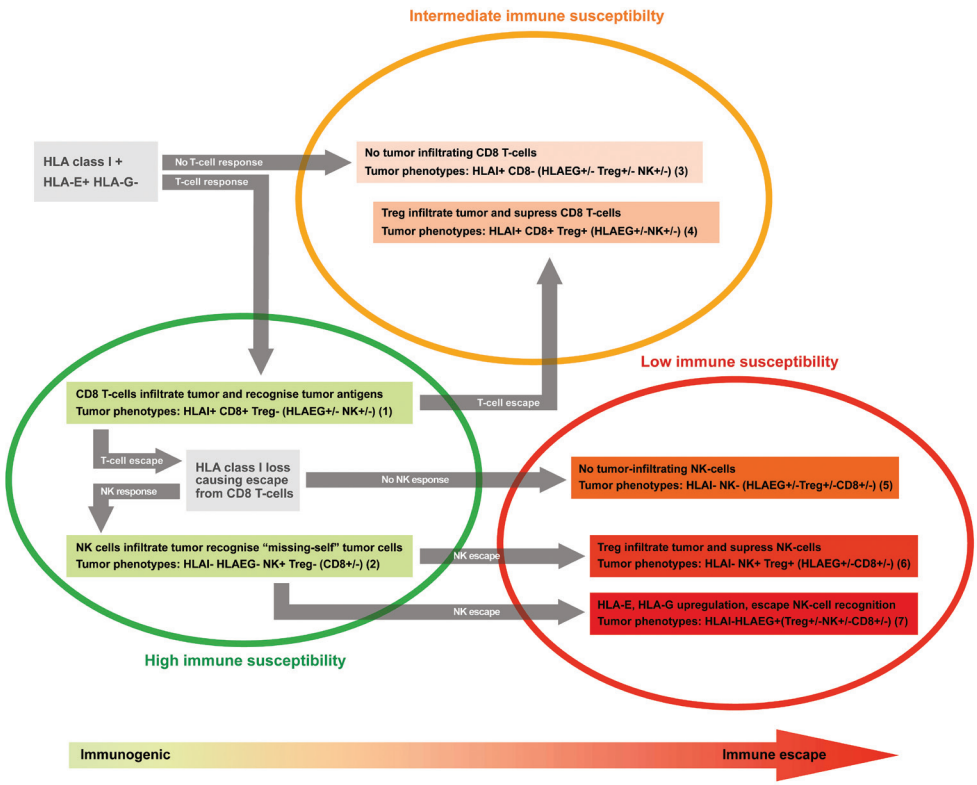

Figure 1 Tumor immune subtypes showing a schematic overview of different stages of immune surveillance and tumor immune escape classified into 7 tumor immune subtypes, graded from (1) to (7) in ascending order from highly immunogenic and therefore high immune susceptibility (green) to high immune escape and low immune susceptibility (red), concerning combinations of CTL infiltration, NK cell infiltration, Treg infiltration, classical HLA class I tumor expression and HLA-EG tumor expression. Tumor immune subtypes were clustered by combining from the original tumor immune subtypes groups as shown in by encircled groups (high immune susceptible) clustered (1) and (2)(green circle), (intermediate immune susceptible) clustered (3) and (4)(orange circle), (low immune susceptible) clustered (5), (6) and (7) (red circle).

T-lymphocytes (CTL) are capable of recognizing tumor-associated antigens presented by classical human leukocyte antigen (HLA) class I (HLA-A, HLA-B, HLA-C) on the tumor cell surface. In order to avoid immune recognition from CTL, cancer cells may lose expression of classical HLA class I[5]. However, this makes them prone to natural killer (NK) cell recognition[6]. Non-classical HLA class I molecules (HLA-E, HLA-G) play a crucial role in immune surveillance by NK-cells. Expression of these molecules on the cell surface causes an inhibitory effect on NK-cell attack[6-8]. Another tumor escape mechanism from immunosurveillance is attraction and induction of immunosuppressive regulatory T cells (Treg) in the tumor microenvironment[9].

A variety of immune reactions have been found to date in breast cancer. Studies have indicated that breast cancer is highly immunogenic and often shows high numbers of tumor-infiltrating lymphocytes[10, 11]. However, as previously reported by our group and others, loss of classical HLA class I expression, upregulation of non-classical HLA-E and HLA-G expression [12-14] and induction and infiltration of Treg in the tumor microenvironment [13, 15-17] are frequent events in breast cancer, indicating that breast tumors are also capable of evading immune recognition. Together, this suggests that complex interactions take place between breast tumor cells and cells from the immune system[18]. Therefore, to get a good perspective on the effects of the immune system on tumor progression and patient outcome, such interactions should be accounted for. Indeed, previous studies of our group and others showed interactions between classical HLA class I and Treg, where loss of HLA class I in combination with presence of Treg in the tumor microenvironment resulted in a worse patient's outcome [16, 18]. This was also the case for classical HLA class I and HLA-E and HLA-G tumor expression, where HLA-E and HLA-G expression resulted in a worse patient outcome exclusively in patients with loss of tumor expression of classical HLA class I[12]. Together, this emphasizes the importance of research on combinations of markers of immune surveillance together with markers of tumor immune escape. We defined tumor immune subtypes, with focus on cellular immune responses, based on tumor expression of classical HLA class I, HLA-E and HLA-G, and tumor infiltration of CTL, NK cells, and Treg. The aim was to investigate the distribution and prognostic effect of the different immune subtypes in a large cohort of breast cancer patients and subsequently validate these effects on a second cohort of breast cancer patients.

PATIENTS AND METHODS

Patients and tumors

The total patient population comprised all retrospectively assessed primarily non-metastasized breast cancer patients primarily treated with surgery in the Leiden University Medical Center between 1985 and 1996 (n=822). 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, time of locoregional/distant tumor recurrence, survival time, and expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)(19). All tumors were graded according to current pathological standards by an experienced breast cancer pathologist. Approval for the study was obtained from the Leiden University Medical Center Medical Ethics Committee. All samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies). The REMARK criteria were respected for analyses of the immune subtypes and writing of this article(32). No statistically significant differences were found in patient or tumor characteristics between the training cohort (1985-1990 (n=440)) and a validation set (1990-1996 (n=382)).

Immunohistochemistry

Mouse antibody against CD8 (ab17147 clone 144B: AbCam, UK) and PEN5 (IM2354, clone 5H10.21.5: Beckman Coulter, NL) were used for immunohistochemical staining of respectively CTL and NK cells in tissue sections cut from intra-operatively derived FFPE tumor material according to previously described standard protocols [16]. Previously described were immunohistochemical stainings for expression of classical HLA class (anti-HLA-A and anti-HLAB/C; Dr. J. Neefjes, Netherlands Cancer Institute, Amsterdam, the Netherlands, HLA-E (ab2216 clone MEM-E/02: AbCam, UK), HLA-G (kindly provided by Prof. Dr. P.J. Van de Elsen) and Treg infiltration (FoxP3, ab20034 clone 236A/E7: AbCam, UK) [12, 16].

Evaluation of immunostaining

Quantification of CD8-positive stained cells and PEN5-positive stained cells in microscopical fields containing tumor was performed by two independent observers in a blinded manner in both training and validation cohorts. CD8 tumor infiltration was classified in two groups: (1) low CTL infiltration, 0-100 CD8 tumor infiltrating cells/mm^2; (2) high CTL infiltration, 100-3000 CD8 infiltrating cells/mm^2. For PEN5, only few positive infiltrating cells were seen. Therefore, any versus none PEN5-positive infiltrating cell were considered as presence and absence of NK cell infiltration respectively. Expression of classical HLA class I, HLA-E and HLA-G and Treg infiltration were previously categorized respectively as loss versus expression, no expression versus expression and absent versus present infiltration [12, 16].

Statistical analysis

Statistical analyses were performed using the statistical packages SPSS (version 16.0 for Windows, Spps Inc, Chicago, IL, USA) and Stata (version 10.0 for Windows, StataCorp, College Station, TX, USA). Cohen's kappa coefficient represented the inter-observer

agreement. The χ^2 test evaluated associations between clinicopathological parameters and tumor immune subtypes. Relapse-free period was defined as the time from date of surgery until any recurrence and was reported as cumulative incidence function, after accounting for death as competing risk. The Kaplan–Meier method was used for survival plotting and log-rank test for comparison of curves. Cox proportional hazard analysis calculated univariate and multivariable analysis for relapse-free period. Relative survival was calculated by the Hakulinen method as the ratio of the survival observed among the cancer patients and the survival that would have been expected based on the corresponding (age, sex, and year) general population. National life tables were used to estimate expected survival. Relative excess risks of death were estimated using a multivariable generalized linear model with a Poisson distribution, based on collapsed relative survival data, using exact survival times. Hazard ratio's and relative risks were served as indications for respectively risk of relapse and relative risk of survival. Variables with a P-value of < 0.10 in univariate analysis were entered in multivariable analysis.

RESULTS

Patient and tumor characteristics

Tumor material was available of 86% (380/440) and 87% (334/382) of the patients in the training cohort and validation cohort respectively. For the training cohort the median age of patients was 58 years (range= 23-96 years) and the median follow-up was 19 years (range= 0.1-22). For the validation cohort the median age and follow-up of patients were respectively 58 years (range= 32-90) and 13 years (range= 0.2-17). Clinicopathological and treatment characteristics are shown in supplementary tables (training cohort table 1A, 1B; validation cohort table 2A, 2B).

Tumor immune subtypes

The Cohen's kappa coefficient for inter-observer agreement of CTL and PEN5 quantification all reached a coefficient of 0.82 or higher. Missing immunohistochemical data was due to tissue damage.

Tumor immune subtypes, representing tumor adaptive immune escape variants were constructed from available data (Figure1). The defined tumor immune subtypes were in ascending order from high immune susceptibility to low immune susceptibility: (1) CTL are able to recognize tumor-associated antigens (TAA) presented by classical HLA class I and anti-tumor immune reaction can take place: Tumors with expression of classical HLA class I, high infiltration of CTL and absence of infiltration of Treg; (2) Tumors with a lack of classical HLA class I expression can escape CTL recognition, but NK cells are able to recognize these cells and anti-tumor immune reaction can take place: Tumors with loss of expression of classical HLA class I, no expression of HLA-EG, present infiltration of NK cells and absent infiltration of Treg; (3) Classical HLA class

I present TAA and could be recognized by CTL, but a low infiltration of CTL results in a limited anti-tumor immune reaction: Tumors with expression of classical HLA class I but low CTL infiltration; (4) Classical HLA class I present TAA and could be recognized by CTL, but immunosuppressive Treg weaken CTL function, resulting in a limited anti-tumor immune reaction: Tumors with expression of classical HLA class I, high infiltration of CTL, but also present infiltration of Treg; (5) Tumors with lack of classical HLA class I escape CTL recognition, but could be recognized by NK cells, which however are not present, resulting in failure of anti-tumor immune reaction: Tumors with loss of expression of classical HLA class I and absent NK cell infiltration; (6) Tumors with lack of classical HLA class I expression escape CTL recognition, but could be recognized by NK cells, however immunosuppressive Treg weaken NK cell function [19], resulting in failure of anti-tumor immune reaction: Tumors with loss of expression of classical HLA class I, present NK cell infiltration, but also present Treg

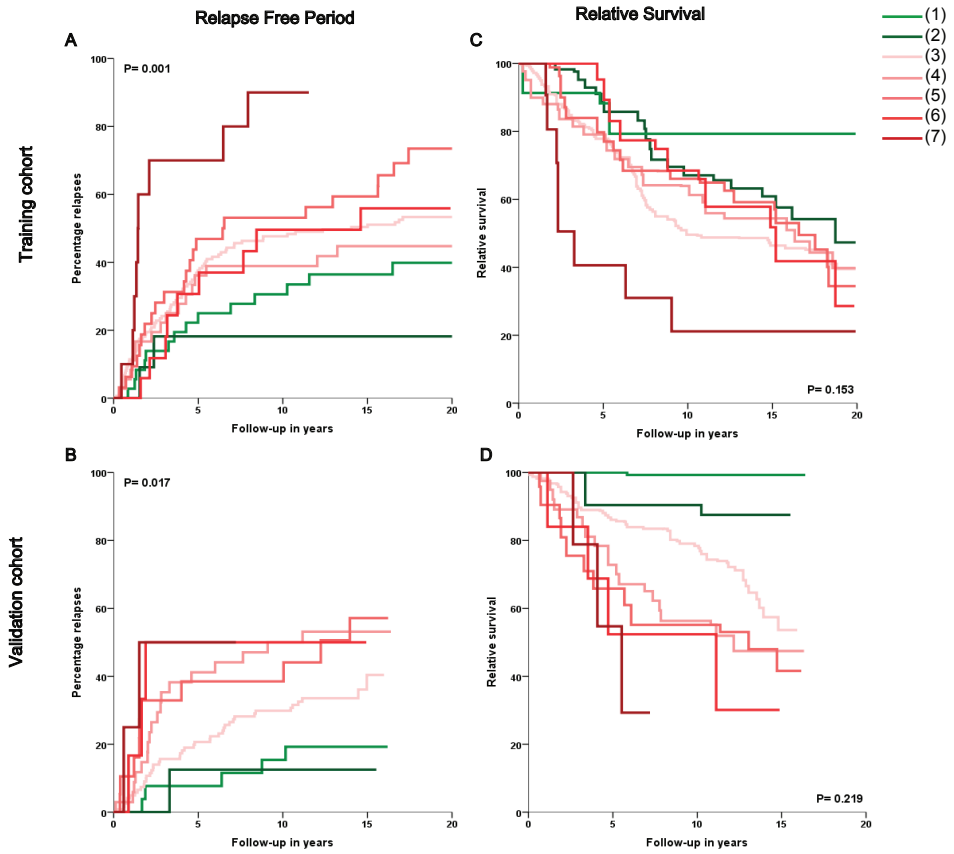

Figure 2 Outcome analyses by tumor immune subtypes for Relapse free period (RFP) **(A, B)** and relative survival (RS) **(C, D)** according to the 7 tumor immune subtypes that are described in the Results section for training cohort patients **(A, C)**, and for validation cohort patients **(B, D)**. Tumor immune subtypes representative for more tumor immune escape resulted in an unfavorable patient outcome concerning RFP and RS compared to more immunogenic tumor immune subtypes. Log-rank P-values are shown in each graph.

infiltration; (7) Tumor with lack of classical HLA class I expression but expression of non-classical HLA-EG escape from both CTL recognition and NK cell recognition: Tumor with loss of expression of classical HLA class I and expression of HLA-EG.
A more simplified tumor immune subtype variable was constructed by joining together tumor immune subtypes: High (subtypes 1-2), intermediate (subtypes 3-4) and low (subtypes 5-7) immune susceptibility (Figure 1, clustered groups shown by circles).

Characteristic		Relapse Free Period					
		Univariate analysis			Multivariable analysis		
	N	HR	95% CI	P	HR	95% CI	P
Age							
<40	74	1.00		0.354			
40-50	92	0.87	0.58-1.33				
50-60	81	1.24	0.82-1.88				
>60	133	0.95	0.64-1.42				
Grade							
I	53	1.00		0.030	1.00		0.293
II	186	1.38	0.86-2.22		1.30	0.73-2.31	
III	136	1.83	1.13-2.96		1.55	1.55-0.86	
Histological type							
Ductal	345	1.00		0.405			
Other	31	1.23	0.76-2.00				
Tumor stage							
pT1	127	1.00		0.001	1.00		0.045
pT2	198	1.34	0.97-1.86		1.03	0.70-1.51	
pT3/4	45	2.56	1.51-3.69		1.75	1.06-2.88	
Nodal stage							
Negative	199	1.00		<0.001	1.00		<0.001
Positive	171	3.09	2.30-4.16		2.78	1.97-3.92	
ER status							
Negative	133	1.00		0.890			
Positive	229	1.02	0.76-1.38				
PgR status							
Negative	155	1.00		0.765			
Positive	201	1.05	0.78-1.41				
HER2 status							
Negative	271	1.00		0.166			
Positive	32	1.42	0.87-2.32				
Immune phenotyope							
High immune susceptibility	48	1.00		0.005	1.00		<0.001
Intermediate immune susceptibility	186	1.80	1.06-3.05		1.95	1.13-3.39	
Low immune susceptibility	59	2.56	1.44-4.57		2.98	1.62-5.48	

Table 1 Cox univariate and multivariate analysis in the training cohort of breast cancer patients for recurrence free period and relative survival for tumor immune subtypes classified into 3 groups that are described in the Results section. **Abbreviations** N number of patients; HR hazard ratio; 95%CI 95% Confidence Interval; ER estrogen receptor; PR progesterone receptor; HER2 human epidermal growth factor receptor 2; ET endocrine therapy; CT chemotherapy.

Associations between clinicopathological patient and tumor characteristics and tumor immune subtypes classified into 7 groups and into 3 groups are shown in supplementary tables 1A, B and 2A, B respectively. No statistically significant validated association was found between patient and tumor characteristics and tumor immune subtypes classified into 7 groups and into 3 groups.

Relative Survival					
Univariate analysis			Multivariable analysis		
RER	95% CI	P	RER	95% CI	P
1.00		0.048	1.00		0.031
0.79	0.49-1.28		0.60	0.32-1.12	
1.51	0.96-2.38		1.49	0.83-2.65	
1.20	0.71-2.03		1.05	0.54-2.05	
1.00		0.005	1.00		0.023
1.74	0.82-3.68		0.62	0.30-1.30	
2.73	1.29-5.75		1.20	0.60-2.41	
1.00		0.333			
1.34	0.74-2.40				
1.00		<0.001	1.00		0.003
1.84	1.18-2.86		1.90	1.10-3.29	
3.69	2.18-6.24		3.40	1.68-6.89	
1.00		<0.001	1.00		<0.001
2.97	2.04-4.33		2.30	1.48-3.56	
1.00		0.157			
0.77	0.54-1.10				
1.00		0.248			
0.81	0.56-1.16				
1.00		0.004	1.00		0.154
2.03	1.25-3.30		1.59	0.84-3.00	
1.00		0.098	1.00		0.006
1.95	0.98-3.98		3.84	1.62-9.09	
2.02	0.97-4.53		4.26	1.70-10.70	

Tumor immune subtypes classified into 7 groups

Distribution in patient training and validation cohort

The tumor immune subtypes classified into 7 groups could be determined for patients with data available for all immune markers: 77% (293/380) of patients in the training cohort; 66% validation cohort. Distributions of immune subtypes and associations with

Characteristic		Relapse Free Period					
		Univariate analysis			Multivariable analysis		
	N	HR	95% CI	P	HR	95% CI	P
Age							
<40	63	1.00		0.147			
40-50	83	0.62	0.38-1.03				
50-60	76	0.57	0.33-0.97				
>60	112	0.68	0.42-1.10				
Grade							
I	63	1.00		0.001	1.00		0.433
II	156	1.45	0.82-2.59		1.68	0.68-4.16	
III	108	2.54	1.43-4.52		1.86	0.72-4.79	
Histological type							
Ductal	293	1.00		0.298			
Other	35	1.35	0.77-2.35				
Tumor stage							
pT1	162	1.00		<0.001	1.00		0.171
pT2	130	2.18	1.46-3.23		1.78	0.98-3.26	
pT3/4	32	2.46	1.34-4.51		1.54	0.63-3.77	
Nodal stage							
Negative	182	1.00		<0.001	1.00		0.01
Positive	142	2.81	1.93-4.08		2.06	1.19-3.57	
ER status							
Negative	155	1.00		0.034	1.00		0.889
Positive	164	0.67	0.46-0.97		1.04	0.60-1.82	
PgR status							
Negative	161	1.00		0.006	1.00		0.184
Positive	150	0.59	0.40-0.86		0.68	0.38-1.20	
HER2 status							
Negative	249	1.00		0.002	1.00		0.934
Positive	27	2.36	1.36-4.09		0.97	0.42-2.22	
Immune phenotyope							
High immune susceptibility	34	1.00		0.005	1.00		0.025
Intermediate immune susceptibility	156	2.66	1.15-6.16		2.45	0.87-6.89	
Low immune susceptibility	29	4.72	1.83-12.18		4.73	1.48-15.06	

Table 2 Cox univariate and multivariate analysis in the validation cohort of breast cancer patients for recurrence free period and relative survival for tumor immune subtypes classified into 3 groups that are described in the Results section. **Abbreviations** N number of patients; HR hazard ratio; 95%CI 95% Confidence Interval; ER estrogen receptor; PR progesterone receptor; HER2 human epidermal growth factor receptor 2; ET endocrine therapy; CT chemotherapy.

known clinicopathological parameters are shown in supplementary tables (training cohort Table 1A; validation cohort Table 2A).

Prognostic associations with patient outcome

The association of tumor immune subtypes classified into 7 groups in the training cohort with relapse-free period and relative survival are shown in Figure 2. Analysis of relapse-

Relative Survival					
Univariate analysis			Multivariable analysis		
RER	95% CI	P	RER	95% CI	P
1.00		0.431			
0.58	0.30-1.10				
0.80	0.42-1.53				
0.77	0.35-1.69				
1.00		0.026	1.00		0.603
1.83	0.64-5.28		1.99	0.50-7.99	
3.27	1.16-9.21		1.69	0.40-7.14	
1.00		0.300			
1.46	0.71-3.01				
1.00		0.002	1.00		0.227
2.57	1.34-4.90		1.96	0.85-4.52	
4.30	1.86-9.96		2.30	0.78-6.79	
1.00		<0.001	1.00		0.208
3.09	1.73-5.13		1.59	0.77-3.25	
1.00		0.008	1.00		0.488
0.44	0.24-0.81		0.78	0.39-1.57	
1.00		0.028	1.00		0.232
0.54	0.31-0.93		0.65	0.31-1.38	
1.00		<0.001	1.00		0.232
3.52	1.91-6.49		1.71	0.71-4.10	
1.00		0.089	1.00		0.040
5.31	0.64-31.33		5.47	0.72-41.70	
11.12	1.12-55.41		10.95	1.31-91.63	

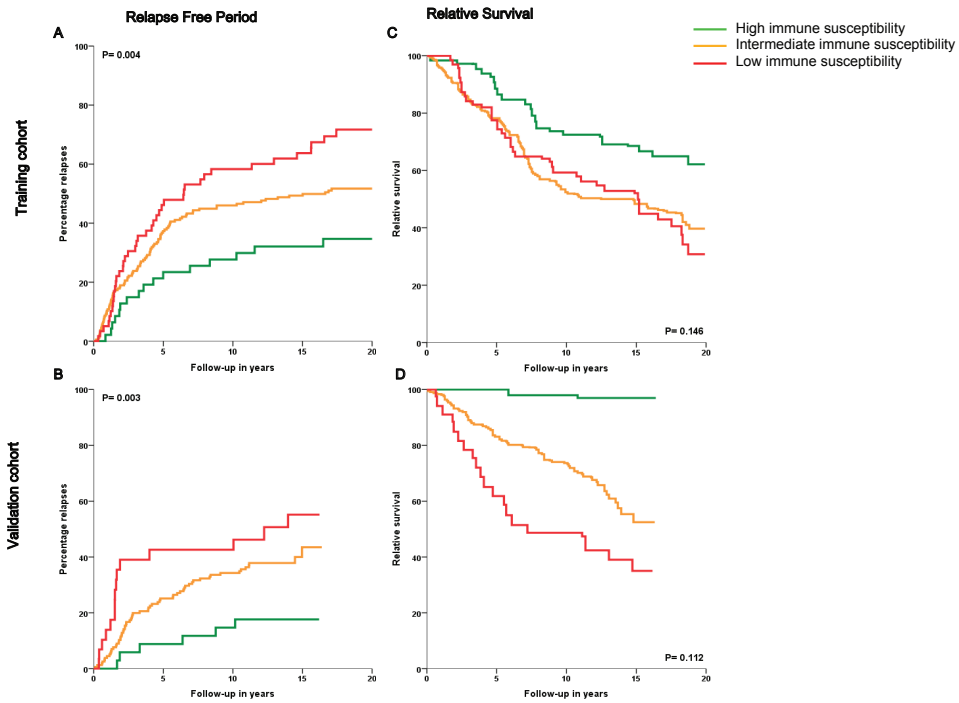

Figure 3 Outcome analyses by tumor immune subtypes for Relapse free period (RFP) **(A, B)** and relative survival (RS) **(C, D)** according to the 3 tumor immune subtypes that are described in the Results section for training cohort patients **(A, C)**, and for validation cohort patients **(B, D)**. Tumor immune subtypes representative for more tumor immune escape resulted in an unfavorable patient outcome concerning RFP and RS compared to more immunogenic tumor immune subtypes. Log-rank P-values are shown in each graph.

free period showed a statistically significant association between the 7 tumor immune subtypes and clinical outcome of patient (RFP p=0.001, Figure 2 A). Tumors that were expected to show lower immune susceptibility resulted in more patient relapses over time compared to tumors that were expected to show higher immune susceptibility. A similar though not significant trend was seen for the association between the 7 immune subtypes and relative survival outcome of patients (RS p=0.153, Figure 2 C). Results for outcome analyses were confirmed in the validation cohort (RFP p=0.017, Figure 2B and RS p=0.219, Figure 2D). Multivariable analyses demonstrated that these 7 tumor immune subtypes were a statistically significant independent prognostic factor in breast cancer patients for both RFP and RS (supplementary Table 3). Though statistical significance was lost in multivariable analyses in the validation cohort, a statistical trend remained for the association between 7 tumor immune subtypes and patient outcome concerning RFP (p=0.055, supplementary Table 4).

Tumor immune subtypes classified into 3 groups

Distribution in patient training and validation cohort

The tumor immune subtypes, consisting of three groups as described above showed the following distribution in the training and validation cohort respectively: High immune susceptible, 16% (48/293) and 16% (34/219); Intermediate immune susceptible, 63% (186/293) and 71% (156/219); Low immune susceptible, 20% (59/293) and 13% (29/219). Associations with known clinicopathological parameters are shown in supplementary tables (training cohort Table 1B; validation cohort Table 2B).

Prognostic associations with patient outcome

The association of the tumor immune subtypes classified into 3 groups with relapse-free period and relative survival is shown in Figure 3. Analysis of relapse-free period showed a significant association between tumor immune subtype and clinical outcome of patients (RFP p=0.004, Figure 3 A). Lower immune susceptible tumor subtypes, resulted in more relapses over time compared to higher immune susceptible tumor subtypes. Again, though not significant a similar associative trend was seen for relative survival outcome of patient and tumor immune subtype (RS p=0.146, Figure 3 C). Results of outcome analyses in the validation cohort were similar to the results found in the training cohort (RFP p=0.003, Figure 3 B and RS p=0.112, Figure 3D).

Multivariable analyses demonstrated that the tumor immune subtypes were a statistically significant independent prognostic factor in breast cancer patients for both RFP (p<0.001, Table 1B) and RS (p=0.006, Table 1B) with high discriminative power; compared to patients with high immune susceptible tumors, patients with intermediate immune susceptible tumors showed an almost twice elevated risk (HR 1.95, 95%CI 1.13-3.39) for developing relapses over time and an almost four times higher relative risk for survival (RR 3.84, 95% CI 1.62-9.09), while patients with low immune susceptible tumors showed an almost three times elevated risk on relapses over time (HR 2.98, 95%CI 1.62-5.48) and a more than four times higher relative risk for survival (RR 4.26, 95%CI 1.70-10.70) (Table 1B). Results of the validation cohort confirmed the associations found in multivariable analyses (RFP p=0.025, Table 2B and RS p=0.040, Table 2B)

DISCUSSION

The impact of the immune response and subsequent tumor immune evasion on tumor progression and patient outcome in breast cancer is poorly understood. Most studies focus on the effect of single parameters, like tumor expression of HLA class I or immune cell tumor infiltration, but separately these do not reflect the multifaceted interaction between immune cells and tumor cells. In order to get a good perspective on the processes involved in these interactions, we defined tumor immune subtypes. These

subtypes were defined based on tumor susceptibility for cellular immune responses using expression of key factors in these responses that reflect local presence of CTL, NK cells, and Treg and tumor expression of classical HLA class I and HLA-E and -G. Outcome analyses of the immune subtypes revealed strong associations with patient outcome where tumors defined as being highly susceptible to immune system attack showed a favorable outcome for breast cancer patients compared to patients with tumors defined having a low immune susceptible profile. These prognostic effects were shown in this study to be independent of known clinicopathological prognostic parameters and were additionally validated in an independent breast cancer patient cohort confirming the high discriminative power on patient outcome stratification.

Prior studies by our group and others have focused on a cellular immune response and its effect on tumor progression and patient outcome in breast cancer [11-16]. DeNardo *et al.* even provides evidence that treatment response is in part regulated by the immune microenvironment [20], again urging the importance of comprehensive determination of the tumor immune status. High tumor infiltration of CD8+ lymphocytes, representative for CTL infiltration, has been found to result in a favorable patient prognosis in one study[11]. However, another study reported high CTL infiltration to be associated with a worse patient outcome[21]. Yet another study could not find a statistically significant prognostic effect for CTL[10]. High Treg infiltration resulted in an unfavorable prognostic factor in a variety of studies[10, 15, 22], while it did not show a statistically significant association with patient outcome in a previous study of our group[16]. Loss of expression of classical HLA class I showed to be a favorable [23] as well as an unfavorable [16] prognostic factor in two different studies and revealed no statistically significant associations with patient outcome in two other studies[24, 25]. Concerning non-classical HLA-E and HLA-G, one study could not find a statistically significant relation with patient prognosis for HLA-G [13, 25] while a study of our group showed tumor expression of HLA-E and HLA-G resulted to be a statistically significant unfavorable prognostic parameter[12]. To our knowledge, the prognostic impact of NK cell infiltration has not been studied in breast cancer, but NK cell presence in the tumor microenvironment has been shown to result in a favorable patient outcome in colorectal cancer[26].
Taken together, these reports show contradictory results and, therefore, do not draw a clear picture of the interaction between breast cancer cells and the immune system. Our present study shows that this may be explained by the simple fact that a successful anti-tumor immune response depends not only on the level of expression of a single marker such as classical HLA class I, but on the variety of factors involved in the multifaceted immune response. Due to the complexity of the balance between immune surveillance and tumor immune escape, it is not a single marker that is able to reflect outcome of the interaction, but a set of key markers. In this study we analyzed a set of such crucial immune markers and defined tumor immune subtypes based on these markers. We demonstrated that a profile that represents tumors that may be more immune susceptible

is predictive for a more favorable clinical outcome for patients with breast cancer. In addition, the prognostic impact with high discriminative power that we found for these tumor immune subtypes, suggests that previous single marker studies are understating or even confounding the impact of the immune system on tumor control. The results found for the tumor immune subtypes are not only concordant with prior evidence on tumor immune biology in breast cancer[4, 18], but additionally join together the conclusions of prior studies by linking single tumor-immune markers to functional tumor-immune interaction. This is the first study providing detailed insight in tumor immune biology in breast cancer, showing that tumor immune surveillance is of crucial importance in the control of tumor progression and therefore in determining patient prognosis.

Many prognostic factors have been identified for breast cancer. Of these, the ASCO guidelines advised the use in clinical practice of urokinases plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1) and gene profiles detected with multiparameter gene expression assays[27]. The clinical value of microarray-based prognostic tools, like the MammaPrint, a 70-gene expression profile, and Oncotype DX, a 21-gene expression profile is currently being debated[28, 29]. One major critique is that these gene prints were constructed using top-down analyses and were not defined based on a biological rationale. Therefore, it is unclear what tumor types are represented by the various patient risk-groups[30]. Contrary to these top-down analyses, the tumor immune subtypes we defined are based on well-founded biological hypotheses. Future research will further improve this function-based approach of prognostic profiling in breast cancer.

REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55(2):74-108.
2. Goldhirsch A, Wood WC, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. Ann Oncol 2007;18(7):1133-1144.
3. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol 2006;6(10):715-727.
4. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004;22:329-360.
5. Algarra I, Garcia-Lora A, Cabrera T, Ruiz-Cabello F, Garrido F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother 2004;53(10):904-910.
6. Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers--an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? Semin Cancer Biol 2007;17(6):459-468.
7. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol 2002;3(11):999-1005.
8. Marin R, Ruiz-Cabello F, Pedrinaci S et al. Analysis of HLA-E expression in human tumors. Immunogenetics 2003;54(11):767-775.
9. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. Proc Natl Acad Sci U S A 2001;98(20):11521-11526.
10. Liu F, Lang R, Zhao J et al. CD8(+) cytotoxic T cell and FOXP3(+) regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. Breast Cancer Res Treat 2011.
11. Mahmoud SM, Paish EC, Powe DG et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol 2011;29(15):1949-1955.
12. de Kruijf EM, Sajet A, van Nes JG et al. 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. J Immunol 2010;185(12):7452-7459.
13. Kleinberg L, Florenes VA, Skrede M et al. Expression of HLA-G in malignant mesothelioma and clinically aggressive breast carcinoma. Virchows Arch 2006;449(1):31-39.
14. Lefebvre S, Antoine M, Uzan S et al. Specific activation of the non-classical class I histocompatibility HLA-G antigen and expression of the ILT2 inhibitory receptor in human breast cancer. J Pathol 2002;196(3):266-274.
15. Bates GJ, Fox SB, Han C et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol 2006;24(34):5373-5380.
16. de Kruijf EM, van Nes JG, Sajet A et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. Clin Cancer Res 2010;16(4):1272-1280.
17. Ladoire S, Arnould L, Apetoh L et al. Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. Clin Cancer Res 2008;14(8):2413-2420.
18. Galon J, Costes A, Sanchez-Cabo F et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313(5795):1960-1964.
19. Ghiringhelli F, Menard C, Terme M et al. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. J Exp Med 2005;202(8):1075-1085.
20. DeNardo DG, Brennan DJ, Rexhepaj E et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. Cancer Discov 2011;1(1):54-67.
21. Matkowski R, Gisterek I, Halon A et al. The prognostic role of tumor-infiltrating CD4 and CD8 T lymphocytes in breast cancer. Anticancer Res 2009;29(7):2445-2451.
22. Gobert M, Treilleux I, driss-Vermare N et al. Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. Cancer Res 2009;69(5):2000-2009.
23. Madjd Z, Spendlove I, Pinder SE, Ellis IO, Durrant LG. Total loss of MHC class I is an independent indicator of good prognosis in breast cancer. Int J Cancer 2005;117(2):248-255.
24. Gudmundsdottir I, Gunnlaugur JJ, Sigurdsson H, Olafsdottir K, Tryggvadottir L, Ogmundsdottir HM. Altered expression of HLA class I antigens in breast cancer: association with prognosis. Int J Cancer 2000;89(6):500-505.
25. Redondo M, Garcia J, Villar E et al. Major histocompatibility complex status in breast carcinogenesis and relationship to apoptosis. Hum Pathol 2003;34(12):1283-1289.

26. Menon AG, Janssen-Van Rhijn CM, Morreau H et al. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. Lab Invest 2004;84(4):493-501.
27. Harris L, Fritsche H, Mennel R et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J Clin Oncol 2007;25(33):5287-5312.
28. Michiels S, Koscielny S, Hill C. Prediction of cancer outcome with microarrays: a multiple random validation strategy. Lancet 2005;365(9458):488-492.
29. van de Vijver M.J., He YD, van't Veer LJ et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002;347(25):1999-2009.
30. Sotiriou C, Pusztai L. Gene-expression signatures in breast cancer. N Engl J Med 2009;360(8):790-800.

A			(1)		(2)		(3)		(4)		(5)		(6)		(7)		p-value
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	
Age																	0.047
<40	74	19.5	11	30.6	1	8.3	21	14.1	10	27.0	8	25.0	3	17.6	4	40.0	
40-50	92	24.2	7	19.4	4	33.3	44	29.5	6	16.2	3	9.4	5	29.4	1	10.0	
50-60	81	21.3	6	16.7	2	16.7	29	19.5	6	16.2	13	40.6	2	11.8	4	40.0	
>=60	133	35.0	12	33.3	5	41.7	55	36.9	15	40.5	8	25.0	7	41.2	1	10.0	
Grade																	0.033
I	53	14.1	4	11.1	4	36.4	17	11.4	1	2.7	6	18.8	4	23.5	3	30.0	
II	186	49.6	16	44.4	5	45.5	78	52.3	19	51.4	17	53.1	10	58.8	1	10.0	
III	136	36.3	16	44.4	2	18.2	54	36.2	17	45.9	9	28.1	3	17.6	6	60.0	
Histological type																	0.578
Ductal	345	91.8	31	86.1	10	90.9	141	94.6	33	89.2	29	90.6	16	94.1	10	100.0	
Lobular	31	8.2	5	13.9	1	9.1	8	5.4	4	10.8	3	9.4	1	5.9	0	0.0	
T-status																	0.305
T1	127	34.3	6	16.7	6	50.0	52	35.4	10	27.8	13	40.6	7	43.8	2	20.0	
T2	198	53.5	25	69.4	5	41.7	70	47.6	22	61.1	14	43.8	9	56.2	6	60.0	
T3/4	45	12.2	5	13.9	1	8.3	25	17.0	4	11.1	5	15.6	0	0.0	2	20.0	
N-status																	0.321
N0	199	53.8	20	57.1	6	50.0	83	57.2	16	44.4	16	51.6	10	58.8	2	20.0	
N1-3	171	46.2	15	42.9	6	50.0	62	42.8	20	55.6	15	48.4	7	41.2	8	80.0	
ER-status																	0.057
Negative	133	36.7	18	50.0	5	41.7	58	39.2	14	37.8	5	16.1	4	23.5	6	60.0	
Positive	229	63.3	18	50.0	7	58.3	90	60.8	23	62.2	26	83.9	13	76.5	4	40.0	
PgR-status																	0.131
Negative	155	43.5	19	52.8	5	41.7	61	41.2	18	48.6	6	20.7	9	52.9	6	60.0	
Positive	201	56.5	17	47.2	7	58.3	87	58.8	19	51.4	23	79.3	8	47.1	4	40.0	
Her2-status																	0.206
Overexpression -	271	89.4	26	86.7	11	100.0.0	105	85.4	28	93.3	25	96.2	16	100.0	8	100.0	
Overexpression +	32	10.6	4	13.3	0		18	14.6	2	6.7	1	3.8	0	0.0	0	0.0	
Local Therapy																	0.714
MAST-RT	132	34.7	11	30.6	6	50.0	55	36.9	15	40.5	11	34.4	7	41.2	1	10.0	
MAST+RT	80	21.1	10	27.8	1	8.3	31	20.8	5	13.5	6	18.8	2	11.8	4	40.0	
BCS	168	44.2	15	41.7	5	41.7	63	42.3	17	45.9	15	46.9	8	47.1	5	50.0	
Systemic therapy																	0.273
CT alone	78	20.5	11	30.6	1	8.3	35	23.5	9	24.3	2	6.2	2	11.8	4	40.0	
HT alone	27	7.1	3	8.3	0	0.0	11	7.4	1	2.7	3	9.4	1	5.9	0	0.0	
CT&HT	4	1.1	0	0.0	0	0.0	1	0.7	1	2.7	2	6.2	0	0.0	0	0.0	
None	271	71.3	22	61.1	11	91.7	102	68.5	26	70.3	25	78.1	14	82.4	6	60.0	
Total	380	100	36	100	12	100	149	100	37	100	32	100	17	100	10	100	

Supplementray Table 1 Correlations between tumor immune subtypes into 7 groups that are described in the Results section in the training cohort of patients **(A)** and in the validation cohort of patients **(B)** and well-established prognostic factors using chi-square test.

Abbreviations N number of patients; % percentage; ER estrogen receptor; PR progesterone receptor; HER2 human epidermal growth factor receptor 2; MAST mastectomy; RT radiotherapy; BCS breast conservative surgery; ET endocrine therapy; CT chemotherapy.

B			(1)		(2)		(3)		(4)		(5)		(6)		(7)		p-value
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	
Age																	0.794
<40	63	18.9	6	23.1	2	25.0	25	20.5	10	29.4	5	26.3	1	16.7	1	25.0	
40-50	83	24.9	6	23.1	2	25.0	29	23.8	12	35.3	4	21.1	1	16.7	0	0.0	
50-60	76	22.8	5	19.2	3	37.5	25	20.5	5	14.7	4	21.1	3	50.0	2	50.0	
>=60	112	33.5	9	34.6	1	12.5	43	35.2	7	20.6	6	31.6	1	16.7	1	25.0	
Grade																	0.420
I	63	19.3	6	24.0	2	25.0	25	20.7	2	6.1	2	11.1	1	16.7	1	33.3	
II	156	47.7	8	32.0	4	50.0	62	51.2	14	42.4	9	50.0	2	33.3	1	33.3	
III	108	33.0	11	44.0	2	25.0	34	28.1	17	51.5	7	38.9	3	50.0	1	33.3	
Histological type																	0.109
Ductal	293	89.3	22	88.0	8	100.0.0	111	91.7	29	87.9	13	72.2	4	66.7	3	100.0	
Lobular	35	10.7	3	12.0	0		10	8.3	4	12.1	5	27.8	2	33.3	0	0.0	
T-status																	0.541
T1	162	50.0	14	56.0	5	62.5	54	45.4	15	45.5	4	25.0	2	33.3	2	50.0	
T2	130	40.1	10	40.0	2	25.0	52	43.7	5	45.5	8	50.0	2	33.3	2	50.0	
T3/4	32	9.1	1	4.0	1	12.5	13	10.9	3	9.1	4	25.0	2	33.3	0	0.0	
N-status																	0.779
N0	182	56.2	17	68.0	5	62.5	61	51.3	18	54.5	9	50.0	3	50.0	1	33.3	
N1-3	142	43.8	8	32.0	3	37.5	58	48.7	15	45.5	9	50.0	3	50.0	2	66.7	
ER-status																	0.411
Negative	155	48.6	13	54.2	3	37.5	46	38.0	20	58.8	8	42.1	3	50.0	2	50.0	
Positive	164	51.4	11	45.8	5	62.5	75	62.0	14	41.2	11	57.9	3	50.0	2	50.0	
PgR-status																	0.046
Negative	161	51.8	15	62.5	2	25.0	52	42.6	24	70.6	8	44.4	4	66.7	2	50.0	
Positive	150	48.2	9	37.5	6	75.0	70	57.4	10	29.4	10	55.6	2	33.3	2	50.0	
Her2-status																	0.316
Overexpression -	249	90.2	15	83.3	6	100.0.0	99	92.5	28	90.3	15	93.8	4	66.7	4	100.0	
Overexpression +	27	9.8	3	16.7	0		8	7.5	3	9.7	1	6.2	2	33.3	0	0.0	
Local Therapy																	
MAST-RT	153	45.8	13	50.0	4	50.0	55	45.1	14	41.2	9	47.4	3	50.0	3	75.0	0.807
MAST+RT	52	15.6	5	19.2	1	12.5	19	15.6	7	20.6	6	31.6	2	33.3	0	0.0	
BCS	129	38.6	8	30.8	3	37.5	48	39.3	13	38.2	4	21.1	1	16.7	1	25.0	
Systemic therapy																	0.594
CT alone	49	14.7	2	7.7	1	12.5	18	14.8	6	17.6	7	36.8	1	16.7	1	25.0	
HT alone	86	25.7	8	30.8	2	25.0	34	27.9	6	17.6	4	21.1	2	33.3	1	25.0	
CT&HT	23	6.9	0	0.0	1	12.5	11	9.0	5	14.7	0	0.0	0	0.0	0	0.0	
None	176	52.7	16	61.5	4	50.0	59	48.4	17	50.0	8	42.1	3	50.0	2	50.0	
Total	334	100	26	100	8	100	122	100	34	100	19	100	6	100	4	100	

A			High immune susceptibility		Intermediate immune susceptibility		Low immune susceptibility		p-value
	N	%	N	%	N	%	N	%	
Age									0.094
<40	74	19.5	12	25.0	31	16.7	15	25.4	
40-50	92	24.2	11	22.9	50	26.9	9	15.3	
50-60	81	21.3	8	16.7	35	18.8	19	32.2	
>=60	133	35.0	17	35.4	70	37.6	16	27.1	
Grade									0.138
I	53	14.1	8	17.0	18	9.7	13	22.0	
II	186	49.6	21	44.7	97	52.2	28	47.5	
III	136	36.3	18	38.3	71	38.2	18	30.5	
Histological type									
Ductal	345	91.8	41	87.2	174	93.5	55	93.2	0.332
Lobular	31	8.2	6	12.8	12	6.5	4	6.8	
T-status									0.534
T1	127	34.3	12	25.0	62	33.9	22	37.9	
T2	198	53.5	30	62.5	92	50.3	29	50.0	
T3/4	45	12.2	6	12.5	29	15.8	7	12.1	
N-status									
N0	199	53.8	26	55.3	99	54.7	28	48.3	0.669
N1-3	171	46.2	21	44.7	82	45.3	30	51.7	
ER-status									0.058
Negative	133	36.7	23	47.9	72	38.9	15	25.9	
Positive	229	63.3	25	52.1	113	61.1	43	74.1	
PgR-status									
Negative	155	43.5	24	50.0	79	42.7	21	37.5	0.437
Positive	201	56.5	24	50.0	106	57.3	35	62.5	
Her2-status									
Overexpression -	271	89.4	37	90.2	133	86.9	49	98.0	0.081
Overexpression +	32	10.6	4	9.8	20	13.1	1	2.0	
Local Therapy									0.928
MAST-RT	132	34.7	17	35.4	70	37.6	19	32.2	
MAST+RT	80	21.1	11	22.9	36	19.4	12	20.3	
BCS	168	44.2	20	41.7	80	43.0	28	47.5	
Systemic therapy									
CT alone	78	20.5	12	25.0	44	23.7	8	13.6	0.508
HT alone	27	7.1	3	6.2	12	6.5	4	6.8	
CT&HT	4	1.1	0	0.0	2	1.1	2	3.4	
None	271	71.3	33	68.8	128	68.8	45	76.3	
Total	380	100	48	100	186	100	59	100	

Supplementary Table 2 Correlations between clustered tumor immune subtypes into 3 groups that are described in the Results section in the training cohort of patients **(A)** and in the validation cohort of patients **(B)** and well-established prognostic factors using chi-square test.

Abbreviations N number of patients; % percentage; ER estrogen receptor; PR progesterone receptor; HER2 human epidermal growth factor receptor 2; MAST mastectomy; RT radiotherapy; BCS breast conservative surgery; ET endocrine therapy; CT chemotherapy

B			High immune susceptibility		Intermediate immune susceptibility		Low immune susceptibility		p-value
	N	%	N	%	N	%	N	%	
Age									
<40	63	18.9	8	23.5	35	22.4	7	24.1	0.842
40-50	83	24.9	8	23.5	41	26.3	5	17.2	
50-60	76	22.8	8	23.5	30	19.2	9	31.0	
>=60	112	33.5	10	29.4	50	32.1	8	27.6	
Grade									
I	63	19.3	8	24.2	27	17.5	4	14.8	0.649
II	156	47.7	12	36.4	76	49.4	12	44.4	
III	108	33.0	13	39.4	51	33.1	11	40.7	
Histological type									0.035
Ductal	293	89.3	30	90.9	140	90.9	20	74.1	
Lobular	35	10.7	3	9.1	14	9.1	7	25.9	
T-status									
T1	162	50.0	19	57.6	69	45.4	8	30.8	0.148
T2	130	40.1	12	36.4	67	44.1	12	46.2	
T3/4	32	9.1	2	6.1	16	10.5	6	23.1	
N-status									
N0	182	56.2	22	66.7	79	52.0	13	48.1	0.253
N1-3	142	43.8	11	33.3	73	48.0	14	51.9	
ER-status									0.740
Negative	155	48.6	16	50.0	66	42.6	13	44.8	
Positive	164	51.4	16	50.0	89	57.4	16	55.2	
PgR-status									0.901
Negative	161	51.8	17	53.1	76	48.7	14	50.0	
Positive	150	48.2	15	46.9	80	51.3	14	50.0	
Her2-status									0.691
Overexpression -	249	90.2	21	87.5	127	92.0	23	88.5	
Overexpression +	27	9.8	3	12.5	11	8.0	3	11.5	
Local Therapy									
MAST-RT	153	45.8	17	50.0	69	44.2	15	51.7	0.345
MAST+RT	52	15.6	6	17.6	26	16.7	8	27.6	
BCS	129	38.6	11	32.4	61	39.1	6	20.7	
Systemic therapy									0.104
CT alone	49	14.7	3	8.8	24	15.4	9	31.0	
HT alone	86	25.7	10	29.4	40	25.6	7	24.1	
CT&HT	23	6.9	1	2.9	16	10.3	0	0.0	
None	176	52.7	20	58.8	76	48.7	13	44.8	
Total	334	100	34	100	156	100	29	100	

Characteristic		Relapse Free Period					
		Univariate analysis			Multivariable analysis		
	N	HR	95% CI	P	HR	95% CI	P
Age							
<40	74	1.00		0.354			
40-50	92	0.87	0.58-1.33				
50-60	81	1.24	0.82-1.88				
>60	133	0.95	0.64-1.42				
Grade							
I	53	1.00		0.030	1.00		0.384
II	186	1.38	0.86-2.22		1.35	0.76-2.41	
III	136	1.83	1.13-2.96		1.51	0.84-2.73	
Histological type							
Ductal	345	1.00		0.405			
Other	31	1.23	0.76-2.00				
Tumor stage							
pT1	127	1.00		0.001	1.00		0.153
pT2	198	1.34	0.97-1.86		1.01	0.69-1.49	
pT3/4	45	2.56	1.51-3.69		1.57	0.94-2.61	
Nodal stage							
Negative	199	1.00		<0.001	1.00		<0.001
Positive	171	3.09	2.30-4.16		2.81	1.98-3.99	
ER status							
Negative	133	1.00		0.890			
Positive	229	1.02	0.76-1.38				
PgR status							
Negative	155	1.00		0.765			
Positive	201	1.05	0.78-1.41				
HER2 status							
Negative	271	1.00		0.166			
Positive	32	1.42	0.87-2.32				
Immune phenotyope							
(1)	36	1.00		0.002	1.00		0.010
(2)	12	0.43	0.10-1.91		0.53	0.12-2.38	
(3)	149	1.60	0.90-2.82		1.82	1.00-3.32	
(4)	37	1.34	0.65-2.75		1.40	0.67-2.94	
(5)	32	2.15	1.11-4.18		2.45	1.20-4.99	
(6)	17	1.48	0.64-3.41		2.18	0.91-5.22	
(7)	10	5.09	2.19-11.82		4.41	1.83-10.62	

Supplementary Table 3 Cox univariate and multivariate analysis in the training cohort of breast cancer patients for recurrence free period and relative survival for tumor immune subtypes classified into 7 groups that are described in the Results section.

Abbreviations N number of patients; HR hazard ratio; 95%CI 95% Confidence Interval; ER estrogen receptor; PR progesterone receptor; HER2 human epidermal growth factor receptor 2; ET endocrine therapy; CT chemotherapy.

Relative Survival					
Univariate analysis			Multivariable analysis		
HR	95% CI	P	HR	95% CI	P
1.00		0.048	1.00		0.006
0.79	0.49-1.28		0.50	0.27-0.96	
1.51	0.96-2.38		1.52	0.84-2.72	
1.20	0.71-2.03		1.00	0.49-2.04	
1.00		0.005	1.00		0.043
1.74	0.82-3.68		0.59	0.28-1.24	
2.73	1.29-5.75		1.11	0.56-2.23	
1.00		0.333			
1.34	0.74-2.40				
1.00		<0.001	1.00		0.002
1.84	1.18-2.86		2.11	1.21-3.68	
3.69	2.18-6.24		3.62	1.77-7.41	
1.00		<0.001	1.00		<0.001
2.97	2.04-4.33		2.30	1.47-3.60	
1.00		0.157			
0.77	0.54-1.10				
1.00		0.248			
0.81	0.56-1.16				
1.00		0.004	1.00		0.135
2.03	1.25-3.30		1.62	0.86-3.07	
1.00		0.098	1.00		0.002
0.12	0.00-62.27		0.001	0-∞	
1.54	0.80-2.97		3.43	1.41-8.32	
1.26	0.54-2.92		2.40	0.86-6.67	
1.39	0.62-3.13		2.33	0.84-6.51	
1.03	0.33-3.21		4.26	1.28-14.15	
3.68	1.44-9.40		11.84	3.86-36.34	

Characteristic		Relapse Free Period					
		Univariate analysis			Multivariable analysis		
	N	HR	95% CI	P	HR	95% CI	P
Age							
<40	63	1.00		0.147			
40-50	83	0.62	0.38-1.03				
50-60	76	0.57	0.33-0.97				
>60	112	0.68	0.42-1.10				
Grade							
I	63	1.00		0.001	1.00		0.61
II	156	1.45	0.82-2.59		1.55	0.62-3.89	
III	108	2.54	1.43-4.52		1.62	0.61-4.30	
Histological type							
Ductal	293	1.00		0.298			
Other	35	1.35	0.77-2.35				
Tumor stage							
pT1	162	1.00		<0.001	1.00		0.113
pT2	130	2.18	1.46-3.23		1.93	1.04-3.56	
pT3/4	32	2.46	1.34-4.51		1.79	0.73-4.39	
Nodal stage							
Negative	182	1.00		<0.001	1.00		0.014
Positive	142	2.81	1.93-4.08		2.03	1.16-3.56	
ER status							
Negative	155	1.00		0.034	1.00		0.728
Positive	164	0.67	0.46-0.97		1.11	0.62-1.97	
PgR status							
Negative	161	1.00		0.006	1.00		0.243
Positive	150	0.59	0.40-0.86		0.70	0.39-1.27	
HER2 status							
Negative	249	1.00		0.002	1.00		0.815
Positive	27	2.36	1.36-4.09		1.11	0.46-2.66	
Immune phenotyope							
(1)	26	1.00		0.031	1.00		0.055
(2)	8	0.58	0.07-4.94		0.77	0.08-7.67	
(3)	122	2.10	0.83-5.31		2.04	0.61-6.89	
(4)	34	3.45	1.28-9.28		3.06	0.85-10.97	
(5)	19	4.09	1.39-12.01		3.67	0.91-1479	
(6)	6	3.82	0.91-16.02		4.16	0.81-21.44	
(7)	4	5.91	1.14-30.67		13.4	2.12-84.86	

Supplementary Table 4 Cox univariate and multivariate analysis in the validation cohort of breast cancer patients for recurrence free period and relative survival for tumor immune subtypes classified into 7 groups that are described in the Results section.

Abbreviations N number of patients; HR hazard ratio; 95%CI 95% Confidence Interval; ER estrogen receptor; PR progesterone receptor; HER2 human epidermal growth factor receptor 2; ET endocrine therapy; CT chemotherapy.

Relative Survival					
Univariate analysis			Multivariable analysis		
HR	95% CI	P	HR	95% CI	P
1.00		0.431			
0.58	0.30-1.10				
0.80	0.42-1.53				
0.77	0.35-1.69				
1.00		0.026			
1.83	0.64-5.28				
3.27	1.16-9.21				
1.00		0.300			
1.46	0.71-3.01				
1.00		0.002			
2.57	1.34-4.90				
4.30	1.86-9.96				
1.00		<0.001			
3.09	1.73-5.13				
1.00		0.008			
0.44	0.24-0.81				
1.00		0.028			
0.54	0.31-0.93				
1.00		<0.001			
3.52	1.91-6.49				
1.00		0.219			
5.2E5	0-∞				
1.5e6	0-∞				
2.5e6	0-∞				
2.6e6	0-∞				
3.7e6	0-∞				
6.5e6	0-∞				