ORIGINAL ARTICLE

c-*myc* Amplifications in primary breast carcinomas and their local recurrences

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Objective: To evaluate the role of c-*myc* oncogene amplifications in the progression of invasive breast carcinomas.
Methods: c-*myc* gene copy number was evaluated in a series of 49 primary breast carcinomas and the corresponding local recurrences using fluorescence in situ hybridisation.
Results: 11 of the primary carcinomas (22%) harboured c-*myc* amplifications; these tumours typically were hormone receptor negative and occurred in younger patients (43 *v* 53 years). At the time of relapse, six additional tumours had acquired a c-*myc* amplification. The mean recurrence-free survival was 24 months; c-*myc* amplified tumours relapsed significantly earlier than carcinomas without amplification (18 *v* 27 months). Univariate analysis showed a worse overall survival in these patients.
Conclusions: While c-*myc* amplifications can be observed in early stage breast cancer, especially in younger patients, they often occur later in tumour development and appear to be associated with disease progression.

Over the last 10 to 15 years, the surgical treatment of invasive breast cancer has undergone major changes. Owing to the increasing quality of radiological examination, breast cancer awareness and the establishment of screening programmes, the typical size of primary breast carcinomas has decreased substantially.[1] On the other hand, encouraging results with breast conserving surgery, in combination with adjuvant therapy, have led to the application of this treatment for larger tumours as well.[2] Currently, data from large follow up studies show local recurrence rates of approximately 5% for breast conserving surgery.[3] While there still is a higher risk of relapse compared with mastectomy, it is commonly acknowledged that the benefits of a less aggressive treatment approach outweigh the possibility of recurrent disease.

Clinical studies focusing on local failure of breast cancer treatment have revealed a large number of cases with distant metastases at the time of intramammary, axillary, or chest wall (following mastectomy) recurrence, and reported a poor overall survival in these patients.[4 5] Addressing these problems, several studies have identified possible clinicopathological or biological predictors of local recurrence.[5–10] However, little is known about biological changes in the tumour during progression of breast carcinomas.

METHODS

Patients

Between 1992 and 1998, 2996 patients with primary breast carcinoma were treated at the Department of Gynaecology and Obstetrics of Heidelberg University. Between 1996 and 2001, 67 of these patients were diagnosed with locally (intramammary, chest wall, or axillary) recurrent breast carcinoma; 14 of the tumours recurred more than once in the given time span. On the basis of the availability of representative paraffin embedded tissue from both primary and recurrent tumours at the Department of Pathology of the University of Heidelberg, 49 individual patients were selected. The tumours were classified according to the World Health Organisation,[11] and pathological staging was based on the fifth edition of the TNM classification. For fluorescence in situ hybridisation (FISH), four tissue microarrays were created containing both primary tumours and local recurrences using 1.5 mm punch needles on a semiautomatic system (Beecher Instruments, Silver Spring, Maryland, USA) as described previously.[12]

Immunohistochemistry

Slides containing full tissue sections of representative tumour areas were dewaxed and rehydrated using xylene and a series of graded alcohols, followed by heat induced antigen retrieval using citrate buffer (DakoCytomation, Glostrup, Denmark) in a microwave oven. Staining was done on an automated staining system (Techmate 500+, DakoCytomation) with the avidin-biotin-complex peroxidase technique using AEC (3-amino-9-ethyl-carbazol) for visualisation and haematoxylin for counterstaining. Dilutions for the primary antibodies were 1:500 for Her2 (A0485, DakoCytomation), 1:200 for Ki-67 (MIB-1, DakoCytomation), 1:50 for oestrogen receptor (1D5, DakoCytomation) and progesterone receptor (PgR636, DakoCytomation), and 1:100 for p53 (DO7, DakoCytomation).

Fluorescence in situ hybridisation

Approximately 5 μm sections of the tissue microarrays were mounted on coated slides (DakoCytomation) and dried overnight at 37°C. Deparaffinised slides were pretreated using 1 M sodium thiocyanate (NaSCN, Merck, Darmstadt, Germany) at 80°C for 30 minutes, followed by digestion with proteinase K (Roche, Mannheim, Germany; 100 μg/ml, 37°C, 17 minutes). DNA obtained from a cosmid clone containing the c-*myc* gene sequence (cos-myc 72)[13] was labelled using tetramethyl-rhodamine-5-dUTP (Roche) with nick translation.[14] Slides and probe DNA (approximately 0.1 μg) were co-denatured in the presence of 10 μg Cot1-DNA (Roche) on an in situ polymerase chain reaction (PCR) thermocycler (Perkin Elmer, Monza, Italy) and hybridised at 37°C for 16 to 24 hours in a humid chamber. Post-hybridisation washes and counterstaining using DAPI (4,6-diamidino-2-phenylindole) were carried out as described previously.[12]

Abbreviations: FISH, fluorescence in situ hybridisation; IRS, immunoreactivity scores; UICC, International Union against Cancer

Table 1 Clinical data on 49 breast carcinomas with local recurrences

Age (years) (median (range))	50 (26 to 85)
Surgical treatment	
Breast conserving surgery	26 (53%)
Mastectomy	23 (47%)
Axillary dissection	47 (96%)
Adjuvant treatment	
Antihormonal therapy	18/37 (49%)*
Radiation therapy	20/37 (54%)*
Cytostatic chemotherapy	29/37 (78%)*
Time to local recurrence (months) (mean (range))	24 (5 to 63)
Local recurrence	
Intramammary	24 (49%)
Chest wall	16 (33%)
Axillary	5 (10%)
Other	4 (8%)

Values are n (%) unless stated otherwise.
*In 12 cases, no information on adjuvant treatment was available.

Statistical evaluation

Data were analysed using the R software package (version 1.7.1, http://www.r-project.org). For count data, Fisher's exact test (two sided) was used; continuous data were analysed using the two sided Wilcoxon rank sum test. Differences between primary tumours and recurrences were calculated using the paired Wilcoxon signed rank test. The Kaplan–Meier method was applied to calculate survival rates for both local recurrences and overall survival; for multivariate analysis, the Cox proportional hazards regression model was used. Univariate survival data were tested for significance using the Mantel–Haenszel log rank test. Probability (p) values less than 0.05 were considered significant.

RESULTS

Clinicopathological data

The clinical characteristics of the primary tumours are summarised in table 1. Twenty six patients were initially treated using breast conserving surgery, 21 by mastectomy, and two by subcutaneous mastectomy. Information on adjuvant treatment was available in 37 cases (table 1). There were 42 invasive ductal, six invasive lobular, and one tubular carcinoma. Tumours were often poorly differentiated (31 cases, 63%). Only 13 carcinomas (27%) measured less than 2 cm (pT1). In 23 cases, multifocal or multicentric tumour growth was observed. Lymphatic vessel invasion was present in 28 cases, and 34 carcinomas had metastasised to the axillary lymph nodes (table 2). Local recurrences occurred after an average of 23.7 months (range 5 to 63 months); there were 24 intramammary, 16 intracutaneous (chest wall), five axillary, two infraclavicular or supraclavicular, and two contralateral recurrences. Twelve of the cohort suffered from a second local relapse between five and 44 months later; one tumour recurred a third time four months after the second relapse. Further follow up information was available in 28 cases (median 15 months, range 3 to 42).

Immunohistochemical characterisation

Immunohistochemical staining results are summarised in table 3. Hormone receptor staining was evaluated using a semiquantitative scoring system, resulting in scores ranging from 0 to 12.[15] Immunoreactivity scores (IRS) greater than 2 were considered positive. Twenty five of the primary tumours (51%) showed expression of the oestrogen receptor (21 cases) or progesterone receptor (15 cases). At the time of recurrence, 14 of these cases were scored negative for both oestrogen receptor and progesterone receptor ($p<0.0003$, both receptors combined). Overexpression of the Her2 protein product was

Table 2 Histopathological characteristics of 49 breast carcinomas with local recurrences

Tumour type	
Invasive ductal carcinoma	42 (86%)
Invasive lobular carcinoma	6 (12%)
Tubular carcinoma	1 (2%)
Histological grading	
G1	1 (2%)
G2	17 (35%)
G3	31 (63%)
Tumour extent	
pT1 (pT1a,pT1b,pT1c)	13 (27%)
pT2	24 (49%)
pT3	6 (12%)
pT4 (pT4a, pT4b, pT4c, pT4d)	6 (12%)
Multifocal	16 (33%)
Multicentric	7 (14%)
Free margins	42 (86%)
Lymph node status	
pN0	13 (27%)
pN1	32 (49%)
pN2	2 (4%)
pNX	2 (4%)
Lymphatic vessel invasion	28 (57%)

Values are n (%).

diagnosed in 17 cases (35%) which showed moderate to strong circumferential membrane staining (Dako score 3+) while the rest of the cases showed no staining or only weak and partial membrane staining (30 cases with Dako score of 0, two cases scored 1). There was no staining difference between primary tumours and recurrences: the same 17 cases showed an overexpression of Her2 at the time of relapse while all other cases remained negative. Nuclear accumulation of the p53 protein in more than 50% of the tumour cells was observed in 18 (37%) of primary tumours and in 20 recurrences (41%). Two tumours with initial p53 overexpression stained negative at the time of relapse; four tumour recurrences which had initially been negative had strong nuclear staining. On average, primary tumours showed a proliferative activity of 51% Ki67 positive nuclei; hormone receptor positive tumours were associated with a lower growth rate (35% *v* 64%, $p = 0.0002$) and overexpression of the p53 protein was associated with an increased proliferative activity (70% *v* 40%, $p = 0.0004$). In addition, tumour recurrences showed a slight reduction in proliferative activity (45% *v* 51%, $p = 0.028$).

c-*myc* Oncogene amplification

FISH results were evaluated by counting the average number of nuclear signals in at least 50 tumour cell nuclei (in 10 cases with only few tumour cell nests included in the tissue array sections fewer nuclei were evaluated). Typical hybridisation results are shown in fig 1. Eleven (22%) of the primary tumours (10 invasive ductal and one invasive lobular carcinoma, nine high grade and two intermediate grade tumours) showed between five and 12 signals per nucleus

Table 3 Immunohistochemical characteristics of 49 breast carcinomas with local recurrences

	Primary tumours	Recurrences	p Value*
Oestrogen receptor expression	21 cases	8 cases	0.0024
Progesterone receptor expression	15 cases	9 cases	0.244
Her2 overexpression	17 cases	17 cases	1
p53 overexpression	18 cases	20 cases	0.300
Average Ki67 expression (% positive nuclei)	51%	45%	0.028

*Calculated using the paired Wilcoxon signed rank test.

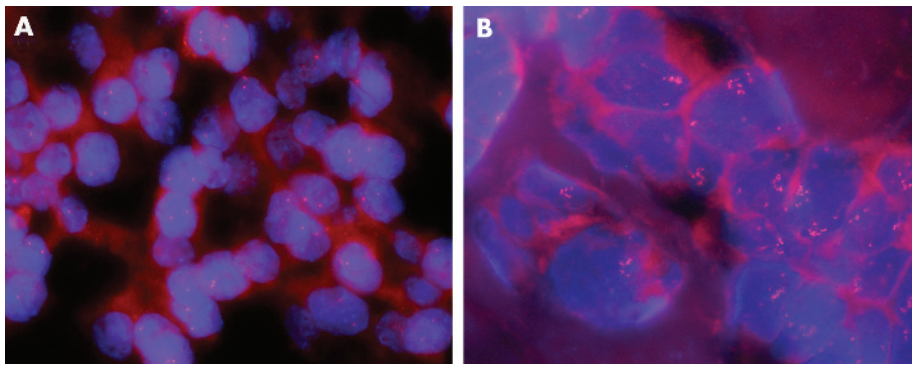

Figure 1 c-*myc* Fluorescence in situ hybridisation. (A) Invasive breast carcinoma showing no c-*myc* amplification; (B) c-*myc* amplified breast tumour with 10 to 12 signals per nucleus.

(7.2 on average) and were considered amplified. These tumours were associated with a lower age at the time of diagnosis (43 *v* 53 years, p = 0.0223) and typically lacked hormone receptor expression (two of 11 cases compared with 16 of 38 cases; p = 0.0373). At the time of recurrence, six additional cases showed c-*myc* oncogene amplifications. With respect to the overall number of cases, this increase (35% *v* 22%) reached significance (p = 0.0197). In primary tumours with c-*myc* amplifications, there was no loss of or further increase in gene copy number. In the six cases with acquired c-*myc* amplification (three chest wall, two intramammary, one axillary recurrence), the average age at the time of diagnosis (51 years) did not vary from that of the remainder of the patients. Three of these six tumours had primarily been oestrogen or progesterone receptor positive; however, all three cases had lost hormone receptor expression at the time of relapse. Patients with c-*myc* amplified primary tumours or recurrences had a shorter recurrence-free survival compared with those with normal gene copy number (18 *v* 27 months, p = 0.0177, fig 2A) and a worsened overall survival following recurrence (p = 0.0332, fig 2B). Using multivariate analysis, c-*myc* amplified primary tumours were associated with earlier local failure (p = 0.041, table 4). However, the only factor independently influencing overall survival following local recurrence was UICC stage (p = 0.039).

DISCUSSION

Locoregional breast cancer recurrences typically occur years after initial treatment and can be a potential source of distant metastases and potentially disease related deaths in these patients.[16] Using comparative genomic hybridisation and analysis of loss of heterozygosity, an increased complexity of karyotypes has been observed during the progression of breast cancer.[17 18] During the time span between initial surgery and locoregional recurrence, residual tumour cells are subjected to different conditions, applying selective pressure including surgery related influences such as local hypoxaemia and inflammatory reactions, as well as radiation induced DNA damage, cytostatic chemotherapy, or antihormonal treatment.

The MYC proto-oncogene functions as a promotor of cell replication by activating positive cell cycle regulators like the cyclin protein family.[19] Activation of the c-*myc* gene in tumours leads to a higher proliferative activity as well as to an increased rate of apoptosis.[20] However, the oncogenetic potential of c-*myc* alterations alone has been found to be low.[21] Different experiments on transgenic mice have shown that additional genetic changes are needed to promote tumour formation. Among these additional alterations are activation of the Her2[22] and BCL2[23] oncogenes, as well as inactivation of the p53 tumour suppressor gene,[21] which are commonly observed in breast cancer.

In our series of locally recurrent breast carcinomas, 11 of the primary tumours (22%) harboured c-*myc* oncogene amplifications. This frequency is slightly higher than in previous studies, which found c-*myc* amplifications in 5–15% of the cases,[24–26] possibly reflecting the selection of a more aggressive subset of breast carcinomas. Most of these 11 *myc*-amplified primary tumours were hormone receptor negative, high grade carcinomas. Interestingly, the average age at

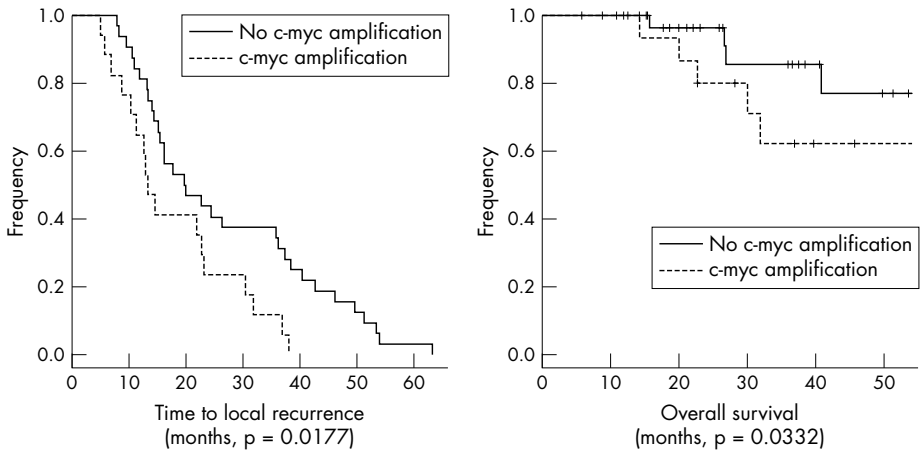

Figure 2 Recurrence free (A) and overall (B) survival stratified by c-*myc* amplification. Crosses indicate follow up end points with patients alive.

Table 4 Cox proportional hazards regression model analysing factors influencing the time to local recurrence

	Hazard ratio (95% CI)	p Value
UICC stage	0.97 (0.52 to 1.79)	0.920
c-*myc* Amplification (primary tumour)	2.39 (1.04 to 5.48)	0.041
c-*myc* Amplification (acquired)	2.66 (0.97 to 7.27)	0.057
High grade	1.22 (0.48 to 3.08)	0.680
Positive margins	3.11 (0.87 to 11.14)	0.081
Lymphatic vessel invasion	1.09 (0.45 to 2.62)	0.850
Positive hormone receptor status	1.03 (0.45 to 2.62)	0.940
Her2 Overexpression	0.91 (0.42 to 1.97)	0.810
p53 Overexpression	1.65 (0.76 to 3.59)	0.210
High proliferative activity (Ki67>25%)	0.97 (0.38 to 2.51)	0.950

UICC, International Union Against Cancer.

diagnosis was only 43 years in these cases, compared with 53 years in the primary tumours without *myc*-amplifications. Recently, Grushko *et al* reported a high frequency of 53% c-*myc* amplifications in BRCA1 associated breast carcinomas.[27] Adem *et al* observed an association of *myc* alterations with both BRCA1 and BRCA2 related tumours.[28] However, as we have no data on BRCA1 or BRCA2 mutations in the patients included in this study, we can only speculate on the reason for the observed difference in patient age.

While amplifications of c-*myc* have been observed in a subset of pure intraductal breast carcinomas,[12 29] Naidu *et al*[30] and Robanus-Maandag *et al*[31] have reported several cases of *myc* amplified invasive ductal carcinoma with an intraductal tumour component lacking c-*myc* amplifications. Based on these findings, an association of c-*myc* with the progression from in situ to invasive carcinoma has been proposed.[31] We have detected amplifications of the c-*myc* oncogene in six local recurrences of primary breast carcinoma with normal *myc* copy number. Contrasting the findings in primarily amplified tumours, these cases were not associated with lack of hormone receptors or younger patient age at the time of diagnosis. Thus it appears likely that in these cases c-*myc* amplifications are based on an increased chromosomal instability and are involved in tumour progression rather than in tumour initiation. In addition, we observed an association between breast carcinomas with increased *myc* copy number, either at the time of primary diagnosis or on recurrence, and a shortened relapse-free time both using univariate and multivariate analysis. This suggests that, owing to hypoxic or adjuvant treatment related cellular damage, an increased selective pressure may lead to the accumulation of genetic alterations such as c-*myc* amplifications that provide a growth advantage or permit survival under cell damaging conditions. Recently, an increased expression of c-*myc* has been found to facilitate human mammary epithelial cells with radiation induced DNA damage to inappropriately enter mitosis by attenuating the G2/M arrest.[32] Furthermore, in another experiment, c-*myc* overexpression was also shown to weaken the G1/S arrest in the same mammary epithelial cells and thus may promote the replication of damaged DNA by an increased S-phase entry.[33] Using MCF-7 breast cancer cells, Venditti *et al* observed that induced c-*myc* gene expression conferred a resistance to antioestrogenic treatment.[34] These results may explain the increased frequency of c-*myc* amplifications both in the progression of in situ to invasive breast carcinomas and in our series of locally recurring tumours.

In conclusion, our results indicate that while c-*myc* amplifications can be observed in early stage breast cancer, especially in hormone receptor negative, high grade tumours in younger patients, they often occur later in tumour development and appear to be associated with disease progression and early local failure.

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