Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Investigating the Correlation Between Long-Term Response in Patients with Metastatic HER2+ Breast Cancer and the Activity of Regulatory T Cells: A Retrospective Study

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Background: Trastuzumab is commonly utilized in the management of metastatic HER2-positive breast cancer. Our main goal was to examine the clinical outcomes and immune markers of patients who received trastuzumab and chemotherapy treatment.

Methods: Between 1995 and 2012, a total of 98 patients diagnosed with metastatic HER2-positive breast cancer were retrospectively analyzed at Ege University Hospital and Tepecik Training and Research Hospital. The clinicopathological characteristics and clinical outcomes of the patients were assessed, and the associations between response rates, survival and the immune profiles of tumor infiltrating lymphocytes were statistically evaluated.

Results: The average age of patients at the time of diagnosis was 50.1±10.3 (ranging from 30 to 79) years. The mean follow-up period for all patients was 97.9±53.8 months. Among the patients, complete response was observed in 24.5%, partial response in 61.2%, and stable disease in 8.2% of cases. The average progression-free survival was 50.3±26.9 months (ranging from 1 to 163 months), and the average overall survival was 88.8±59.4 months (ranging from 12 to 272 months). After analyzing all cases, it was found that patients who were younger (p=0.006), exhibited higher CD3-positivity (p=0.041), presented with higher FOXP3-positivity (p=0.025), showed complete or at least partial response to treatment (p=0.008), and experienced a long-term response to trastuzumab (and chemotherapy) treatment had longer survival (p=0.001).

Conclusion: Patients with HER2-positive breast cancer, who initially respond positively to palliative trastuzumab and chemotherapy treatment, can achieve long-term tumor remission lasting for several years.

Keywords: metastatic breast cancer, trastuzumab, forkhead box p3, long-term response

Introduction

About 20% of breast cancer patients develop metastases, and in cases of metastatic breast cancer, prognostic factors including visceral organ involvement, time since the first diagnosis, and hormonal receptor status play a significant role.[1] Among these receptors, HER-2 overexpression occurs in approximately 15% of patients. This overexpression is linked to a less favorable prognosis.[2] Trastuzumab, a monoclonal antibody targeting the HER-2 receptor, triggers antibody-dependent cellular cytotoxicity by binding to the HER-2 receptor on tumor cell surfaces. The inclusion of trastuzumab in chemotherapy has led to noteworthy improvements in response rates and survival outcomes.[3–5] Although the majority of patients with metastatic breast cancer treated with trastuzumab-based regimens progress within one year,[6,7] few patients experience long-term remission.[8,9]

Received: 6 June 2024
Accepted: 12 September 2024
Published: 27 September 2024

Median overall survival was 25 months in the first reported trial of trastuzumab in advanced disease,[10] and exceeded 37 months in a recent study.[7]

Some of these women remain alive and disease-free after five to ten years from the diagnosis of metastases, which leads to hypothesize that cure could be possible in a small subset of patients. Long-term survivor (LTS) was defined as overall survival (OS) being equal or greater than 5 years, and non-long-term survivor (NLTS), if OS was less than 5 years.[11] Studies in patients with extended survival after first-line trastuzumab define long-term response on the basis of progression-free survival (PFS) or overall survival. Moreover, these studies have selected different cut-offs to define PFS and OS, either 2, 3 or 5 years.[9,11–16] This 2-year cut-off was considered to be sufficiently long to distinguish an adequate proportion of patients with long-term response to trastuzumab with respect to the PFS and OS observed in the overall population treated with trastuzumab.[6,10,17] While most of these studies described the features of a single long-term response group, few of them have compared long-term and short-term responders[11,12] Limited data, usually from case reports or studies with a small number of patients, have been published on the long-term effects of trastuzumab treatment.[18–20] A number of studies have been carried out to explore the connection between the clinical and molecular disparities and the long-term response and survival of patients undergoing combination chemotherapy with trastuzumab.[12,21]

Genetic and immunological factors have been the primary focus of exploration, with genetic studies uncovering prevalent mutations such as PI3K activation, PTEN loss, and PI3KCA mutation in HER-2 positive breast cancer patients, obtaining different clinical responses to the combination of trastuzumab and chemotherapy.[12,22,23] These studies, however, have faced challenges in establishing a clear association between these genetic mutations and clinical outcomes due to constraints related to tissue samples and the intricacy of the interrelation.

HER-2-positive breast cancer is categorized as an immune-infiltrating cancer, akin to triple negative breast cancer, characterized by the prevalence of type 1 T cells.[24] The process of antigen presentation is augmented by cytokine release and activates antigen-presenting cells. The presence of Th1-type cytokines such as IL-2 and IFN-gamma synergistically elicits cytotoxic T lymphocytes (CTL) and facilitates NK-cell-mediated regression of cancer cells.[25] Notably, the majority of tumor-infiltrating lymphocytes (TILs) are represented by prominent CD8 T cells, which serve as the primary effector cell type and are linked to a favorable prognosis. Meanwhile, Type 2 CD4+ T helper cells encompass forkhead box p3 (FOXP3) CD4+ regulatory T cells. Infiltration of Foxp3 T cell or PD-1 T cell indicates impaired immunity and a poorer prognosis.[26] The presence of Th2 type cytokines like IL-4 and IL-10 inhibits Th1 and CTL cells and supports Treg within the tumor, thereby promoting tumor growth.[27] While the correlation between total TILs and clinicopathological characteristics and clinical outcomes in breast cancer remains inconclusive, isolated studies suggest that highly differentiated breast cancers lacking hormone receptors and expressing HER-2 are correlated with higher TIL levels, although this requires further investigation due to limited case data and incomplete detail.[28,29]

Furthermore, chemotherapy has been discerned to induce immunogenic cell death, chiefly by elevating the release of IL-2 and IFN-gamma, consequently enhancing the permeability of tumor cells to granzyme B and rendering them susceptible to CTL-mediated lysis even in the absence of CTL-recognizable antigens. Additionally, chemotherapy exhibits the potential to hinder immunosuppressive cytokines such as IL-4, IL-10, and IL-13 while fostering antitumor immunity.[30] Several studies have indicated that the presence of tumor-infiltrating lymphocytes in the tumor microenvironment is a predictive indicator of the effectiveness of chemotherapy and trastuzumab.[31,32] The specific factors within these immunological mechanisms that play a role in an extended response in patients undergoing trastuzumab and chemotherapy combination treatment are not fully understood.

Our research aimed to examine the clinical outcomes and immune markers of patients who received trastuzumab and chemotherapy treatment.

Material and Method

Clinicopathological Features

The study included patients diagnosed with HER-2 positive metastatic breast cancer at Ege University Hospital and Tepecik Training and Research Hospital from 1995 to 2012, who underwent combination therapy with trastuzumab and chemotherapy. Inclusion criteria stipulated an age range of 18 to 80, HER-2 positive metastatic breast cancer, absence of

autoimmune diseases, non-use of immunosuppressive drugs, and long-term responders (2 years or more) for the treatment group; short-term responders (less than 2 years) were designated as the control group. Exclusion criteria comprised diagnosis of a second primary tumor. This descriptive study retrospectively evaluated demographic data and medical information of 98 cases with metastatic stage HER-2 positive breast carcinoma treated at these institutions from 1995 to 2012. The cases were further assessed regarding tumor type and grade, lymphovascular and perineural invasion, lymph node involvement, location of metastases and treatment modalities. The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the local Hospital Ethics Committee.

Immunohistochemical Examination

The paraffin block chosen for immunohistochemical (IHC) evaluation was deemed most suitable Slides with 5-µm sections were obtained and then subjected to overnight heating at 60°C, followed by dewaxing in xylene and hydration with decreasing concentrations of alcohol. All slides underwent heat-induced epitope retrieval in the microwave. This involved a 20-minute treatment with EDTA buffer at pH 8.0 for the FOXP3 antibody and sodium citrate buffer at pH 6.0 for the CD3 antibody. Subsequently, the slides were cooled at room temperature for 20 minutes and then blocked to recover endogenous peroxidase and biotin. The IHC tests were manually conducted using the streptavidin-biotin peroxidase method (Invitrogen, Camarillo, 85–9043, USA). Purified monoclonal mouse antibodies against FOXP3 (Anti-FOXP3) antibody (236A/E7) (ab20034, at 1/ 300 dilution) and against CD3 (Anti-CD3 antibody, ab5690, at 1/100 dilution) were utilized. The Anti-CD3 antibody was employed for assessing T lymphocytes in tumor microenvironments. Two pathologists, unaware of the patients' clinical characteristics, carried out the histopathological assessments. The immune reactivity for FOXP3 was evaluated using a scoring system. The FOXP3 positive lymphocytes in the tumor microenvironment were enumerated, and the positivity rates were classified as <20 cells/high-power field (HPF) for low and ≥20 cells/HPF for high. Additionally, all CD-positive T lymphocytes infiltrating the tumors were counted in each high-power field. A dense infiltration was considered present if there were 50 or more CD3-expressing T lymphocytes in a HPF (Figure 1). A positive HER-2 status was defined as 3+ immunohistochemistry staining or 2+ immunohistochemistry staining, along with in situ fluorescence hybridization positivity (FISH, HER-2/CEP17 ratio>2.2). With IHC staining, estrogen receptor staining of 10% or more defines hormone-positive disease, staining between 1% and 10% indicates weak hormone-positive disease, and staining below 1% defines hormone-negative disease.

Statistical Analysis

Statistical analysis was performed using SPSS 25.0 statistical package. Quantitative data were compared using the chi-square test in the statistical analysis. Nonparametric data, on the other hand, were compared using the Mann–Whitney *U*-test. The comparison of measurements across more than 2 groups involved the utilization of the nonparametric Kruskal–Wallis test. Overall Survival (OS) was the defined duration between the initiation of first-line trastuzumab and the date of death from

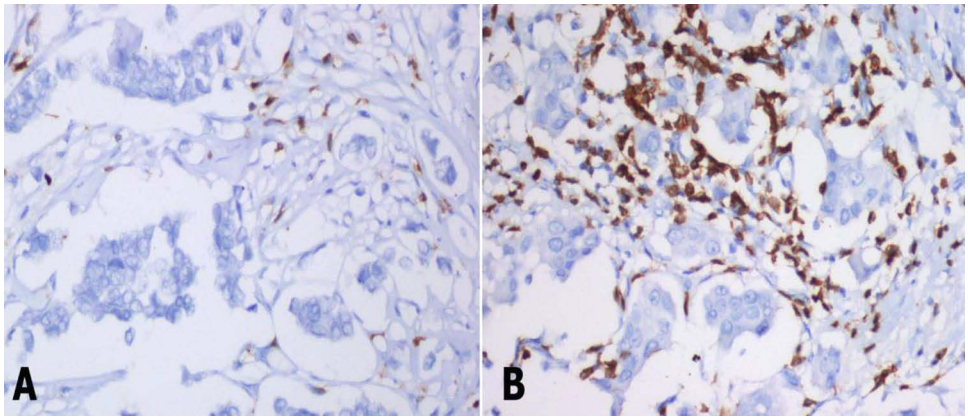

Figure 1 (**A**) A sample of low CD3-positive T cell infiltrations (DAB x 200). (**B**) There was a high density of CD3-positive T cells (DAB x 200).

breast cancer or last visit if the patient is still alive. Progression-free survival (PFS) was the duration between the initiation of first-line trastuzumab and the first progression and/or death. For those still alive, their status was assessed and noted as of January 2023. Long-term responders (LTR) were defined as patients with non-progressive disease for at least 2 years on first-line trastuzumab. Long-time survivors were patients who survived for over 5 years (60 months). The estimation and comparison of OS and PFS were carried out using the Kaplan–Meier analysis along with the Log rank test. Covariates assessed for association with PFS and OS were utilized in the construction of both logistic regression and Cox proportional hazard models, employing forced entry and forward stepwise methods. The multivariable Cox proportional hazards model was then used to determine adjusted hazard ratios (HR), with a significance level set at $P < 0.05$.

Ethics Statement

Ethics committee approval was received from Çanakkale Onsekiz Mart University Faculty of Medicine/Turkey for the conduct of the research on 02.03.2016 (Clinical Research Ethics Committee). Patients provided written consent so that their samples and clinical data could be used for investigational purposes.

Results

All 98 patients included in the study were diagnosed with stage IV metastatic HER2-positive breast cancer. The average age of these individuals was 50.1±10.3 years, ranging from 30 to 79 years, with 51% being postmenopausal. The mean follow-up duration was 97.9±53.8 months (12–237.4 months). Out of the patients, 26.5% survived, while 73.5% had succumbed at the time of data analysis. The distribution of tumor localization was as follows: 45 cases in the right breast, 50 in the left, and 3 cases (3.1%) had bilateral breast tumors. The surgical procedures performed included breast-conserving surgery with axillary dissection in 34 patients (35%), modified radical mastectomy with axillary dissection in 29 patients (29.6%), tumor excision in 33 patients (%33.4) and toilet mastectomy in 2 patients (%2). The predominant type of tumor was invasive ductal carcinoma (76.5%), with 4.1% classified as grade 1, 57.1% as grade 2, and 38.8% as grade 3. Axillary lymph node dissection was carried out in 73.5% of cases, revealing lymph node metastasis in 65.2% of these situations. Among cases with lymph node metastasis, capsular invasion was present in 75.5% of them. Metastases were predominantly found in bone and soft tissue (n = 50; 51%), liver (n = 20; 21%), and lung (n = 28; 29%). Brain metastases were present in only 5% of documented patients (n = 5) (Table 1). In the clinical response analysis of metastatic first-line treatment, it was shown that 24.5% of patients had a complete response, 61.2% had a partial response, and 14.3% had stable disease or progressive disease. Long-term response was observed in 75% of patients (n:74). Kaplan Meier Survival analyses identified a statistically significant relationship between overall survival and complete response to treatment, with a log-rank p-value of 0.008 (Figure 2). The mean progression-free survival (PFS) was 50.3±26.9 months (1–163 months), and the mean overall survival (OS) was 88.8±59.4 months (12–272 months). A 68% of the patients (n=67) survived for more than 5 years, while 25% (n=24) survived for more than 10 years. A correlation was found between younger age and improved overall survival (p=0.006) (Figure 3).

In the immunohistochemical examinations, ER-positivity was identified in 67 cases (68.3%) and PR-positivity in 51 cases (52%). In 68 cases (69.4%), either ER or PR positivity was confirmed. C-erbB2 positivity rate ranged from 20% to 100%, with a mean of 76.1% ± 22.7%, while the Ki-67 proliferation index ranged from 15% to 90%, with a mean of 34.7% ± 22. Evaluation of immune profiles of TILs could be conducted in only 39 cases, all of which exhibited CD3 expressed T lymphocytes and at least one FOXP3 positive regulatory T cell (TREG) in the TILs. In a subgroup of 39 cases, CD3 and FOXP3 staining was performed (Table 2). Fourteen patients (35.8%) were classified as living for more than 5 years, and 28 patients (71.7%) were identified as having a long-term response. Statistical analysis revealed that long-term survivors had higher levels of CD3 expressed T cells and a denser presence of FOXP3 positive TREGs within the TILs, with both demonstrating statistical significance. Conversely, there was no association observed between long-term response and CD3 or FOXP3 expressions.

The results of the Cox proportional hazards model, displaying the impact of various parameters on LTR-associated outcomes, are summarized in Table 3. Upon conducting statistical analyses, it was observed that factors including menopausal status (p=0.925), visceral/non-visceral involvement (p=0.710), presence of local recurrence (p=0.668), adjuvant chemotherapy (p=0.189), adjuvant radiotherapy (p=0.255), adjuvant hormonotherapy (p=0.792), and type of

Table 1 Demographic and Histopathologic Features of Patients

Parameters	Status	N	%
Prognosis	Survived	26	26.5
	Exited	72	73.5
Tumor Location	Right	45	45.9
	Left	50	51
	Bilateral	3	3.1
Diagnosis	IDC	75	76.5
	ILC	4	4.1
	IDC and ILC	5	5.1
	Invasive Papillary Carcinoma	4	4.1
	Other histologic variants	10	10.2
Grade	Grade 1	4	4.1
	Grade 2	56	57.1
	Grade 3	38	38.8
Type of Surgery	Breast conserving surgery with axillary dissection	34	35
	Modified radical mastectomy with axillary dissection	29	29.6
	Tumor excision	33	33.4
	Toilet mastectomy	2	2
Adjuvant Radiotherapy	Yes	51	52.0
Axillary lymph node dissection	Yes	72	73.5
Therapy response	Complete response	24	24.5
	Partial response	60	61.2
	Stable disease	8	8.2
	Progressive disease	6	6.2
Non-visceral organ metastasis	Lymph node	48	49
	Skin (cervical and abdominal location)	3	3
	Bone	41	42
	Soft tissue	9	9
Visceral organ metastasis	Lung	28	29
	Liver	20	21
	Brain	5	5
Histopathologically confirmed lymph node metastasis	Present	47	47.9
Capsular invasion in the metastatic lymph nodes	Present	37	37.7
Lymphovascular invasion	Present	38	38.8

Abbreviations: IDC, Invasive Ductal Carcinoma; ILC, Invasive Lobular Carcinoma.

surgery (excision/breast preservation/radical mastectomy p=0.715) did not demonstrate significance. Notably, in cases showing long-term response, only complete and partial responses to the initial multidisciplinary treatment were deemed significant influencers of survival (p=0.008). It is interesting to note that out of 24 patients displaying a complete response to the first-line treatment, 17 were alive (70.8%), while out of 57 patients with a partial response, only 16 were alive (28%). Overall, individuals who were younger (p=0.006), exhibited higher CD3-positivity (p=0.041), (Figure 4) displayed elevated FOXP3-positivity (p=0.025) and demonstrated complete or partial response to treatment (p=0.008), as well as long-term response to trastuzumab treatment (p=0.001), were associated with longer survival (Figure 5).

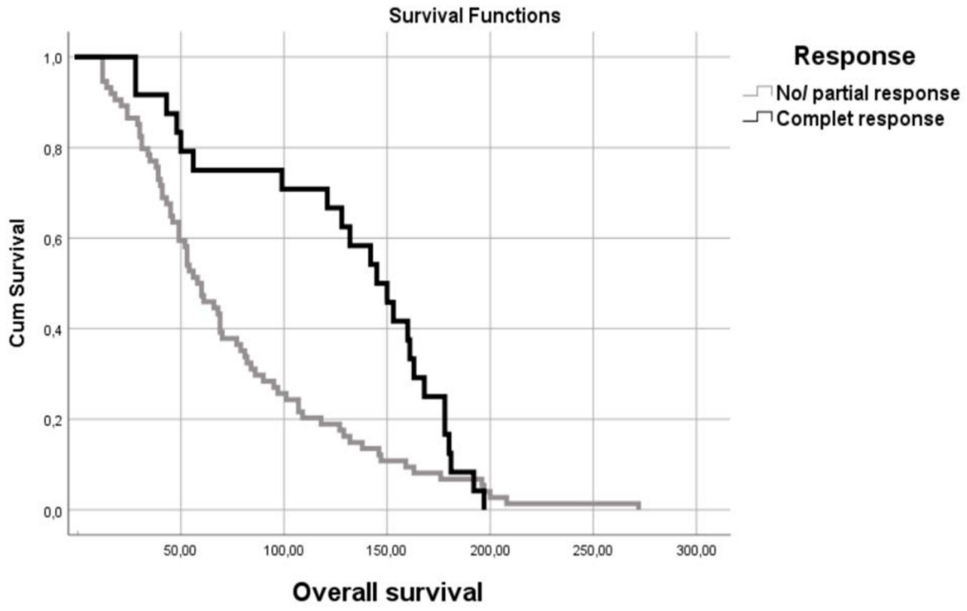

Figure 2 The survival curve according to the response to first-line trastuzumab treatment (Log rank, p=0.008).

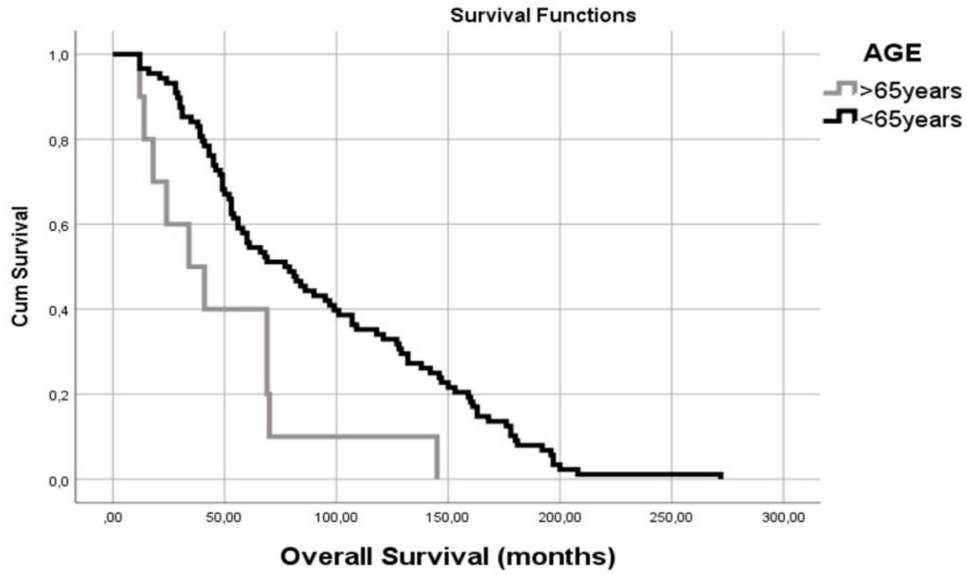

Figure 3 The survival curve according to the age of patients (Log rank, p=0.006).

Discussion

The prognosis of metastatic breast cancer patients significantly depends on HER-2 positivity. Effective chemotherapy and trastuzumab treatment have shown to increase survival rates in HER-2 positive breast cancer patients.[33] Pivotal Phase III trials assessing first-line trastuzumab treatment have reported PFS of around 7–11 months and OS of 25–30 months.[10] While most patients with metastatic breast cancer respond transiently to conventional treatments, the majority develop evidence of progressive disease within 12 to 24 months of first-line therapy.[34,35] However, a subset of patients continue to respond well to trastuzumab for an extended period. Limited research has explored whether the clinicopathological characteristics of metastatic HER-2 positive breast cancer patients with a long-term response to trastuzumab differ from those without adequate response.[36]

Table 2 Immunohistochemical and Molecular Findings

Parameters	Status	N	%
ER status	Positive	67	68.3
PR status	Positive	51	52.0
ER and/ or PR positivity	Yes	68	69.4
C-erbB2 expression	2+ 3+	9 89	9.2 90.8
HER2 amplification (by FISH)	Positive	10	10.2
Ki67 proliferation index	15–29 ≥30	81 17	82.7 17.3
CD3 expressed T cells (in 39 cases)	Low High	18 21	46.2 53.8
FOXP3 expression (in 39 cases)	<20/ HPF ≥20/ HPF	28 11	72 28

Abbreviations: ER, Estrogen Receptor; PR, Progesterone Receptor; HER2 (also known as c-erbB-2), Human Epidermal Growth Factor Receptor 2; CD3, Cluster of Differentiation 3; FOXP3, Forkhead box p3.

Table 3 Cox Proportional Hazards Model of LTR Patients (N=53, 54.1%)

Parameters Method: Enter	HR (95% CI)	p value
Age 65+years <65 years	 Reference 2.7 (1.3–5.3)	 0.004
Complete response Yes No	 Reference 0.058 (0.028–0.121)	<0.001
Trastuzumab treatment >2 years <2 years	 Reference 0.078 (0.018–0.337)	0.001
Hormone receptor-positive Positive Negative	 Reference 1.2 (0.729–2.048)	0.444
CD3-positive cells Low High	 Reference 1.025 (0.984–1.067)	0.241
FOXP3-positive cells Low High	 Reference 0.964 (0.881–1.056)	0.434

Abbreviations: LTR, Long-term response; CD3, Cluster of Differentiation 3; FOXP3, Forkhead box p3.

In this study, 24.5% of patients experienced complete response, while 61.2% had a partial response. The average PFS was 50.3 months, and the average OS was 88.8 months across all patients. A 75% of the patients (n:74) showed a long-term response. Those who live for more than 5 years constitute 54%, and those who live for more than 10 years constitute 31%. In patients with long-term response, the average PFS was 57.3 months, and the average OS was 97 months. Among patients with a long-term response,

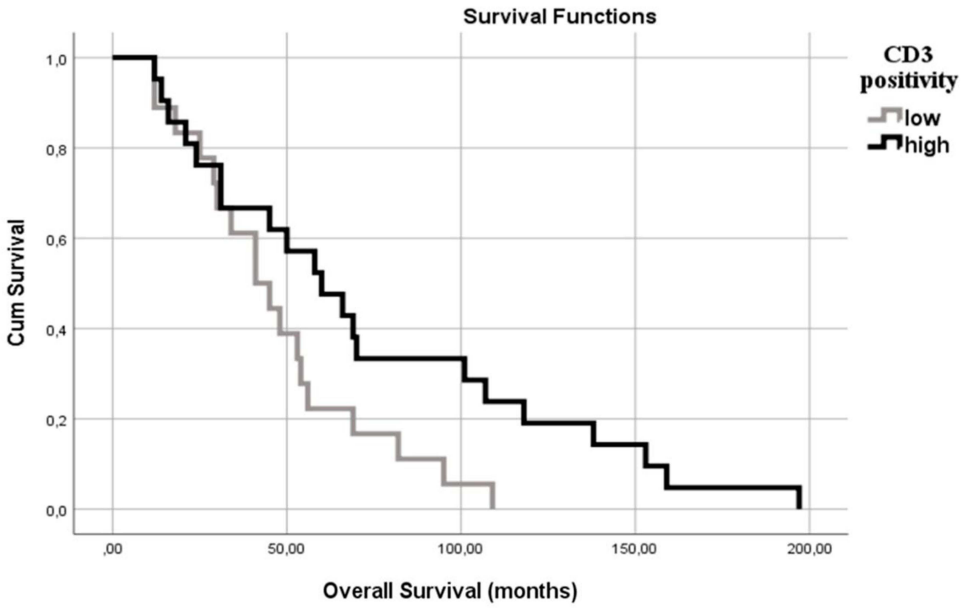

Figure 4 The survival curve according to the presence of CD3-positive T cells (Log rank, p=0.041).
Abbreviation: CD3, Cluster of Differentiation3.

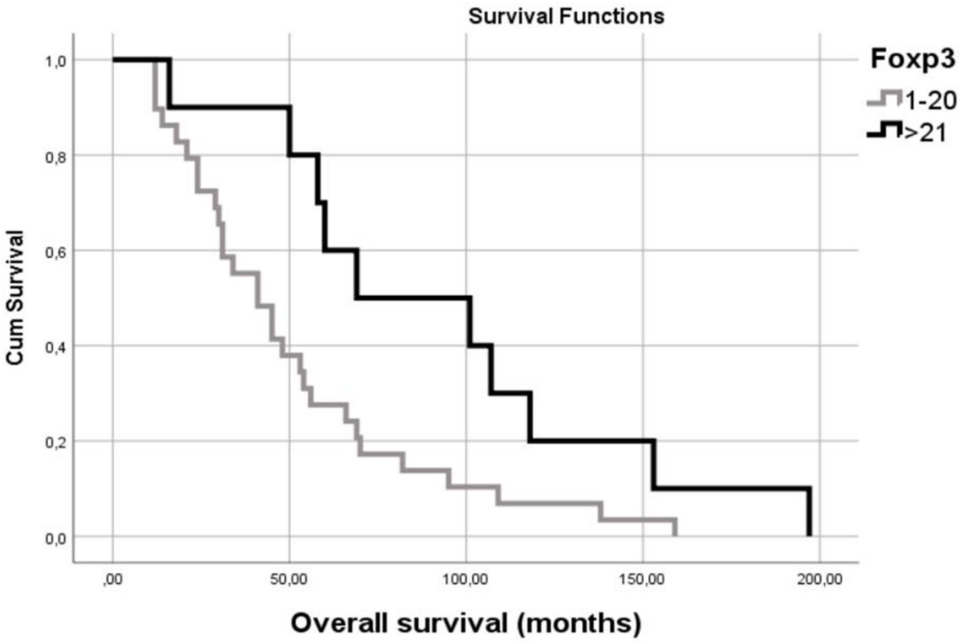

Figure 5 The survival curve according to the presence of FOXP3-positive T cells (Log rank, p=0.025).
Abbreviation: Foxp3, Forkhead boxp3.

70.8% achieved complete response and 28% had a partial response during the 98-month follow-up. Those with the longest survival among long-term responders were those who achieved complete response and younger individuals. In a study by Witzel et al, the median PFS was 54 months with an average follow-up period of 41 months. In patients who achieved long-term remission, the complete response rate was 37.8% and the partial response rate was 32%.[13] A similar aspect was observed in our study, where long-term responses and complete responses were noted. Survival increased in young patients who achieved a complete response. There have been rare cases in the literature where patients with complete responses have reached survival times of over 20 years.[37,38] Notably, liver metastases were found to be a prevalent site of metastasis, with 25% of long-term responder group patients having liver metastases, showing no correlation with LTR status. This is in contrast to 35% of LTR

patients in the LHORA study and 32.8% of LTR patients in the HER-OS study.[16] However, the distribution of metastatic locations did not show significant differences among patients with long-term responses in this study. Some studies have indicated improved clinical outcomes for patients with non-visceral metastases, contrary to the findings of the present study.[39] No correlation was found between hormone receptor (HR) positivity and LTR status in this study. The conclusions of relevant studies are conflicting. While some studies have reported long-term response and survival for patients with HR-positive and HER-2 positive tumors, contradicting studies have shown that patients with HR-negative and HER-2 positive tumors exhibited better response and survival.[40,41] Montemurro et al highlighted a weaker response to trastuzumab plus chemotherapy in the presence of high HR positivity (more than 30% of cancer cells) compared to the response rate observed in tumors with lower or absent HR expression.[42] These varying results suggest that HR positivity may play a role in the different anti-HER-2 responses and sensitivity to chemotherapy.

In long-term follow-up studies of breast cancer patients with HER-2 positive status, researchers have explored the impact of immunological parameters, either in relation to or independent of clinical characteristics.[25] While limited literature is available on the integration of the immune system in treatment response, preclinical data suggests that the immune system may be involved in the processes of antibody-dependent cellular immunity via natural killer cells and cytotoxic T lymphocytes, both of which are crucial in the mechanism of trastuzumab action.[33] Although components of innate immunity seem to play a primary role, adaptive immune cells have been observed to actively contribute to prolonged treatment efficacy. Studies indicate that regulatory T cells, through the secretion of TGF-Beta and IL-10, inhibit the antitumor effect by affecting CD4, CD8, natural killer cells, and dendritic cells. Clinical studies have revealed that in patients with locally advanced invasive breast cancer, a higher rate of complete pathological response was achieved through neoadjuvant therapy characterized by lymphocytic infiltration >50% compared to those without.[31]

Additionally, improved clinical outcomes have been linked to tumors with a microenvironment containing increased CD8+ T cells.[43] Conversely, breast cancer cases featuring a dense presence of Th2 cells with Treg or FOXP3+ exhibited less favorable clinical results.[44] These findings are consistent with and substantiated by other studies.[15]

In our study, we utilized an immunohistochemical technique to conduct CD3 and FOXP3 stainings on 39 patients' pathological samples who exhibited a long-term response leading to a substantial improvement in survival rates. Among these individuals, 28 demonstrated a long-term response, while 10 did not. Through statistical analysis of clinicopathological traits and IHC findings, we observed that individuals with an extended response tended to be younger with significant infiltration of CD3- and FOXP3-positive T cells. Contrary to existing literature, our findings revealed decreased levels of FOXP3 in non-responding patients, suggesting a potential involvement of other immune system cells and cytokines in addition to Tregs in influencing cancer cells and their microenvironment. It is crucial to replicate and expand upon these findings through larger-scale studies.

Conclusion

In our study, among patients with HER2-positive metastatic breast cancer, those who were younger and had strong FOXP3 positivity showed better responses. Additionally, younger patients with long response durations achieved longer survival. Further, comprehensive research is necessary to validate immune or pathological markers that influence the patient group with extended response times and increased survival.

Patients with HER2-positive metastatic breast cancer who have long response durations and survival are rarely encountered in daily practice. The limited number of pathology blocks available for immunomarker staining among the included patients can be considered a limiting factor of our study.

Data Sharing Statement

All data generated or analyzed during this study are provided in this article. For further inquiries, please contact the corresponding author.

Statement of Ethics

All activities involving human participants adhered to the ethical standards of the Institutional Review Board of The First Affiliated Hospital of USTC and were conducted in accordance with the 1964 Declaration of Helsinki and its later amendments or similar ethical standards. Informed consent was obtained from all individuals participating in the study.

Acknowledgments

This paper has been uploaded to research square as a preprint: https://www.researchgate.net/publication/379069153.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

There are no funding sources to declare.

Disclosure

The authors all affirm that they do not have any conflicts of interest.

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