Research article

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C-*myc*, not *HER-2/neu*, can predict recurrence and mortality of patients with node-negative breast cancer

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Abstract

Background: At present, node-negative, high-risk breast cancer patients cannot be identified with sufficient accuracy. Consequently, further strong prognostic factors are needed.

Methods: Among 181 node-negative breast cancer (NNBC) patients, c-*myc* and *HER-2/neu* oncogenes were identified prospectively using double differential PCR. The possible impact of amplification of those oncogenes on disease-free survival (DFS) and overall survival was examined. Furthermore, the possible effects of adjuvant therapies on rate of recurrence and mortality in oncogene-amplified NNBC patients were investigated.

Results: The prevalence rates for amplification of c-*myc* and *HER-2/neu* were 21.5% and 30.4%, respectively. On univariate analysis, c-*myc*-amplified NNBCs were associated with significantly shorter DFS at 36 months after the initial diagnosis (85.3% versus 97.3%). As compared with nonamplified cancers, *HER-2/neu*-amplified NNBCs did not exhibit any significant differences after 36 months and 95 months. Multivariate analysis indicated that c-*myc* amplification and tumour size, in contrast to *HER-2/neu* amplification, oestrogen receptor status, grading and age, were the only independent parameters for DFS. During the period of observation, we found no evidence for an impact of amplification of the oncogenes on overall survival in all cases. With respect to various adjuvant systemic therapies such as chemotherapy (cyclophosphamide, methotrexate, 5-fluorouracil; fluorouracil, epirubicin, cyclophosphamide) and endocrine therapy (tamoxifen), no significant differences were identified in oncogene-amplified NNBC patients in terms of DFS and overall survival. However, those c-*myc*-amplified NNBC patients who did not receive adjuvant systemic therapy exhibited significantly shorter DFS and overall survival as compared with c-*myc*-nonamplified patients.

Conclusion: C-*myc* amplification appears to be a strong prognostic marker with which to predict early recurrence in NNBC patients. C-*myc*-amplified NNBC patients without adjuvant systemic therapy experienced shorter DFS and overall survival.

Keywords: amplification of oncogenes, c-*myc*, disease-free survival, *HER-2/neu*, mortality, node-negative breast cancer, overall survival, recurrence

Introduction

Breast cancer is the most common form of cancer in women from developed countries. Axillary lymph node status is widely accepted as an important parameter for assessing prognosis in breast cancer patients. However, recurrence and death also occur in patients with node-negative breast cancer (NNBC). A recurrence rate of 30% may be expected during the first 5 years after diagnosis. Prognostic factors such as tumour size, tumour grading, hormone receptor status, age, histology, ploidy and proliferation index are used to define subgroups of high-risk NNBC patients [1–8]. Despite the availability of these prognostic markers, high-risk NNBC patients cannot be identified with sufficient accuracy. This has led to a search for new and possibly stronger prognostic markers in order to define new subgroups and to facilitate decision-making with respect to appropriate therapy [2,3,9].

AGCN = average gene copy number; CART = Classification and Regression Trees; CMF = cyclophosphamide, methotrexate, 5-fluorouracil; DFS = disease-free survival; NNBC = node-negative breast cancer; FEC = fluorouracil, epirubicin, cyclophosphamide; OS = overall survival; PCR = polymerase chain reaction.

Numerous reports have described the correlation between amplification of oncogenes and its impact on the course of breast cancer disease [4–7,10–32]. In a meta-analysis in which 29 studies were evaluated [33], c-*myc* amplification exhibited significant but weak associations with tumour grade, lymph node metastasis, negative progesterone receptor status and postmenopausal status. Furthermore c-*myc* amplification was significantly associated with risk for recurrence and death. However, studies in recent years have further shown that the c-*myc* gene participates in most aspects of cellular function, including replication, growth, metabolism, differentiation and apoptosis [34]. Amplification of the oncogene *HER-2/neu* has also been shown to be indicative of poor prognosis in breast cancer. Studies revealed that the prognostic effect of *HER-2/neu* is stronger for survival than for recurrence [16,19,20,22, 27,29]. Furthermore, increased HER-2/neu levels in primary tumours were associated with a poor response to endocrine therapy [5,12,15,32].

A drawback of many studies of oncogenes in human breast cancer is that usually only one oncogene was evaluated. Based on a series of unselected cases, in the present study we examined the possible influence of the amplification of the oncogenes c-*myc* and *HER-2/neu* on disease-free survival (DFS) and overall survival (OS). Furthermore we studied whether adjuvant therapies such as chemotherapy and endocrine treatment or no treatment at all had any impact on DFS and OS among oncogene-amplified NNBC patients [1–3,8,9,32,35–38].

Patients and methods

Among 181 NNBC patients who had undergone breast-conserving therapy or modified mastectomy combined with axillary lymphadenectomy of level 1 and 2 (at least 10 lymph nodes per patient had been removed), c-*myc* and *HER-2/neu* oncogenes were assessed prospectively using double differential PCR.

Table 1 shows some clinical, histological and molecular parameters. The median follow-up period was 42 months. Postoperatively, the following therapies were administered in addition to radiotherapy: tamoxifen 20 mg/day for 5 years in 54.7% of patients; and chemotherapy (six cycles of cyclophosphamide, methotrexate, 5-fluorouracil [CMF] or four cycles of fluorouracil, epirubicin, cyclophosphamide [FEC]) in 33.7% of patients. Of the NNBC patients, 11.6% did not receive any further systemic therapy.

After tissue preparation, malignant and normal tissues were kept fresh and transported to the pathologist (U.B.). The pathologist dissected samples for assessment of oncogenes and hormone receptors. A positive receptor status was defined as the presence of more than 10 fmol/mg cytosol protein. The histopathological grading was performed according to the method of Bloom and Richardson [39]. Lymph node sections were stained with haemotoxylin and eosin; immunohistochemical investigations were not performed. The tumour tissues were stored at −70°C and the DNA was isolated using the Fast Prep System (Bio 101 Savant, Savant Instruments, Inc., Holbrook, NY, USA). Less than 200 mg (10–200 mg) tissue was homogenized using FastDNA Kit in the Fast Prep Machine. After preparing the DNA, the content was measured and the isolated DNA was stored at −20°C. General details for the double differential PCR technique, reproducibility and clinical significance were described previously [17,18,28,40]. A cutoff point of more than 2.0 average gene copy number (AGCN) was considered to be positive for *HER-2/neu* amplification [40]. To calcu-

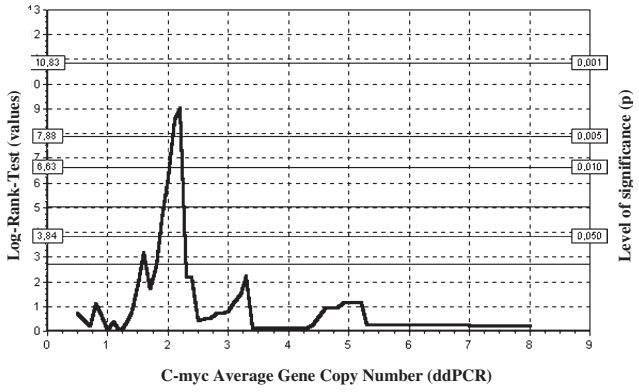
Table 1

Clinical, histological and molecular parameters of 181 node-negative breast cancer patients

Parameter	Number of patients (%)
Tumour size	
T1	118 (65.2)
T2	54 (29.8)
T3/4	9 (5.0)
Age (years)	
<40	14 (7.7)
40–55	46 (25.4)
>55	121 (66.9)
Histopathological grading	
G1	5 (2.8)
G2	105 (58)
G3	71 (39.2)
ER status	
ER negative	64 (35.4)
ER positive	115 (63.5)
Unknown	2 (1.1)
PR status	
PR negative	66 (36.5)
PR positive	112 (61.9)
Unknown	3 (1.7)
Oncogenes	
C-*myc* nonamplified	142 (78.5)
C-*myc* amplified	39 (21.5)
HER-2/neu nonamplified	126 (69.6)
HER-2/neu amplified	55 (30.4)
C-*myc* and *HER-2/neu* coamplified	22 (12.2)

ER, oestrogen receptor; PR, progesterone receptor.

Figure 1



Classification and Regression Trees (CART) analysis of c-*myc* among 181 node-negative breast cancer patients. dd, double differential.

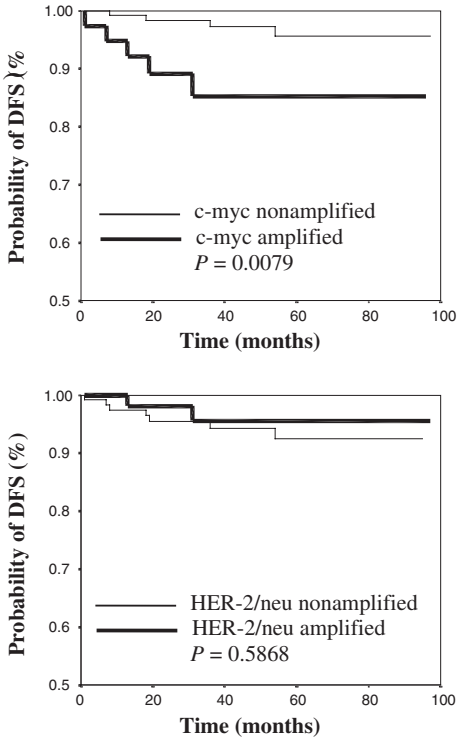
late the cutoff point for c-*myc* amplification, all NNBC patients were subjected to Classification and Regression Trees (CART) analysis. A cutoff value of 2.1 AGCN for c-*myc* oncogene led to the best distinction between patients. Fig. 1 shows the CART analysis for all c-*myc* values [41].

Results were evaluated using the SPSS system (SPSS GmbH Software, Munich, Germany). The monoparametric survival curves were determined using the Kaplan–Meier method in order to estimate the impacts of intratumoural c-*myc* and *HER-2/neu* oncogene amplification on DFS and OS. Statistical deviations were defined using the log-rank test. Recurrence of disease was found at the following locations: local ($n = 2$), contralateral ($n = 1$), axilla ($n = 2$), lung ($n = 1$), brain ($n = 1$), liver ($n = 1$) and skin ($n = 1$). During the period of observation, 14 patients died. In order to derive relevant information regarding the effects of oncogene amplification on the course of breast cancer disease, the accumulated values were determined after postoperative periods of 36 and 95 months. We applied the multivariate Cox model to enable us to identify independently predictive parameters [42]. Parameters considered included the oncogenes c-*myc* and *HER-2/neu*, tumour size, histopathological grading, oestrogen receptor status and age (<40 or ≥ 40 years). $P < 0.05$ was considered statistically significant.

Results

The prevalence rates for amplification of c-*myc* and *HER-2/neu* were 21.5% and 30.4%, respectively. Coamplification was found in 22 patients (12.2%; Table 1). Among those patients with c-*myc* amplification five recurrences were identified (5/39 [12.8%]), and among those with *HER-2/neu* amplification two recurrences (2/55 [3.6%]) were identified. Among those patients in whom neither c-*myc* nor *HER-2/neu* were amplified four recurrences were found (4/109 [3.6%]), and among those with coamplification of both oncogenes two recurrences (2/22 [9.1%]) were observed. Four patients with c-*myc* amplification (4/39 [10.3%]) and three patients with *HER-2/neu* amplification (3/55 [5.5%]) died. Nine patients who lacked amplification of either oncogene (9/109 [8.3%]) and two patients with coamplification (2/22 [9.1%]) died.

Figure 2



Kaplan–Meier estimation of disease-free survival (DFS) among 181 node-negative breast cancer patients.

Using univariate analysis, c-*myc*-amplified cancers were associated with a significantly lower DFS of 85.3%, as compared with 97.3% ($P = 0.0290$) among c-*myc*-nonamplified breast cancers. Ninety-five months after diagnosis (operation), the estimated DFS of c-*myc*-amplified cancer patients was only 85.3%, as compared with 95.6% among c-*myc*-nonamplified patients ($P = 0.0079$). Comparison of nonamplified cancers with *HER-2/neu*-amplified cancers did not reveal any significant differences with regard to DFS (Fig. 2).

Multivariate analysis revealed that c-*myc* amplification and tumour size, in contrast to oestrogen receptor status, grading and age, were the only independent parameters impacting on DFS (Table 2). With regard to OS, no independent parameters were identified among the prognostic markers referred to above.

Table 2

Tumour parameters and disease-free survival in 181 node-negative breast cancer patients in univariate and multivariate analyses

	DFS after 36 months		DFS after 95 months	
Parameter	Univariate *P* value	Multivariate *P* value	Univariate *P* value	Multivariate *P* value
Tumour size	0.0223*	0.024*	0.0047*	0.002*
c-*myc*	0.0290*	0.013*	0.0079*	0.004*
HER-2/neu	0.2943	0.252	0.5868	0.166
Oestrogen receptor	0.6797	0.192	0.5363	0.622
Tumour grade	0.0998	0.128	0.0255*	0.663
Age < 40, ≥ 40	0.2330	0.965	0.0920	0.717

*$P < 0.05$.

Table 3

Recurrences and disease-free survival in 181 node-negative breast cancer patients after different adjuvant systemic therapies or no therapy

Subgroup		*n*	Recurrence (*n* [%])	DFS (*P*)
Chemotherapy (*n* = 61)				
c-*myc*	Amplified	19	3 (15.8)	0.1748
	Nonamplified	42	2 (4.8)	
HER-2/neu	Amplified	26	1 (3.8)	0.2552
	Nonamplified	35	4 (11.4)	
Endocrine therapy (*n* = 99)				
c-*myc*	Amplified	15	1 (6.7)	0.3325
	Nonamplified	84	2 (2.4)	
HER-2/neu	Amplified	22	1 (4.5)	0.6641
	Nonamplified	77	2 (2.6)	
No therapy (*n* = 21)				
c-*myc*	Amplified	5	1 (20.0)	0.0209*
	Nonamplified	16	0 (0)	
HER-2/neu	Amplified	7	0 (0)	0.4969
	Nonamplified	14	1 (7.1)	

*$P < 0.05$ (log-rank test). DFS, disease-free survival.

Table 4

Mortality and overall survival in 181 node-negative breast cancer patients after different adjuvant systemic therapies or no therapy

Subgroup		*n*	Mortality (*n* [%])	OS (*P*)
Chemotherapy (*n* = 61)				
c-*myc*	Amplified	19	1 (5.3)	0.7442
	Nonamplified	42	3 (7.1)	
HER-2/neu	Amplified	26	0 (0.0)	0.1042
	Nonamplified	35	4 (11.4)	
Endocrine therapy (*n* = 99)				
c-*myc*	Amplified	15	1 (6.7)	0.9846
	Nonamplified	84	6 (7.1)	
HER-2/neu	Amplified	22	2 (9.1)	0.8307
	Nonamplified	77	5 (6.5)	
No therapy (*n* = 21)				
c-*myc*	Amplified	5	2 (40.0)	0.0098*
	Nonamplified	16	1 (6.3)	
HER-2/neu	Amplified	7	1 (14.3)	0.5649
	Nonamplified	14	2 (14.3)	

*$P < 0.05$ (log-rank test). OS, overall survival.

Regarding various adjuvant systemic therapies, such as chemotherapy (CMF, FEC) and endocrine therapy (tamoxifen), no significant differences were observed with respect to DFS and OS in c-*myc*-amplified and *HER-2/neu*-amplified NNBC patients in comparison with nonamplified patients. However, significantly shorter DFS and OS were observed among c-*myc*-amplified patients who did not receive systemic adjuvant therapy. No corresponding associations were found among *HER-2/neu*-amplified NNBC patients as compared with nonamplified patients (Tables 3 and 4).

Discussion

Numerous clinical studies have proved axillary lymph node status to be the dominant factor for prognosis and prediction of DFS and OS in breast cancer patients. The fact of the matter is that even among NNBC patients 25–30% can be expected to progress or even die. The advantage

conferred by adjuvant chemotherapy or endocrine therapy was examined in randomized studies [2,3,8,9,35–38]. Because adjuvant therapies may positively impact on outcome in only around 15%, the costs of these therapies make their routine application in all NNBC patients inappropriate [1]. For this reason, predictive factors that accurately define subgroups of NNBC patients who may benefit from adjuvant systemic therapy would be a great advantage.

In the present study the oncogenes c-*myc* and *HER-2/neu* were examined with regard to their ability to predict DFS, OS and rate of recurrence, as well as mortality. All patients were randomly selected from one department (Frauenklinik Klinikum Ibbenbueren, Ibbenbueren, Germany). C-*myc* amplification was identified in 21.5% and *HER-2/neu* amplification in 30.4%. Berns and coworkers [12–14] reported amplification of c-*myc* in 20% and of *HER-2/neu* in 24% using a standard southern blot technique. In a selected high-risk cohort of NNBC patients, Roux-Dosseto *et al*. [26] applied the same method and found prevalence rates for c-*myc* and *HER-2/neu* amplification of 25% and 31%, respectively. Those oncogenes were assessed in the present study using a double differential PCR technique [17,18,40,43]. Using this method, Brandt *et al*. [17] found c-*myc* to be amplified in 19.7% and *HER-2/neu* in 16.7% of breast cancers, without consideration of nodal status; coamplification of those oncogenes was present in 7%. In the present study, simultaneous amplification of both oncogenes was observed in 12.2%. As in the present investigation, Roux-Dosseto *et al*. [26] found that c-*myc* amplification among NNBC patients was significantly associated with earlier recurrence in univariate and multivariate analyses. However, *HER-2/neu*-amplified NNBC patients did not have the same outcome. Accordingly, c-*myc* amplification appears to be an independent prognostic marker, which has greater predictive power than does oestrogen receptor status and tumour grade. As early as 1992, Berns and coworkers [11,12,14] reported that patients with c-*myc*-amplified breast cancers had an unfavourable prognosis.

The first study to mention possible prognostic importance of *HER-2/neu* gene amplification was reported in 1987 by Slamon *et al*. [27]. That study included 187 patients with NNBC and node-positive breast cancer; by univariate and multivariate analyses, it revealed that *HER-2/neu* amplification correlated very closely with shorter DFS and OS in a subgroup of 87 node-positive patients. In 1993, in an analysis of 210 patients, Press *et al*. [7] found that amplification of *HER-2/neu* correlated with unfavourable prognosis with respect to DFS.

Attempts by other investigators to confirm these findings were met with various degrees of success. Some studies claimed that *HER-2/neu* status was an independent predictive factor in the case of breast cancer, whereas other studies could not confirm this [6,14,16,19,29,30]. It is certainly of great interest to the clinician that only two out of five studies including more than 100 patients and with a follow-up period of at least 3 years attributed some prognostic value to *HER-2/neu* amplification among patients with NNBC [6,7,16,19,28]. Almost all of the studies that dealt with *HER-2/neu* status and DFS, as well as OS, showed no benefit of this oncogene in the prognosis of NNBC patients. In a survey conducted by Ravdin and Chamness [24], only one [22] of 11 studies concerning immunohistochemical overexpression of *HER-2/neu* indicated a significant result with respect to OS in multivariate analysis.

In the present study, 19 out of 39 c-*myc*-amplified patients received chemotherapy, 15 patients received endocrine therapy and five patients received no further therapy. The greatest recurrence rates were noted in the group of patients who received no therapy (20%) and in those who received chemotherapy (16%). The lowest recurrence rate of 7% was seen in patients treated with tamoxifen. All patients who received endocrine therapy ($n = 99$) were characterized by positive oestrogen and/or progesterone receptor status. The proto-oncogene c-*myc* can be upregulated by oestrogen stimulation of hormone-dependent breast cancer cells. Endocrine therapy with the antioestrogen tamoxifen can mediate the downregulation of c-*myc*, culminating in cell cycle arrest [44]. Berns *et al*. [12] reported that 38% of patients with amplification of c-*myc* or with coamplification of c-*myc* and *HER-2/neu* profited from endocrine therapy. C-*myc*-amplified patients affected by metastatic disease showed a tendency toward longer DFS with endocrine therapy, as compared with shorter DFS following second-line chemotherapy. However, the minor rate of response after chemotherapy did not correlate with OS. The poorer responses to chemotherapy among patients with c-*myc*-amplified tumours in the present study (recurrence rate 16%) may be in agreement with experimental findings in erythroleukaemia cells that the degree of cis-platinum resistance correlated directly with the level of c-*myc* expression [45].

In the present study, 26 out of 55 *HER-2/neu*-amplified patients received chemotherapy, 22 patients received endocrine therapy and seven patients received no further therapy. In total, low recurrence rates of 3.8%, 4.5% and 0% were found in the above-mentioned therapy groups, respectively (Table 3). Compared with nonamplified patients administered the same therapies, no significant differences with regard to DFS and OS were observed. In contrast to that finding, c-*myc*-amplified patients not administered adjuvant systemic therapy exhibited a significantly shorter DFS and OS than did nonamplified patients who also received no adjuvant systemic therapy. However, the therapy groups analyzed in the present study are

small, and therefore we recommend careful interpretation of the findings.

Higher recurrence rates or poor response to endocrine treatment with tamoxifen in patients with *HER-2/neu*-amplified hormone receptor positive tumours, as reported in studies in node-positive or metastatic breast cancer [12,15,32], were not observed in the present study. Although deregulated *HER-2/neu* activity can strongly stimulate cytoplasmic signalling pathways, which in turn impinge on c-*myc* at multiple levels causing its deregulated expression [46], this scenario does not appear to be active in NNBC because the recurrence rate of 22 c-*myc* and *HER-2/neu* coamplified patients was only 9%. However, among those patients in whom coamplification of c-*myc* and *HER-2/neu* was absent the recurrence rate was lower (4/109 [3.6%]). Differences in the numbers of recurrences and deaths can be accounted for by the fact that some deaths were not related to tumours.

Conclusion

C-*myc* amplification appears to represent a prognostic marker with which early recurrence may be predicted in NNBC patients. C-*myc*-amplified NNBC patients who were not administered adjuvant systemic therapy suffer shorter DFS and OS. C-*myc* could be used together with the tumour-associated protease urokinase-plasminogen activator and its inhibitor (i.e. plasminogen activator inhibitor-1), after further randomized studies have been conducted, to confirm whether NNBC patients should receive adjuvant systemic therapy [47–49].

Competing interests

None declared.

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