Chapter

Prognostic value of a mRNA immune infiltrate signature on histopathological breast cancer subtypes of lymph node-negative primary operable breast cancer

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Abstract

Introduction Cancer related inflammation plays a key role in cancer progression and has been reported to be able to both promote and inhibit tumour growth. In breast cancer the prognostic value of a general tumour inflammatory cell infiltrate is controversial. This can in part be explained by the use of small heterogeneous patient groups and varying methodologies for assessing tumour cell infiltrates. We have defined a gene expression immune infiltrate signature representing a general tumour inflammatory cell infiltrate which can easily be applied to large genome wide expression datasets to identify relatively poor from highly immune infiltrated breast cancer samples.

Methods We used the mRNA immune infiltrate signature as a standardized assessment of general tumour inflammatory cell infiltrate to investigate its association with patient survival. First, distant metastasis-free survival time (MFS) was used as an endpoint for survival analysis in a discovery cohort of 344 lymph node negative primary breast cancer patients who did not receive any adjuvant therapy. Second, the results were validated in a large dataset consisting of three combined public cohorts of patients which also did not receive any adjuvant therapy (n=523). Finally, all samples were assigned to groups representing the main routine pathological factors (ER, PR, and Her2Neu receptor status) to assess the association between tumour inflammatory cell infiltrates, MFS and breast cancer molecular subtypes.

Results A significant positive association between MFS and amount of inflammatory cell infiltrate measured by the mRNA immune infiltrate signature as an objective measure, was observed in both the discovery and validation datasets. Samples with a high TIL immune infiltrate signature were found more than expected in the ER-/HER2- and especially in the HER2+ subtype. Furthermore, the association between MFS and immune infiltrate was found to be strongest in the HER2+ group.

Conclusions In lymph node negative primary breast cancer, high levels of tumour inflammatory cell infiltrates as determined by gene expression analysis, are associated with better metastasis-free survival, especially in HER2+ breast cancer patients.

Introduction

Recently, cancer related inflammation (CRI) has been described as the seventh hallmark of cancer, playing a key role in the tumour microenvironment and cancer progression [1]. Features of CRI include infiltration of white blood cells, macrophages and the presence of polypeptide messengers of inflammation such as cytokines, interleukins and chemokines (mediators of inflammation). Two pathways regarding CRI have been suggested; intrinsic (oncogene driven) and extrinsic (micro-environment driven). In the intrinsic pathway an inflammatory response is initiated by genetic events in neoplastic cells, while in the extrinsic pathway inflammation itself promotes cancer development [2].

The interplay between the immune system and cancer is complex and an inflammatory component is present in the microenvironment of most neoplastic tissues and is reported to both promote and inhibit tumour growth depending on type, location and density of the inflammatory cells involved [3-5].

In breast cancer, the host systemic inflammatory response is primarily associated with poor patient outcome [6,7]. In contrast, there have been conflicting reports regarding the prognostic value of the local inflammatory response. A review by Mohammed et al. [8] reports 13 published studies in which a pronounced general inflammatory cell infiltrate is associated with improved outcome in primary breast cancer. In contrast, 7 studies reported association with poorer outcome, while 4 studies reported no associations between general inflammatory infiltrate and patient survival. More detailed analyses indicated that particular inflammatory cell types, such as T-lymphocytes, are associated with recurrence and cancer-specific survival.

The majority of these studies have been conducted in small, heterogeneous tumours groups, regardless of tumour type and pathological factors such as estrogen (ER), progesteron (PR) and Her2Neu receptor status. In addition, different methodologies are used to define tumour inflammatory cell infiltrates, and no standardized assessment is defined. It therefore seems that the presence of a general inflammatory cell infiltrate has prognostic value in breast cancer patients, but the role of these infiltrates in predicting patient survival is controversial and may vary according to breast cancer subtypes.

In previous work on genomic profiling of *BRCA1* and *CHEK2**1100delC-mutated breast carcinomas, large numbers of tumour infiltrating lymphocytes (TILs) were found in a substantial fraction of basal-

like breast carcinomas (BLCs) [Massink et al] and a relatively smaller fraction of luminal breast carcinomas [Ref Massink 2]. In these studies, histopathological analysis of TIL percentages was performed on 96 primary breast cancer samples for which full-face frozen sections were available for H&E-staining. For these samples genome wide gene expression data was also available and by differential gene expression analysis we constructed an immune infiltrate gene expression signature correlating well with the reported TIL percentages. In the current study, we use this immune infiltrate gene expression signature as a standardized assessment of general tumour inflammatory cell infiltrate, predominantly T-lymphocytes, to investigate its association with patient survival within different groups of breast cancer based on molecular subtypes.

Materials and Methods

Sample collection

Fresh-frozen specimens of primary breast tumours from female familial breast cancer cases were selected from the tissue bank of the Erasmus Medical Center Rotterdam. All cases had been screened for germline mutations in *BRCA1*, *BRCA2* and for the *CHEK2**1100delC mutation. The complete breast cancer cohort consists of 155 primary tumours and includes 26 tumours with a *CHEK2**1100delC mutation, 47 *BRCA1*-mutated tumours, 6 *BRCA2*-mutated tumours, and 76 non-*BRCA1*/*BRCA2*/*CHEK2**1100delC mutated (BRCAX) tumours. These BRCAX breast cancer cases all originated from families with at least two breast cancer cases in first or second degree relatives of which at least one had been diagnosed before the age of 60. The entire cohort is described in detail by Nagel et al. [9]. In this study, 96 tumour samples for which both H&E stained sections and gene expression data is available were used for further analyses. The gene expression data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE54219.

Gene expression microarrays

For gene expression analysis .CEL files of the individual samples as deposited in GEO 54219 were used. The data was analyzed in Partek Genomics Suite (v6.6, Partek Inc.). Detection of differential gene expression was performed by ANOVA analysis, genes with FDR-stepup (false discovery rate) p-values smaller than 0.05 were considered to be statistically significant differentially expressed.

mRNA signature for immune infiltrate

To identify samples with low and high number of tumour infiltrating lymphocytes (TILs), hierarchical clustering of expression data was used. This approach has largely been described in previous work [ref Massink et al.]. In short, the proportion of lymphocytic nuclei of 96 tumour samples was assessed on H&E-stained frozen sections. This was done by assessing the number of tumour, stromal and TIL nuclei for multiple representative areas on the H&E stained slides. Microarray expression data were available for these samples (Affymetrix HG-U133_plus_2.0 array) and in the subsequent mRNA analysis, the luminal and basal samples were processed separately. These samples were divided in two groups based on the TIL percentages (high and low TIL count, median split) on which ANOVA analysis was performed to find differentially expressed probe sets passing a FDR p-value <0.05. Finally, the overlapping probe-sets (n=156) for the luminal and basal sample sets were determined to create the final mRNA immune infiltrate signature, see Additional File 1. In previous work, the correlation between immune infiltrate signature mRNA values and TIL percentages as determined on H&E stained slides was found to be highly significant (r_s=0.74, p-value < 0.001) [ref Massink et al CHEK2]. Survival analysis was not performed in this familial cohort, because patients received varying treatment regimes.

Discovery dataset

To investigate the relationship between the quantities of TILs based on mRNA immune infiltrate signature expression levels with clinical outcome we used a group of 344 lymph node–negative breast cancer patients who did not receive any adjuvant systemic treatment for which gene expression data (HG-U133-A array) are available. The study was approved by the Medical Ethics Committee of the Erasmus MC Rotterdam, the Netherlands (MEC-02.953) and was performed in accordance to the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl). The gene expression data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession numbers GSE2034 and GSE5327 [10]. The mean age at time of surgery was 53 years (SD, 12); 221 patients (64%) were ER-positive and 123 patients (36%) were ER-negative; 198 patients were premenopausal and 146 were postmenopausal. T1 tumours (<=2 cm) were present in 168 patients (49%), T2 tumours (>2–5 cm) in 163 patients (47%), T3/4 tumours (>5 cm) in 12 patients (3%), and 1 patient with unknown tumour stage. Of the complete group, 226 patients (66%) did not have a metastasis at a

distant organ during follow-up (median follow-up time of patients still alive was 101 months; range, 61–171 months). Of the 156 HG-U133_plus_2.0 array immune infiltrate signature probe-sets, 120 were found to overlap with the HG-U133_A array and were used to divide the samples in a high and low TIL group based on hierarchical clustering analysis.

Validation datasets

To validate our findings we used three public datasets of primary operable, lymph node-negative (LNN) breast cancers for which gene expression (all HG-U133_A platform) and survival data (distant metastasis free survival time) were available resulting in a combined data-set of 523 samples. These datasets comprise: GSE11121 (n=200) [11], GSE2990 (n=125), selection of LNN-untreated patients out of the n=189 total cohort [12], and GSE7390 (n=198) [13]. In the combined cohort, 387 patients (74%) remained relapse free for a distant metastasis (median follow-up time of 124 months), 404 patients were ER-positive (77%) and 119 ER-negative (23%). Additional clinical characteristics were reported in the respective studies. To significantly reduce the level of inter-experimental variation and minimize possible biases in the cohorts, these datasets were first combined by batch mean centering as described [14]. Data of the complete cohort were again used for hierarchical clustering to divide the samples in a high and low TIL group.

Breast cancer molecular subtypes

Samples were assigned to groups according to gene modules as described by Desmedt et al. [15] using the Bioconductor R package genefu [16]. The resulting 4 groups represent the ER-/HER2-, HER2+, and ER+/HER2- tumours, were the ER+/HER2- group is subdivided in a high and low proliferating group. These molecular subtypes are reported to correspond well with clinicopathological characteristics [15].

Statistical analysis

All cohorts consist of LNN patients, who did not receive systemic (neo-) adjuvant therapy. This makes a pure prognostic analysis possible. Distant metastasis-free survival was used as endpoint and log-rank tests were used to test the equality of survivor functions. Kaplan-Meier curves were used to plot differences in survival times. Stata v13 (StataCorp, College Station, Texas, USA) was used and 2-sided p-values of < 0.05 were considered significant.

Results

Association of TILs with prognosis

We used a gene expression immune infiltrate signature for assessment of general tumour inflammatory cell infiltrate. This signature was found by differential gene expression analysis of breast cancer samples with low and high TIL percentages based on pathological review. To investigate the putative prognostic role of local tumour inflammatory cell infiltrates, the immune infiltrate signature was used to assign samples to high and low infiltrate groups in a large cohort of primary lymph-node negative sporadic breast cancers who did not receive systemic (neo-)adjuvant therapy (n=344). Figure 1A shows the hierarchical clustering of mean centered, standardized gene expression data for these 344 samples. Figure 1B and C show a detailed close-up of a H&E-stained slide of breast cancer samples with low and high TIL breast cancer sample respectively. A significant association between distant metastasis-free survival (MFS) and infiltrate group was observed; high TIL is associated with better survival with a Cox proportional hazards ratio (HR) of 0.58 (95% Confidence Interval (CI) 0.38 - 0.88), p-value 0.009. Figure 2A.

To validate our findings, we performed similar analyses in a combined dataset consisting of three public datasets of primary operable, lymph node-negative breast cancers for which gene expression (all HG-U133_A platform) and survival data are available (n=523). Again, a significant association between MFS and infiltrate group was observed, the HR was 0.62 (95% CI 0.42 - 0.92), p-value 0.016, Figure 2B.

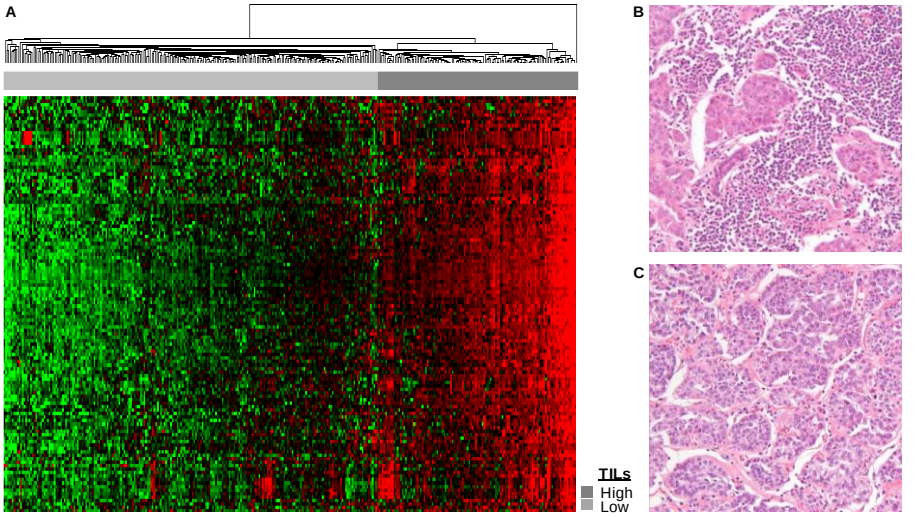

Figure 1. High and Low TIL sample assignment. A, Hierarchical clustering of standardized gene expression values of the immune infiltrate gene expression signature in breast cancer samples from the 344 samples discovery data-set. Sample assignment is as follows: dark-grey marks high-TIL samples, light-grey marks low-TIL samples. Figures **B** and **C** show high-magnification close-ups (100X) of H&E stained slides of a high and low TIL sample respectively. TILs can be seen as small round dark stained cells amidst the cancerous tissue.

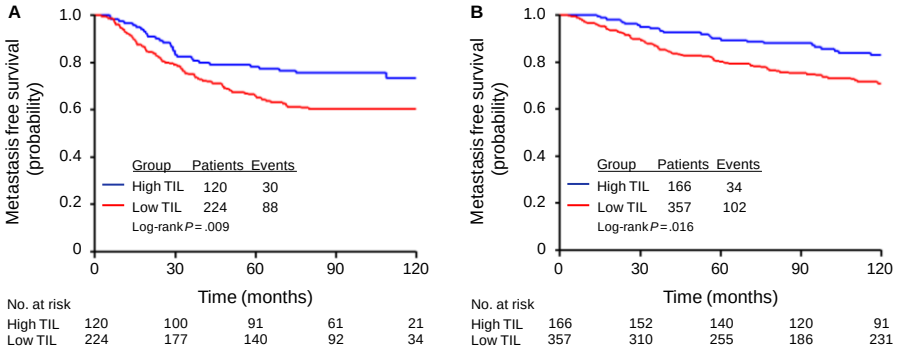

Figure 2. Prognostic ability of the immune infiltrate gene expression signature in LNN breast cancer. Kaplan-Meier curves of estimated metastasis free survival **(A)** for all patients in the discovery data-set. (**B)** for all patients in the validation data-set (3 public data-sets combined). A significant association between prognosis and TILs was found in both data-sets.

Association of breast cancer subtypes, TILs and prognosis.
To investigate the relationship between molecular subtypes, TILs, and prognosis, all samples of both discovery and validation cohorts were assigned to four groups according to gene modules as described by Desmedt et al. [15]. The resulting groups represent ER-/HER2-, HER2+, and ER+/HER2- (high and low proliferating groups) breast tumours. Distant metastasis-free survival was again used as endpoint to assess the prognostic value of the mRNA immune infiltrate signature (high and low TIL group) within these groups. Table 1 shows the grouping of TIL among the molecular subtypes, which is not random according to the Chi-sq test ($p<0.001$).

Table 1. Grouping of high and low TIL breast cancer samples among molecular subtypes in the combined data-sets.

mRNA Cluster	Subtype				
	ER+/HER2- High	ER+/HER2- Low	ER-/HER2-	HER2+	Total
High	73 (90.7)	67 (96)	83 (60.4)	63 (38.9)	286
Low	202 (184.3)	224 (195)	100 (122.6)	55 (79.1)	581
Total	275	291	183	118	867

Observed and expected (in brackets) numbers of high and low TIL samples amongst the different molecular subtypes. High TIL samples are found more than expected in the ER-/HER2- and especially in the HER2+ subtype. Chi-square p-value <0.001.

Samples with a high TIL immune infiltrate signature are found more than expected in the ER-/HER2- and especially in the HER2+ subtype. Kaplan-Meier survival curves in the subgroups showed a similar trend in all breast cancer subtypes and a significant difference in the HER2+ group ($p<0.001$). This finding is supported by a significant interaction P test results between TILs and the HER2 molecular subgroup: 0.003. Figure 3 shows the Kaplan-Meier curves for all groups. All HRs are shown in Table 2.

As the HER2+ group contains both ER- and ER+ samples, we examined the distribution of ER- and ER+ samples amongst the HER2+ high and low TIL groups. According microarray expression levels [17], the HER2+ high TIL group consists of 59% ER- and 41% ER+ samples, the HER2+ low TIL group contains 51% ER- and 49% ER+ samples.

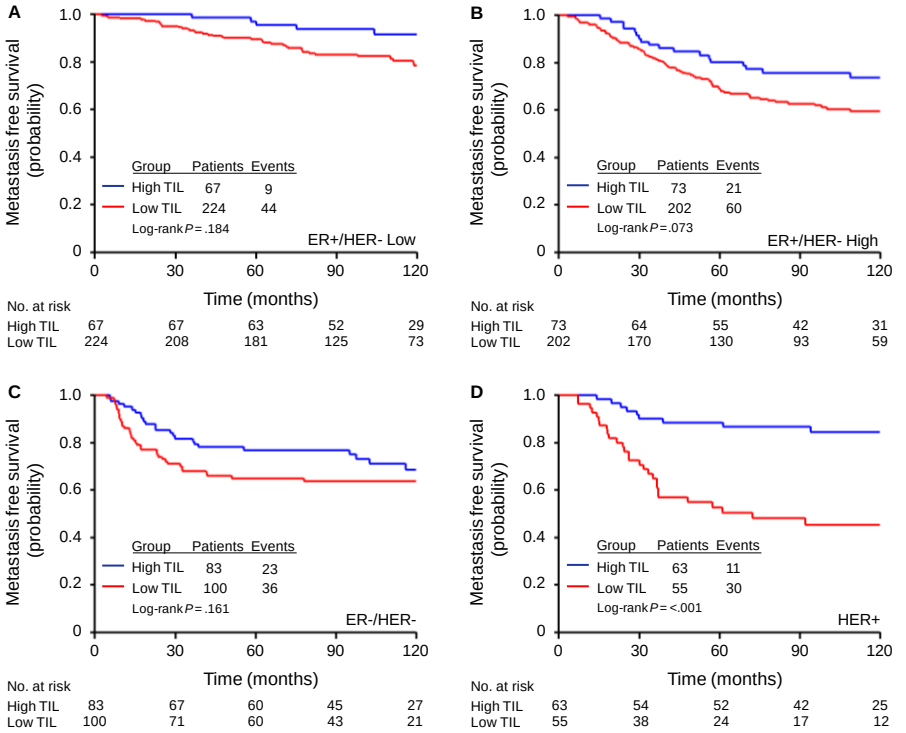

Figure 3. Prognostic ability of the immune infiltrate gene expression signature for molecular subtypes in the combined (discovery and validation) breast cancer data-set. Kaplan-Meier curves of estimated metastasis free survival **(A)** for the ER+/HER- low proliferation group, (**B)** for the ER+/HER- high proliferation group, **(C)** for the ER-/HER- group and **(D)** for the HER+ group. A significant association between prognosis and TILs was found for the HER2+ molecular subgroup.

Table 2. Prognostic value of the immune infiltrate gene expression signature for molecular subtypes in the combined data-sets.

Molecular group	HR	95% CI	p-value
HER2+	0.24	.12 to .47	<.001
ER+/HER2- High	0.64	.40 to 1.04	.073
ER+/HER2- Low	0.61	.30 to 1.26	.184
ER-/HER2-	0.69	.41 to 1.16	.161

The table summarizes the hazard ratio (HR), 95% confidence interval (CI), and log-rank test *P* values of univariate Cox proportional hazards regression between TIL groups and breast cancer molecular subtypes.

Discussion/Conclusion

In this study, using 867 lymph node-negative breast tumour samples in total, we found that an immune infiltrate gene expression signature used as a measure of general tumour inflammatory cell infiltrate, confirms that high numbers of tumour infiltrating lymphocytes are associated with good prognosis. This was particularly evident for the HER2+ tumour subgroup. As the HER2+ group is a mixture of ER+ and ER- breast cancer samples we examined whether ER status could be the underlying causative factor associated with prognosis. This hypothesis was rejected as ER+ and ER- cases were observed in near equal numbers amongst the HER2+ high and low TIL groups.

The strength of this study is that the mRNA signature was derived from TIL counts, which are generally highly heterogeneous distributed, determined on full face frozen tissue sections which are more likely to be representative for the whole tumour as compared to core biopsies or tissue microarrays used in earlier studies [18]. Furthermore, using a mRNA-based immune infiltrate signature as a standardized tool to assess the amount of tumour infiltrating lymphocytes (TILs), enabled us to combine large public datasets thereby increasing power for analyses.

Our findings partly contrast, but mostly agree with micro-array experiment based observations made by other groups: were we find TILs to be associated with good prognosis in all tumour subgroups, most notably in the HER2+ group, a quantitative microarray based analysis by Calabro et al. reported an association between high TIL and improved survival in patients with ER- tumours and worse survival in patients with ER+ tumours [19]. Rody et al. [20], found that a T-cell metagene predicts a favorable prognosis in ER- and HER2+ breast cancers in a combined cohort of 12 independent data-sets. Furthermore, Teschendorff et al. [21] showed that an immune response gene expression module identifies a good prognosis subtype in ER- breast cancer, which they claim to be a heterogenous group of breast cancers. However, a direct comparison between our results and these reports is not easily made as our analyses are restricted to lymph node-negative patients who did not receive any systemic adjuvant therapy, whereas the above mentioned cohorts also include lymph-node positive patients and varying treatment regimes. Indeed, in the study by Teschendorff et al. lymph node status itself was the most significant predictor of distant metastasis in univariate Cox regression analysis. The patients in our cohort follow a more natural course of disease as compared to these other studies,

in which treatment may be considered a confounding factor and could explain the contrasting observations.

In this study, high levels of TILs are found to be significantly associated with breast cancer subtypes, samples with a high TIL signature are observed more than expected in the ER-/HER2- and especially in the HER2+ subtype. This is in line with the hypothesis that these poorly differentiated, genomically instable breast cancer subtypes, might be more antigenic. In these tumours, large numbers of tumour associated antigens could stimulate a strong antitumour response by the host's immune system [1,22]. Of importance for HER2+ tumours, a recent study demonstrated that TILs from a patient with metastatic cholangiocarcinoma contained CD4+ T helper 1 (T(H)1) cells recognizing a specific mutation in erbb2 interacting protein (ERBB2IP) expressed by the cancer [23].

The presence of large numbers of TILs in the ER-/HER2- and HER2+ subtype also indicates that proficient T-cell activition, expansion and recruitment has taken place. However, generating an anti-tumour immune response is a multi-step process that is executed by effector T cells that can recognize and kill tumour targets. Multiple strategies are employed by tumours to attenuate the effectiveness of T-cell-mediated attack interfering with nearly every step required for effective immunity. Tumours can deregulate antigen-presenting cells, establish a physical barrier at the vasculature that prevents homing of effector tumour-rejecting cells and suppress effector lymphocytes through the recruitment and activation of immunosuppressive cells such as myeloid-derived suppressor cells, tolerogenic monocytes, and T regulatory cells [24].

Partially confirming our findings, in a recent phase III randomized adjuvant breast cancer trial in lymph node-positive breast cancer patients, higher lymphocytic infiltration was found to be significantly associated with the ER-/HER2- breast cancer subgroup only, but not with the HER2+ breast cancer subgroup. However, a significant association between increasing lymphocytic infiltration and magnitude of benefit with particular chemotherapy regimens was shown for HER2+ breast cancer. As HER2 is a potent tumour antigen, it has been suggested that immuno-editing is vital for HER2+ breast cancers to escape host immune-mediated elimination [25]. The larger amount of immunogenic cell death under specific chemotherapeutic regimens could be critical for successful treatment outcome. Nevertheless, it is unclear what makes the contrasting observation between the prognostic value of TILs in HER2+ lymph node-positive [25] and HER2+ lymph node-negative breast cancers in our study.

It is suggested that the aforementioned host immune antitumour response may not affect primary tumour regressions, but rather that adaptive memory could play a role in preventing recurrence [22]. Furthermore, a role of the immune system in controlling metastatic processes in breast cancer is well supported [26]. The presence of TILs at diagnosis could indicate that adaptive immunity has been generated in the patient. If this adaptive immunity is established before the primary tumour has metastasized, it could stall tumour progression and prevent recurrence and thereby lead to better overall survival. In contrast, this effect is not seen if metastases has occurred before adaptive immunity is established, explaining the differences seen between HER2+ lymph node-negative and positive tumours.

In conclusion, we found that high levels of tumour inflammatory cell infiltrates as determined by gene expression analysis, are frequently observed in ER- and HER2+ breast tumours, and are associated with better metastasis-free survival In lymph node-negative primary breast cancer, especially in HER2+ breast cancer patients.

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