Impact of c-erb2 status on survival after high dose chemotherapy in high-risk breast cancer patients

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ABSTRACT

Objectives: To investigate the impact of c-erb2 status on survival after high-dose chemotherapy.

Methods: Between March 1997 and June 2004, a total of 54 women with breast cancer who has at least 8 metastatic lymph nodes underwent high-dose chemotherapy with hematopoietic stem cell transplantation in Gülhane Military Medical School, Ankara, Turkey. Archival specimens were analyzed by fluorescent in situ hybridization to determine the impact of c-erb2 status after peripheral blood stem cell transplantation on survival. The patients were divided into c-erb2 negative (n=20) and positive (n=11) groups.

Results: No statistically significant differences were detected between c-erb2 negative and positive groups regarding 5-year disease-free survival (41 and 27%, log rank p=0.11), and overall survival (60 and 45%, p=0.33). Transplant related mortality did not differ between groups.

Conclusion: We found no differences between c-erb2 negative and positive groups regarding disease-free and overall survival. To clarify the value of the c-erb2 status in predicting outcome after high-dose chemotherapy, prospective randomized studies are needed.

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Following conventional cytotoxic chemotherapy, long-term disease-free survival (DFS) is only achieved in 15-30% of the patients with primary breast cancer who has tumor involvement in more than 9 axillary lymph nodes.[1,2] This relatively inferior survival rate in high risk primary breast cancer justifies the conduction of experimental studies including high-dose chemotherapy (HDC) supported by autologous stem-cell transplantation. The results from randomized controlled studies that explored the use of marrow-supported HDC as treatment for metastatic or high risk primary breast cancer, with the possible exception of the most recent Dutch study,[3] has not supported the concept of marrow-supported high-dose therapy as consolidation results in survival advantage, which was confined to c-erb2 negative subgroup of patients. The human epidermal growth factor receptor 2 (HER2) has gained increasing clinical importance over the last few years, particularly in breast cancer, not just as potential prognostic or predictive factor but also as a target for tumor biologic therapy.[4,5] Thus, this study was designed to clarify the value of the c-erb2 status in predicting outcome after HDC.

Methods. A total of 54 patients underwent HDC and peripheral blood stem cell transplantation (PBSCT) between March 1997 and July 2004, according to a study design evaluating the status of HDC in high-risk primary breast cancer patients. Patients with surgical diagnosis of breast carcinoma, with at least 10 metastatic lymph nodes, without distant metastasis proven by physical examination, abdominal ultrasounography, and bone scintigraphy, with good performance status (ECOG ≤1), with white blood cell count over 4000/mm^3 and platelet count over 100000/mm^3, and with no liver or hepatic dysfunction, underwent PBSCT after 4 cycles of induction chemotherapy. Written informed consent was obtained from all patients, and the study was approved by the local ethics committee. To determine the impact of c-erb2 status after PBSCT on survival, their archival specimens were analyzed by fluorescent in situ hybridization

(FISH). Patients whose archival specimens were not available for analysis were excluded from this analysis (n=23). The patients were divided into c-erb2 negative (n=20) and positive (n=11) groups.

Induction chemotherapy. Four cycles of CEF (cyclophosphamide 600 mg/m^2 on day one, epirubicin 75 mg/m^2 on day one, 5-fluorourasil 600 mg/m^2 on day one, every 21 days) were administered after healing of surgical wounds.

Stem cell mobilization and apheresis. Recombinant human granulocyte- colony stimulating factor (G-CSF) (Filgrastim, Neupogen, Roche, Basel, Switzerland) was given at a total dose of 10-15 µg/kg/day twice-daily subcutaneous injections, beginning 14 days after the completion of the last cycle of induction chemotherapy, and continuing until the completion of apheresis. Using a continuous flow cell separator (COBE Spectra, Lakewood, CO, USA), the leukapheresis procedure was performed with a 3-way central catheter on days 4, 5 or 6. Harvested autologous plasma was mixed with dimethylsulfoxide (DMSO) to yield a final DMSO concentration of 5%. The final suspension was transferred into freezing bag and frozen to -100°C using a computerized freezing device (R 201 Planar) and then stored in liquid nitrogen at -196°C following standard methods as described before.[6] The CD34+ cells in the leukapheresis product were enumerated by flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany) using direct CD34+ immunoflourescence. A minimum of 1x10^6 mononuclear cells were incubated for 30 min at 4°C with fluorescence conjugated to fluorescein isothiocyanate (Becton Dickinson, Heidelberg, Germany). The gated percentage of CD34+ cells was multiplied by the absolute count of mononuclear cells in the apheresis product to yield the absolute CD34+ cell count for each apheresis.

High-dose chemotherapy regimens. Conditioning regimens included CNV (n=28): Cyclophosphamide 2.4 g/m^2, mitoxantrone 35 mg/m^2, etoposide 250 mg/m^2/day times 6 days; ICE (n=1): Ifosfamide 2.5 g/m^2/day times 6 days, carboplatin 250 mg/m^2/day times 6 days, etoposide 250 mg/m^2/day times 6 days; TCM (n=1): Thiotepa 250 mg/m^2/day times 2 days, melphalan 50mg/m^2/day times 2 days, carboplatin 450 mg/m^2/day times 3 days; CNP (n=1): Cyclophosphamide 60 mg/kg/day times 2days, mitoxantrone 35mg/m^2, carboplatin 200 mg/m^2/day times 6 days.

Stem cell transplantation (SCT) and posttransplant supportive therapy. At the time of transplantation (day 0), stem cell bags were quickly thawed at bedside and immediately infused intravenously through a central catheter. After transplantation, 19 patients received recombinant human G-CSF at a dose of 5 µg/kg/day once a day by intravenous route; 7 patients received recombinant human granulocyte-monocyte colony stimulating factor (Molgramostim, Leucomax; Sandoz-Schering-Plough Laboratories, Paris, France) at a dose of 5 µg/kg/day once daily by intravenous route; the remaining 5 received no growth factor (GF). The GF administration continued until leukocyte counts exceeded 1x10^9/L for 3 consecutive days. Single donor thrombopheresis was performed as needed to keep the platelet number above 10x10^9/L. Erythrocyte transfusion was performed to keep the hemoglobin level above 8 g/dL. All blood products were irradiated (2000 cGy) and transfused via leukocyte filter. Fever was defined as fever over 38.3°C or fever over 38°C lasting at least one hour. Patients were discharged if leukocyte count greater than 1x10^9/L, neutrophil count greater than 0.5 x10^9/L, and platelet count grater than 50 x10^9/L on 3 consecutive days. Posttransplant hospitalization duration was documented as the period elapsed from reinfusion to discharge.

Posttransplant management and follow-up. All patients underwent locoregional radiotherapy (chest wall or breast parenchyma; axillary, parasternal, and peripheral lymph nodes) as soon as possible after HDC. Radiation therapy was delivered daily in 2 Gy fractions 5 times per week to a cumulative dose of at least 50 Gy. After completion of chemotherapy and radiotherapy, patients with hormone receptor positive tumors were prescribed tamoxifen 20 mg per Os daily for 5 years. They were checked with physical examination, hemogram, routine biochemistry, chest roentgenography at least every 4 months, with mammography, abdominal ultrasonography, and bone scintigraphy every year.

C-erb2 overexpression determined by FISH. Unstained, 4 µm thick, formalin fixed, paraffin-embedded breast cancer sections were mounted onto plus slides (Fisher Scientific, Pittsburg, PA) and processed using the Ventana chromosome in situ hybridization kit (Ventana Medical Systems, Tucson, AZ) on the Ventana Gen (Ventana Medical Systems) automated in situ hybridization instrument. After deparaffinization in xylene, transfer through 2 changes of 100% ethanol, and rinsing in aqua distilled slides were placed on the Ventana Gen instrument. The slides were incubated for 30 minutes in 30% Ventana pretreatment solution at 45°C followed by 45 minutes in 30% Ventana Protein Digesting Solution at 45°C. Ventana Unique Sequence Digoxigenin-labeled HER2 DNA probe was prewarmed for 5 minutes at 37°C before manual application. The amount of probe hybridization mixture was calculated relative to the target area (10 mL of probe mixture per 22 x 22 mm^2 of tissue area). Denaturation was performed at 69°C for 5 minutes

before slides were incubated overnight at 37°C with the hybridization probe. After overnight hybridization and 3 posthybridization stringency washes, fluorescein-labeled antidigoxigenin detection reagent was manually applied for 28 minutes at 37°C. After removal of the slides from the instrument, each slide was counterstained with 18 mL of propidium iodide antifade (1:2) and covered with a glass coverslip. Slides were evaluated for HER2 gene copy numbers using a Zeiss Axioskop 50 fluorescence microscope at a magnification x100. Scoring of amplification was performed as follows: the probe displays a single fluorescent for each HER-2/neu gene copy. The expected number of HER2 spots per normal or unampflified tumor cell is 2, or 4 in dividing cells. A minimum of 100 tumor cells were evaluated in each specimen. Tumors were considered amplified for HER2 gene when at least 20 cells displayed 5 or more spots per cell.

Statistical analysis. Mann-Whitney's U test was used for comparison of distribution of values for unpaired series such as age, number of patients enrolled, time to transplantation, number of previous chemotherapy cycles, number of total nucleated cells, number of CD34+ cells harvested, time to leukocyte and platelet engraftment, number of febrile days, number of days with parenteral antibiotherapy, number of days in hospital, number of erythrocyte and platelet units transfused. Chi-square test was used for comparison of categorical variables such as gender, history of previous radiotherapy, preparative regimens and type of malignancy, hormone receptor status, family history of breast cancer, type of growth factor used in the posttransplant period, CD34+ selection, menopausal status, and presence of lymphatic invasion in tumor. If the expected frequency in table cells was under 5 or total sample size was under 20, Fisher's exact test was used. All p values are based on 2-sided tests. The p value was considered statistically significant if <0.05.The main end points for the comparison of both groups were DFS and overall survival (OS). Disease-free survival was calculated from transplantation to the initial appearance of a relapse of disease or to death from any cause. Data on patients known to be alive and without a relapse at the time of an analysis were censored at the time of their last follow-up visit. Overall survival was calculated from transplantation to death from any cause; data on patients known to be alive at the time of an analysis were censored at the time of their last follow-up visit. The Kaplan-Meier method was used to estimate curves for disease-free and OS, and comparisons were made with use of log-rank test. Statistical analysis was performed using statistical software (SPSS for Windows, version 9.0, SPSS Inc., USA).

Results. The characteristics of patients in c-erb2 negative (n=20) and c-erb2 positive (n=11) groups are shown in **Table 1**. Both groups were comparable regarding the age, number of metastatic axillary lymph nodes, number of previous chemotherapy cycles, time to transplantation, number of patients with history of breast cancer in family, and tumor characteristics.

Supportive care requirements and engraftment kinetics. Blood cultures revealed coagulase negative *Staphylococcus aureus* at 9 cases, *Escherichia coli* (*E.coli*) at 2 cases, meticilline resistant *Staphylococcus aureus* at 3 cases, *Pseudomonas aeruginosa* at 2 cases, both *Staphylococcus aureus* and *Pseudomonas aeruginosa* at one case. Between c-erb2 negative and positive groups there were differences regarding leukocyte and platelet engraftment, number of febrile days, hospitalization duration and supportive care requirements (**Table 2**).

Transplant related mortality and long-term complications. One patient died of *E. coli* sepsis on the third day of transplantation. On 23th day one patient died of myocardial infarct. The transplant

Table 1 - Characteristics of patients.

Characteristics	C-erb2 negative	C-erb2 positive	*P* value
No. of patients	20	11	
Age (years)			
Median	43.6	43	0.982*
Min-max	27.4 - 66.6	29.1 - 55.7	
Tumor size (cm)			0.306*
Median	2.5	3,7	
Min-max	0.6 - 10	1.1 - 8	
No. of metastatic nodes			0.275*
Mean	15.7	17.6	
Min-max	8 - 30	9 - 33	
Mean of metastatic/total node ratio	0.73	0.80	0.334*
Interval between diagnosis and tx (days)			0.825*
Median	131	131	
Min-max	105 - 282	102 - 265	
No. of total chemotherapy cycles			0.938*
Mean	4	4	
Min-max	4 - 7	4 - 7	
No. of patients with family history of breast cancer	4	2	1†
No. of premenapousal patients	14	7	0.633†
No. of patients with CD34 selection	15	4	0.281†
CD34 number (x10^6/kg)			0.233*
Mean	3.49	5.11	
Min-max	1.25 - 7.14	1.712 - 20.45	
Apheresis (no of patients)			0.121‡
1 section	16	10	
2 sections	4	-	

* Mann Whitney U test, † Fisher Exact test, ‡ Pearson Chi-square test

related mortality did not differ between groups. Three patients suffered from vaginal bleeding, which caused the discontinuance of tamoxifen at one case.

Disease-free survival and overall survival. For all patients (n=54), the median duration of follow-up was 1244 days (min-max: 5-3336). The 5-year OS rate was 53.9%. After 5 years, 45.7% of patients were disease-free. After a median follow-up of 41.4 months, 8 patients died in the c-erb2 negative group whereas 7 patients died in the c-erb2 positive group. Between the c-erb2, negative and positive groups there was no difference regarding the projected OS rates at 5 years (60 and 45% log rank p=0.33) (**Figure 1**). Current health statuses of patients are shown in **Table 3**. Between the groups there was no difference regarding projected DFS rates at 5 years (41 and 27% log rank p=0.11) (**Figure 2**).

Discussion. The cochrane collaboration published the results of a meta-analysis of 13 prospective randomized trials (ACCOG 2004,[7] GALGB 2005, Dutch pilot 1998,[8] Dutch Intergp 2003,[3] ECOG 2003,[9] GABG 2004,[10] MDACC 2000,[11] PEGASE 01 2003,[12] ICCG 2005,[13] IBCSG 2003, JGOG 2001, MCG 2001,[14] and WSG 2003). In total, 2535 eligible woman were randomized to receive HDC with autograft and 2529 were randomized to receive conventional treatment. There was a statistically significant benefit in event-free survival (EFS) for woman in the high dose group at 3 years (RR 1.12 [95% confidence interval (Cl), 1.19]) and at 4 years (RR 1.30 [95% Cl, 1.16, 1.45]). At 5 and 6 years there was no statistically significant difference

Table 3 - Current health status of patients in C-erb2 negative and positive groups.

Patient's status	C-erb2 negative (n=20) n (%)	C-erb2 positive (n=11) n (%)
Exitus	8 (40)	7 (64)
Alive with relapse	2 (10)	2 (18)
Alive without relapse	10 (50)	2 (18)

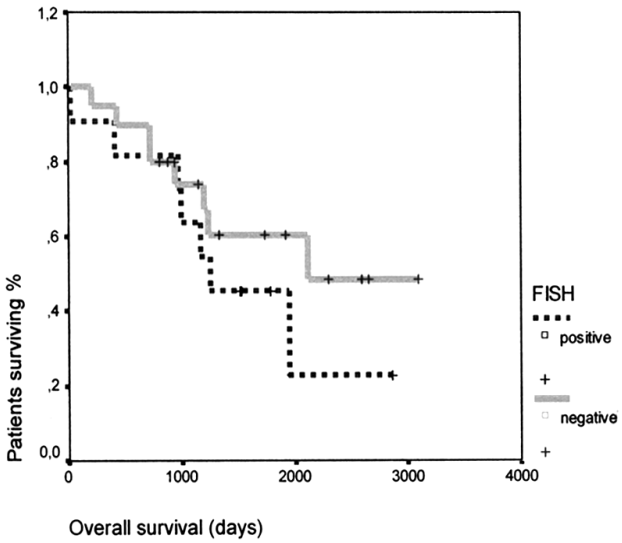

Figure 1 - Overall survival rates of c-erb2 negative and positive groups. Fish - fluorescent in situ hybridization

Table 2 - Data on supportive care requirements and engraftment kinetics of c-erb2 negative and positive groups.

Parameters	C-erb2 negative	C-erb2 positive
Growth factor (no of patients)		
G-CSF	13	6
GM-CSF	5	2
No GF	2	3
Leukocyte engraftment (mean day ± SD)	10.63 ± 2.03	11.73 ± 3.0
Platelet engraftment (mean day ± SD)	12.11 ± 3.78	12.09 ± 2.66
Erythrocyte transfusion (unit)	2.95 ± 2.01	2.36 ± 1.21
Platelet transfusion (unit)	0.85 ± 0.88	0.64 ± 0.81
Febrile days (mean ± SD)	5.0 ± 3.31	3.82 ± 2.09
Hospitalization (mean ± SD)	12.88 ± 3.03	13.90 ± 3.5

G-CSF - granulocyte colony stimulating factor,
GM-CSF - granulocyte-monocyte colony stimulating factor

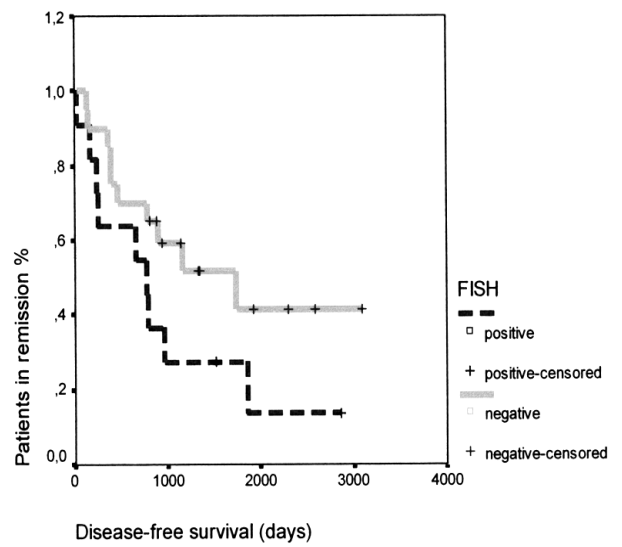

Figure 2 - Disease-free survival rates of C-erb2 negative and positive groups. Fish - fluorescent in situ hybridization

between the groups in EFS. With respect to OS, there was no statistically significant difference between the groups at any stage of follow-up.[15] Randomized studies addressing the value of HDC in high-risk breast cancer indicate that if HDC proves to benefit patients with breast cancer, it is quite likely that this benefit will be limited to a subgroup of patients. Most studies addressing the value of c-erb2 as a predictive factor unite in the conclusion that c-erb2 overexpression is associated with high relapse rates. Zemzoum et al[16] evaluated the independent clinical relevance of HER2 status in lymph node negative breast cancer patients who had no adjuvant systemic therapy and were followed for more than 10 years. Although HER2 amplification and HER2 expression did not reach significance for DFS, they were significant for OS, even in the multivariate analysis in this study (HER2_AMP: p=0.004; RR, 3.7; 95% CI, 1.5 - 9.2; HER2_EXP: p=0.009; RR, 3.4; 95% CI, 1.4 - 8.7).[16] Nieto et al[17] performed a retrospective analysis of 146 patients who were previously enrolled at another program onto clinical trials of HDCT for 4 - 9 involved axillary lymph nodes, for more than 10 involved axillary nodes, or inflammatory carcinoma. All patients received the same HDC regimen, with cyclophosphamide, cisplatin, and carmustine (STAMP-I), followed by autologous stem-cell transplantation. Median follow-up was 42 months (range 5 - 90 months). They found that positivity for c-erb2 was significantly associated with increased risk of relapse and death.[17]

In a study of Bitran et al,[18] 25 patients with more than 10 positive axillary nodes were treated with 6 cycles of standard-dose chemotherapy (5-FU, doxorubicin and cyclophosphamide) followed by HDC (2.5 g/m^2 cyclophosphamide for 3 days and thiotepa 225 mg/m^2 for 3 days) with autologous stem cell support. They reported that 4 patients relapsed systemically between 6th and 18th months and that all 4 patients who relapsed had breast cancers that overexpressed c-erb2. They concluded that patients with HER2 overexpression appear to be at a high risk for relapse, even when treated with HDC and SCT (p=0.00004).[18] Rodenhuis et al[3] randomized 885 patients with at least 4 tumor positive axillary lymph nodes to high-dose group (n=442) and to conventional-dose group (n=443). They found that HDC improves relapse-free survival in the group with 10 or more positive nodes. In the c-erb2 negative subgroup (n=620), relapse-free survival was significantly longer after HDC than after conventional-dose chemotherapy (hazard ratio for relapse, 0.66; 99% Cl, 0.46 - 0.94; p=0.002). Reportedly there was a trend toward an OS benefit after HDC (p=0.07). Patients with c-erb2 positive tumors (n=181) in the high-dose group had a higher frequency of relapse than those in the conventional-dose group, though the difference was not statistically significant.[3]

Studies mentioned above are difficult to compare because of the differences in the methods (FISH versus Immunohistochemistry [IHC]) and assay variability, particularly concerning the types of anti-c-erb2 antibodies used (monoclonal versus polyclonal). Even studies with the same antibody type are difficult to compare because of the arbitrary definition of positive staining. Shortly IHC for c-erb2 has not been standardized yet. In our study, we used FISH method which has been claimed to be most accurate technique to evaluate c-erb2. We found no significant differences between c-erb2 negative and positive groups regarding OS (60 versus 45%, log rank p=0.33) and DFS (41 versus 27%, log rank p=0.11). Relapse characteristics were not different too. Although not significant statistically, OS and DFS seems to be superior in patients with c-erb2 negative tumors. Patients with c-erb2 positive tumors have a worse prognosis. This result is in accordance with the studies mentioned above. It can be concluded that the prognosis of patients with c-erb2 positive tumors is so unfortunate that they do not benefit even from HDC.

In conclusion, it is necessary to perform prospective randomized studies with more patients and longer follow-up to clarify the value of the c-erb2 status in predicting outcome after HDC.

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