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Association between *c-myc* amplification and pathological complete response to neoadjuvant chemotherapy in breast cancer

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ABSTRACT

Background: The aim of this study was to investigate whether *c-myc* amplification in human breast cancer is associated with response to neoadjuvant chemotherapy comprising paclitaxel followed by 5-FU/epirubicin/cyclophosphamide (P-FEC).

Methods: Tumour tissue samples were obtained before neoadjuvant chemotherapy (P-FEC) from 100 primary breast cancer patients (stage II/III). *C-myc* and *HER2* amplification were examined by FISH, and oestrogen receptor (ER), progesterone receptor (PR), Ki67, and topoisomerase 2α (TOP2A) expression were examined immunohistochemically. Pathological complete response (pCR) was defined by a complete loss of tumour cells in the breast without any lymph node metastasis.

Results: *C-myc* amplification was observed in 40% (40/100) of breast tumours, and was significantly associated with ER-negative tumours (23/40 for ER(–) versus 17/60 for ER(+), $P = 0.004$), high histological grade tumours (11/18 for grade 3 versus 29/82 for grades 1 + 2, $P = 0.043$) and TOP2A-positive tumours (28/51 for TOP2A(+) versus 12/49 for TOP2A(–), $P = 0.002$). pCR rate was 20% for total patients (10.0% for ER(+) and 35.0% for ER(–)). Further, breast tumours with *c-myc* amplification (*c-myc*(+)) showed a significantly ($P = 0.041$) higher pCR rate (12/40) than those without such amplification (*c-myc*(–)) (8/60). This association between pCR and *c-myc* amplification was observed in ER-positive tumours (4/17 for *c-myc*(+) versus 2/43 for *c-myc*(–), $P = 0.048$) but not in ER-negative tumours (8/23 for *c-myc*(+) versus 6/17 for *c-myc*(–), $P = 0.973$).

Conclusion: Our results suggest that *c-myc* amplification is significantly associated with a high pCR rate to P-FEC in breast tumours, especially in ER-positive tumours.

1. Introduction

The *c-myc* gene encodes nuclear DNA binding proteins that regulate the expression of a variety of genes implicated in cell proliferation, apoptosis, metabolism, stemness, invasiveness and inhibition of differentiation.[1,2] Since amplification of *c-myc* has been observed in a significant proportion (8–37%) of primary human breast cancers, it is thought that *c-myc* is

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involved in the development and progression of breast cancer.[3,4] Rose-Hellekant and Sandgren have reported that *c-myc* transgenic mice develop mammary tumours[5], a direct indication of an important role for *c-myc* in mammary carcinogenesis.

Since *c-myc* promotes tumour cell proliferation and invasiveness, both of which are typical features often associated with biological aggressiveness, it is speculated that breast tumours with *c-myc* amplification have a poor prognosis. In fact, it has been reported by several investigators that breast tumours with *c-myc* amplification are more likely to be oestrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and to have a high histological grade and a significant association with poor prognosis.[6–8]

Interestingly, *c-myc* has a dual function, i.e. it promotes cell apoptosis as well as proliferation.[9] Since rapidly proliferating cells are generally more sensitive to chemotherapy and since it has been reported that *c-myc* sensitises tumour cells to apoptosis induced by chemotherapy,[10,11] it is hypothesised that breast tumours with *c-myc* amplification are more sensitive to chemotherapy than those without such amplification. The association between *c-myc* amplification and response to chemotherapy has been mostly studied *in vitro*, and greater sensitivity of breast tumour cells with *c-myc* amplification to paclitaxel has been reported by Olah et al.[12] and to doxorubicin by Fornari et al.[13,14]

These *in vitro* results regarding the association between *c-myc* amplification and response to chemotherapy prompted us to investigate whether such an association can be observed in human breast cancer, for such investigation has rarely been conducted.[15,16] First, the development of clinically useful predictors for response to chemotherapy in breast cancer is very important since no such predictors are currently available. In the study presented here, we investigated whether *c-myc* amplification can predict the response to neoadjuvant chemotherapy consisting of paclitaxel followed by 5-FU/epirubicin/cyclophosphamide, which is one of the most commonly used chemotherapeutic regimens for human breast cancer.

2. Materials and methods

2.1. Patients and tumour samples

During the period of from 2004 to 2009, 125 patients (stage II/III) were treated with neoadjuvant chemotherapy [paclitaxel (80 mg/m^2) q1w × 12 cycles followed by 5-FU (500 mg/m^2)/epirubicin (75 mg/m^2)/cyclophosphamide (500 mg/m^2) q3w × 4 cycles] (P-FEC) followed by surgery. Before neoadjuvant chemotherapy, each patient underwent tumour biopsy with a vacuum-assisted core biopsy (8G) (Mammotome®; Ethicon Endosurgery, Cincinnati, OH, USA) under ultrasonographic guidance. Of these 125 patients, Mammotome specimens were available for the immunohistological and FISH analysis from 100 patients who were retrospectively included in this study. Of these 100 patients, fresh tumour specimens were also available from 75 patients for the DNA microarray analysis. Mammotome specimens for histological examination were fixed in 10% buffered formaldehyde and those for RNA extraction were snap frozen in liquid nitrogen and kept at −80 °C until use. Informed consent for the study was obtained from each patient before tumour biopsy.

2.2. Evaluation of response to chemotherapy

Clinical tumour response to P-FEC was evaluated on enhanced MRI images which were obtained at three time points, i.e. before neoadjuvant chemotherapy, after paclitaxel therapy and after P-FEC therapy. Tumour size was determined as tumour length × width (cm^2). Reduction rates after paclitaxel only were calculated according to the following formula: reduction rate after paclitaxel = (tumour size before paclitaxel – tumour size after paclitaxel)/tumour size before paclitaxel. After P-FEC, the patients underwent breast conserving surgery or mastectomy. Pathological response to P-FEC was evaluated by using the surgical specimens obtained at surgery. The specimens were cut at 5-mm intervals and haematoxylin and eosin sections were examined for the presence or absence of tumour cells. A complete loss of both invasive and non-invasive tumour cells in the primary tumour site without any lymph node metastasis was defined as a pathological complete response (pCR). Primary end-point in the present study was pCR rate to P-FEC since pCR is the most frequently adopted end-point in the evaluation of response to chemotherapy in breast cancer. Secondary end-point was clinical response to paclitaxel only.

2.3. RNA extraction and gene expression profiling

RNA was extracted with TRIzol® (Invitrogen, Carlsbad, CA, USA) from tumour biopsy samples obtained with the Mammotome®. RNA (50 ng; RIN value >6) was subjected to be used for the generation of second-strand cDNA, and cRNA was amplified with a random primer (WT-Ovation™ FFPE System V2; NuGEN, San Carlos, CA, USA). Next, the amplified cRNA was biotinylated and fragmented with FL-Ovation™ cDNA Biotin Module V2 (NuGEN), and hybridised to a DNA microarray (Human Genome U133 Plus 2.0 Array; Affymetrix, Santa Clara, CA, USA) overnight (17 h) according to the manufacturer's protocol. The hybridised DNA microarray was then fluorescence stained with GeneChip Fluidics Station 450 (Affymetrix), and scanned with the Scanner 3000 (Affymetrix).

2.4. Microarray data processing

For intrinsic subtype analysis, raw data were MAS5-normalised, log2-transformed and mean-subtracted. For subtypes, the five subtypes of breast cancer, luminal A, luminal B, HER2-enriched, basal-like and normal breast-like defined by Sorlie et al. were used.[17] The dataset downloaded from their web site was used for computing the centroids of those subtypes. Within all probe sets on the array, 435 UniGene IDs could be matched to the intrinsic genes of the cDNA microarray used by Sorlie et al. for their study. If multiple probe sets were found for the same UniGene ID, those signals were averaged. Each tumour sample was assigned to one subtype corresponding to the nearest centroid in terms of Pearson's correlation coefficient. These procedures were

computed with statistical software R, version 2.10.1 (http://www.R-project.org/), except for MAS5-normalisation, which was computed with Affymetrix Expression Console software.

2.5. Immunohistological and FISH examination

Immunohistochemical examination of oestrogen receptor-α (ER), progesterone receptor (PR), topoisomerase 2α (TOP2A) and Ki67 in tumour samples obtained with Mammotome® before neoadjuvant chemotherapy was performed according to a previously described method.[18,19] ER and PR were defined as positive when 10% or more of tumour cells immunohistochemically stained positive (ER: Clone 6F11, Ventana, Tucson, AZ, USA; PR: Clone16, SRL, Tokyo, Japan). TOP2A (clone Ki-S1, monoclonal, IgG isotype, 1:100; DAKO, Tokyo, Japan) and Ki67 (Clone MIB-1, DAKO) were defined as positive when 20% or more of tumour cells were stained positive because this cutoff value (20%) was optimal for differentiating the sensitive (pCR) and non-sensitive (non-pCR) tumours to P-FEC for both biomarkers.

HER2 amplification was determined with FISH using the PathVysion HER-2 DNA Probe Kit (Abbott Molecular, Chicago, IL, USA) according to the manufacturer's instructions. The ratio of *HER2* gene signals to chromosome 17 centromere signals was calculated for each tumour, which was considered to be *HER2*-amplified if the FISH ratio was $\geqslant 2.0$. *C-myc* amplification was determined with FISH using the *MYC*/CEN-8 FISH Probe Mix (Dako A/S, Glostrup, Denmark) according to the manufacturer's instructions. FISH scoring was performed by counting fluorescence signals in at least 50 malignant tumour cells with the aid of WinROOF imaging software (Mitani Corporation, Tokyo, Japan). The ratio of *c-myc* gene signals to chromosome 8 centromere signals was calculated for each tumour, which was considered to be *c-myc* amplified if the FISH ratio was $\geqslant 1.5$.[20] The histological grade was determined according to the Scarff–Bloom–Richardson grading system.

2.6. Statistics

We used SPSS software (SPSS Inc., Chicago, IL) for all statistical analysis. The relationship between clinicopathological parameters and *c-myc* amplification was analysed by the chi-square test (or Fisher's exact test). The difference in the reduction rate between *c-myc* amplified and non-amplified tumours and that in Ki67-index or ratios of *c-myc* to chromosome 8 centromere signals among the intrinsic subtypes were evaluated by Mann–Whitney's *U* test. Difference in *c-myc* mRNA expression between *c-myc* amplified and non-amplified tumours was evaluated by Welch's *T*-test. The relationship between pCR rates and various parameters was evaluated using the logistic regression model. Relapse-free survival rates were calculated with the Kaplan–Meier method and differences in relapse-free survival rates were evaluated with the log-rank test. All recurrences other than the ipsilateral in-breast recurrences after breast conserving surgery were counted as events. There were no deaths from other causes than recurrences. A median follow-up period was 31.6 months with a range from 3.2 to 73.0 months. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. C-myc amplification and clinicopathological parameters

Representative results of FISH examination of *c-myc* are shown in Fig. 1, and the relationships between *c-myc* amplification and the various clinicopathological parameters of breast tumours are listed in Table 1. Breast tumours with *c-myc* amplification were significantly more likely to be ER-negative tumours ($P = 0.004$) and tended to be PR-negative ($P = 0.059$). Further, breast tumours with *c-myc* amplification were significantly more likely to be of a high histological grade ($P = 0.043$) and TOP2A-high ($P = 0.002$) and tended to be Ki67-high tumours ($P = 0.060$). No other parameters were significantly associated with *c-myc* amplification.

C-myc mRNA expression levels were compared between *c-myc* amplified and non-amplified tumours in Fig. 2. *C-myc* amplified tumours showed significantly ($P < 0.05$) higher *c-myc* mRNA expression levels than *c-myc* non-amplified tumours.

3.2. C-myc amplification and intrinsic subtypes

RNA was available from 75 Mammatome specimens, and was subjected to DNA microarray analysis for the determination of intrinsic subtype (Table 2). Thirty-four tumours were classified as luminal A, 21 as luminal B, 7 as HER2-enriched, 12 as basal-like and one as normal breast-like subtypes (one normal breast-like tumour was excluded from further analysis). Luminal A subtype showed very high ER positivity (94%) followed by luminal B subtype (48%). HER2-enriched subtype showed low ER positivity (14%) and none of the basal-like subtype tumours were ER-positive. Similar trends were observed for PR. PR positivity was highest in luminal A (76%), followed by luminal B (24%), and was negative in both HER2-enriched and basal-like subtypes. On the other hand, *HER2* amplification was highest in HER2-enriched, followed by luminal B, while it was very low in luminal A and basal-like subtypes.

Percentages of Ki67-positive tumour cells in each of the tumours are shown in Fig. 3. The percentage of Ki67-positive tumour cells for luminal A (median: 14.7) was lower than that for luminal B (median: 27.3), though the difference was not statistically significant, but it was significantly lower than that for basal-like subtype (median: 44.1). The ratio of *c-myc* to centromere signal for each tumour is also shown in Fig. 3. The ratios seem to be similar for luminal A, luminal B and HER2-enriched subtypes but for the basal-like subtype it was significantly higher than that for the luminal A subtype.

3.3. C-myc amplification and response to neoadjuvant chemotherapy

First, the relationship between *c-myc* amplification and clinical responses to paclitaxel only was studied (Fig. 4A). Breast tumours with *c-myc* amplification showed a significantly

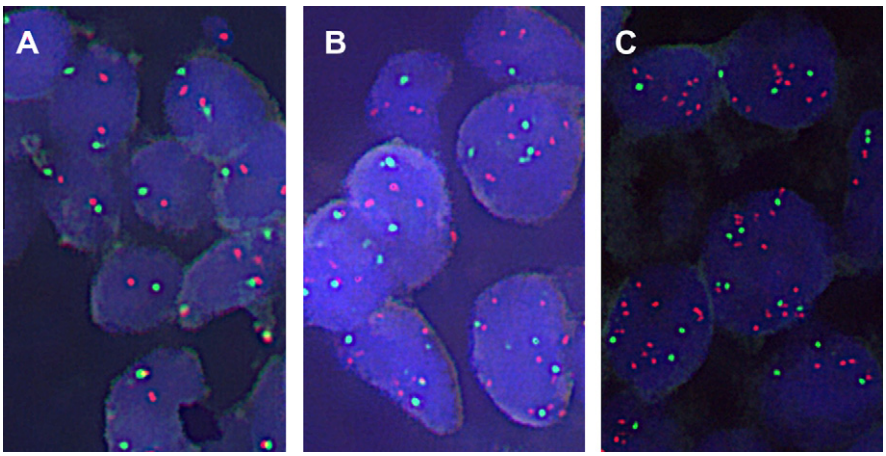

Fig. 1 – Representative results of FISH for *c-myc*. *C-myc* amplification was analysed by means of FISH using the probes for *c-myc* (red) and chromosome 8 centromere (green). Representative results are shown for breast tumours without amplification (Panel A: ratio of *c-myc* to centromere signals = 1.0) and those with amplification (Panel B: ratio = 1.8; and Panel C: ratio = 3.0).

Table 1 – Relationship between *c-myc* amplification and clinicopathological parameters of breast tumours.

	Total	Amplification		P-value
		c-myc–	*c-myc*+	
Menopausal status				
Premenopausal	53	32	21	0.935
Postmenopausal	47	28	19	
Tumour size				
T1	5	4	1	
T2	66	37	29	0.443
T3	29	19	10	
Lymph node status				
N1, 2	69	44	25	0.251
N0	31	16	15	
Histological grade				
1, 2	82	53	29	0.043
3	18	7	11	
Histological type				
Invasive ductal	93	55	38	0.415
Invasive lobular	7	5	2	
ER				
Positive	60	43	17	0.004
Negative	40	17	23	
PR				
Positive	44	31	13	0.059
Negative	56	29	27	
HER2 amplification				
Present	29	18	11	0.787
Absent	71	42	29	
Ki67				
Positive	51	26	25	0.060
Negative	49	34	15	
TOP2A				
Positive	51	23	28	0.002
Negative	49	37	12	

Abbreviations: *c-myc*–, without *c-myc* amplification; *c-myc*+, with *c-myc* amplification; ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TOP2A, topoisomerase 2 alpha.

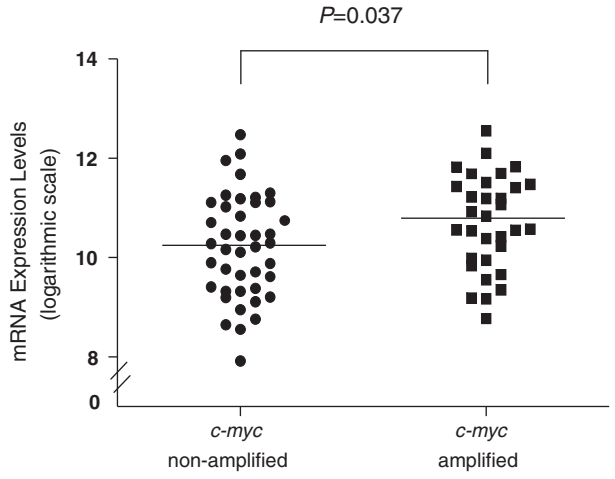

Fig. 2 – Comparison of *c-myc* mRNA expression between *c-myc* amplified and non-amplified tumours. *C-myc* mRNA expression levels in tumour tissues were determined by DNA microarray, and were compared between *c-myc* amplified ($n = 32$) and non-amplified tumours ($n = 42$) after logarithmic conversion by Welch's *T*-test. Bars, median.

($P = 0.039$) larger reduction rate than those without such amplification. Subset analysis according to ER status showed that breast tumours with *c-myc* amplification tended to show a higher reduction rate than those without it in the ER-positive ($P = 0.08$) but not in the ER-negative subset ($P = 0.68$).

Next, the relationship between *c-myc* amplification and pathological responses to P-FEC was studied (Fig. 4B). The findings were very similar to those for clinical responses. That is, breast tumours with *c-myc* amplification showed a significantly ($P = 0.041$) higher pCR rate than those without such amplification. The univariate analysis showed that *c-myc* amplification was significantly associated with pCR but the multivariate analysis failed to show such an association (Table 3).

Subset analysis according to ER status showed that breast tumours with *c-myc* amplification showed a significantly higher pCR rate than those without it in the ER-positive ($P = 0.048$) but not in the ER-negative subset ($P = 0.973$). Relationships between pCR and clinicopathological parameters according to ER status are shown in Table 4. In the ER-positive subset, only *c-myc* amplification was significantly associated with pCR, while in the ER negative subset, menopausal status, Ki67 status, and tumour size, but not *c-myc* amplification, showed a significant association with pCR.

3.4. *C-myc amplification and clinicopathological parameters according to ER status*

The relationships between *c-myc* amplification and the clinicopathological parameters of breast tumours were analysed according to ER status (Table 5). In the ER-positive subset, breast tumours with *c-myc* amplification were significantly more likely to be node-positive ($P = 0.040$), to have a high histological grade ($P = 0.032$) and to be TOP2A-high tumours ($P = 0.040$). However, such significant associations were not observed in the ER-negative subset.

Relapse-free survival rates of the patients with and without c-*myc* amplification were compared in Fig. 5. There was no significant difference in relapse-free survival rates between them.

4. Discussion

In the study reported here, we first examined the relationship between *c-myc* amplification and the various clinicopathological characteristics in breast tumours, and detected associations between *c-myc* amplification and ER negativity, PR negativity, high histological grade, TOP2A positivity and Ki67 positivity. These results are essentially consistent with those previously reported,[8,21] suggesting that breast tumours with *c-myc* amplification harbour biologically aggressive phenotypes. In fact, it has been reported that breast tumours with *c-myc* amplification show a poorer prognosis than those without such amplification.[6–8]

Recent gene expression analysis by means of DNA microarray has revealed that breast tumours can be classified into five intrinsic subtypes, i.e. luminal A, luminal B, HER2-enriched, basal-like and normal breast-like, which are all biologically distinct.[17] We were able to confirm in our study the previously reported associations of ER, PR, *HER2* and Ki67 with these intrinsic subtypes.[22,23] We also investigated the associations of *c-myc* amplification with these intrinsic subtypes

Table 2 – Relationship between intrinsic subtypes and ER, PR, *HER2*.

	Intrinsic subtypes			
	Luminal A	Luminal B	HER2-enriched	Basal-like
ER				
Positive	32(94%)	10(48%)	1(14%)	0(0%)
Negative	2(6%)	11(52%)	6(86%)	12(100%)
PR				
Positive	26(76%)	5(24%)	0(0%)	0(0%)
Negative	8(24%)	16(76%)	7(100%)	12(100%)
HER2 amplification				
Present	5(15%)	10(48%)	5(71%)	2(17%)
Absent	29(85%)	11(52%)	2(29%)	10(83%)

Abbreviations: ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

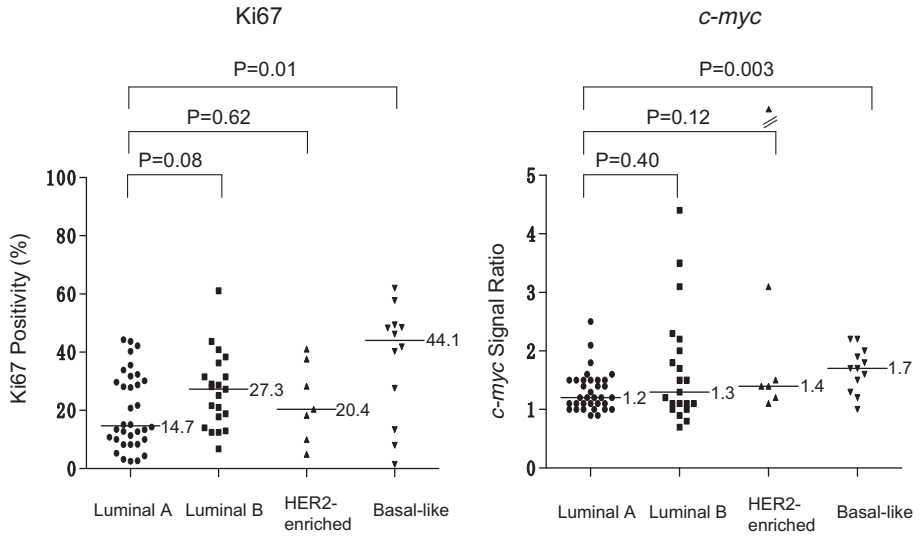

Fig. 3 – Ki67 and *c-myc* amplification according to intrinsic subtype. Intrinsic subtypes were determined by gene expression profiling as described under Section 2. The graphs show a comparison of percentages of Ki67 positive tumour cells (left) and ratios of *c-myc* to chromosome 8 centromere signals (right) for the intrinsic subtypes (Mann–Whitney's *U* test).

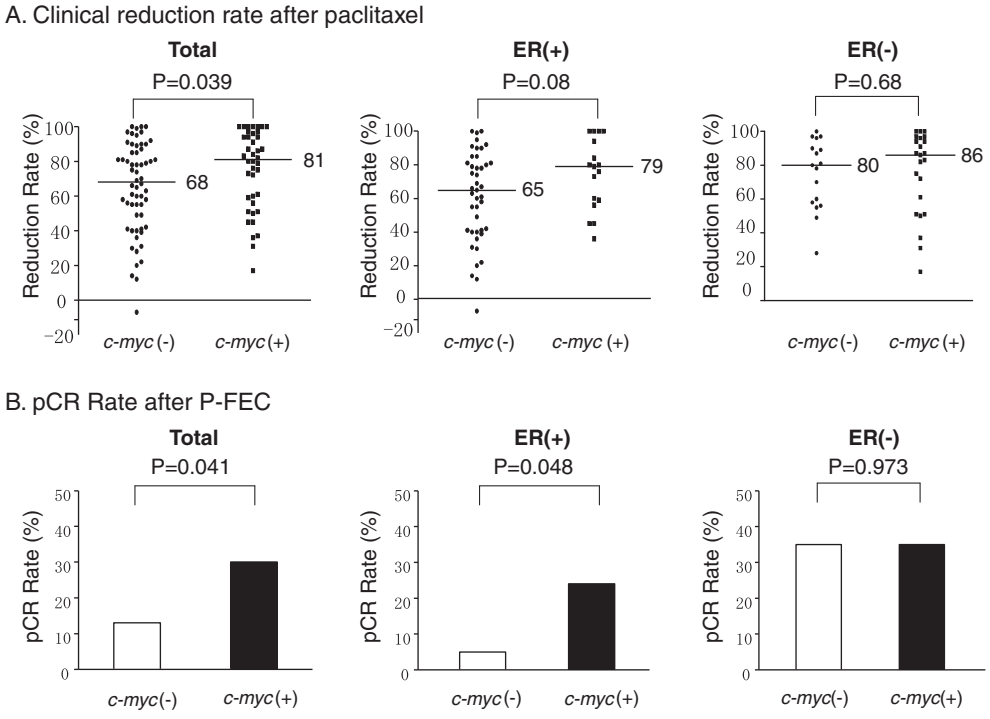

Fig. 4 – Clinical and pathological response to neoadjuvant chemotherapy according to *c-myc* amplification status. (Panel A) Tumour reduction rates in response to paclitaxel and determined by MRI were compared between breast tumours with (*c-myc*(+)) and without (*c-myc*(–)) amplification of *c-myc* (Mann–Whitney's *U* test). (Panel B) Pathological complete response (pCR) rates in response to paclitaxel followed by 5-FU/epirubicin/cyclophosphamide (P-FEC) were compared between *c-myc*(+) and *c-myc*(–).

because reports on such associations have been rare.[3,24] *C-myc* amplification in luminal A was lower than in luminal B, though the difference was statistically not significant, but was significantly lower than in the basal-like subtype. Similarly, the Ki67 index (percentage of Ki67-positive tumour cells) in luminal A was lower than in luminal B, though statistically but not significantly so, and again was significantly lower than in the basal-like subtype. These results seem to suggest

Table 3 – Univariate and multivariate analysis of various parameters in their correlation with pathological response.

	Univariate analysis			Multivariate analysis		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Menopausal status (post versus pre)	4.50	1.49–13.61	0.005	9.90	1.04–90.91	0.046
Tumour size (T1, T2 versus T3)	10.20	1.30–83.33	0.008	5.05	1.35–18.94	0.016
Lymph node metastasis (+/–)	1.44	0.47–4.41	0.517			
Histological grade (G3 versus G1, G2)	1.72	0.53–5.56	0.270			
ER (–/+)	4.85	1.67–14.08	0.002	2.36	0.37–15.15	0.364
PR (–/+)	4.00	1.23–12.99	0.016	1.15	0.15–8.77	0.896
HER2 amplification (+/–)	1.87	0.67–5.21	0.225			
Ki67 (+/–)	7.67	2.08–28.27	0.001	5.01	1.10–22.86	0.038
TOP2A	2.71	0.95–7.77	0.057			
C-myc amplification (+/–)	2.79	1.02–7.62	0.041	2.77	0.76–10.15	0.123

Abbreviations: OR, odds ratio; CI, confidence interval; ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TOP2A, topoisomerase 2 alpha.

Table 4 – Relationship between pCR and clinicopathological parameters according to ER status.

	ER-positive			ER-negative		
	Non-pCR	pCR	*P*-value	Non-pCR	pCR	*P*-value
Menopausal status						
Premenopausal	34	2	0.167	14	3	0.048
Postmenopausal	20	4		12	11	
Tumour size						
T1, T2	36	6	0.105	16	13	0.036
T3	18	0		10	1	
Lymph node status						
N1, 2	34	3	0.420	20	12	0.412
N0	20	3		6	2	
Histological grade						
G1, G2	50	6	0.649	17	9	0.605
G3	4	0		9	5	
Histological type						
Invasive ductal	49	6	0.579	24	14	0.417
Invasive lobular	5	0		2	0	
PR						
Positive	40	4	0.512	0	0	
Negative	14	2		26	14	
HER2 amplification						
Present	12	2	0.430	9	6	0.608
Absent	42	4		17	8	
Ki67						
Positive	19	4	0.145	15	13	0.021
Negative	35	2		11	1	
TOP2A						
Positive	20	3	0.420	17	11	0.385
Negative	34	3		9	3	
C-myc amplification						
Present	13	4	0.048	15	8	0.973
Absent	41	2		11	6	

Abbreviations: pCR, pathological complete response; ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TOP2A, topoisomerase 2 alpha.

that *c-myc* amplification may play a more important role in the proliferation of luminal B and basal-like than in that of the other subtypes.

C-myc has been implicated in the regulation of cell proliferation and apoptosis, both of which are very important determinants for sensitivity to chemotherapy. In fact, it has been

Table 5 – Relationship between *c-myc* amplification and clinicopathological parameters according to ER status.

	ER positive			ER negative		
	c-myc–	*c-myc*+	*P*-value	*c-myc*–	*c-myc*+	*P*-value
Menopausal status						
Premenopausal	26	10	0.907	6	11	0.428
Postmenopausal	17	7		11	12	
Tumour size						
T1, T2	29	13	0.492	12	17	0.816
T3	14	4		5	6	
Lymph node status						
N1, 2	30	7	0.040	14	18	0.537
N0	13	10		3	5	
Histological grade						
G1, G2	42	14	0.032	11	15	0.973
G3	1	3		6	8	
Histological type						
Invasive ductal	39	16	0.666	16	22	0.826
Invasive lobular	4	1		1	1	
PR						
Positive	31	13	0.730	0	0	
Negative	12	4		17	23	
HER2 amplification						
Present	11	3	0.386	7	8	0.680
Absent	32	14		10	15	
Ki67						
Positive	14	9	0.143	12	16	0.944
Negative	29	8		5	7	
TOP2A						
Positive	13	10	0.040	10	18	0.185
Negative	30	7		7	5	

Abbreviations: *c-myc*–, without *c-myc* amplification; *c-myc*+, with *c-myc* amplification; ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TOP2A, topoisomerase 2 alpha.

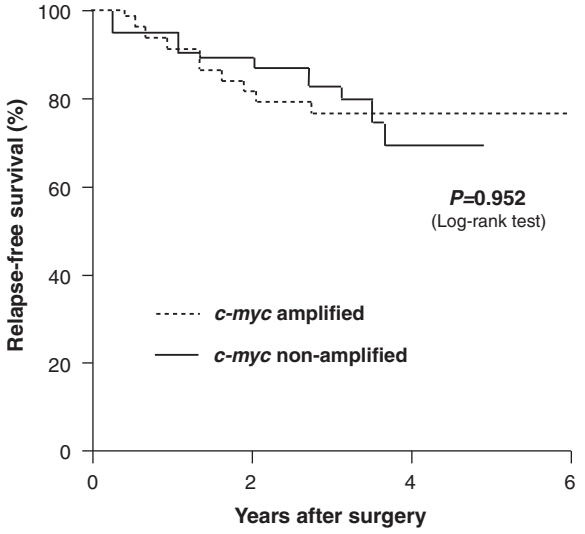

Fig. 5 – Prognosis according to *c-myc* amplification status. Relapse-free survival rates of patients with *c-myc* amplified tumours (*n* = 40) and those with *c-myc* non-amplified (*n* = 60) tumours were compared (Kaplan–Meier curves).

shown *in vitro* that tumour cells with *c-myc* amplification are associated with a high sensitivity to chemotherapy including the use of paclitaxel and anthracyclines.[12–14] However, the part that *c-myc* amplification plays in chemosensitivity in human breast cancer has yet to be studied clinically. We, therefore, investigated the association of *c-myc* amplification with response to P-FEC in the neoadjuvant setting. Our study is unique in that paclitaxel monotherapy was administered and MRIs were obtained both before and after paclitaxel administration so that the association between *c-myc* amplification and response to paclitaxel could be determined accurately. We were able to show that there was significant association between *c-myc* amplification and a higher response to paclitaxel, and interestingly the subset analysis showed that such an association pertains to ER-positive but not to ER-negative tumours. As for pCR rates to P-FEC, similar findings were obtained in that they were significantly higher for breast tumours with *c-myc* amplification than for those without such amplifications in ER-positive tumours but not in ER-negative tumours. In addition, of the various clinicopathological parameters, only *c-myc* amplification was significantly associated with pCR for ER-positive tumours (Table 4), indicating a possibility that *c-myc* amplification could be useful for the prediction of response to P-FEC in breast tumours, especially in ER-positive tumours.

The exact reason why *c-myc* amplification is associated with pCR in ER-positive but not ER-negative tumours remains to be clarified. It is of interest, however, that we found that *c-myc* amplification was significantly associated with TOP2A-positivity in ER-positive but not ER-negative tumours. Such an association seems to explain, at least in part, the reason why *c-myc* amplification was significantly associated with pCR in ER-positive tumours because TOP2A-positive tumours, which rapidly proliferate, are thought to be more sensitive to chemotherapy.

Recently, *c-myc* amplification is more commonly assayed by means of FISH using formalin-fixed and paraffin-embedded (FFPE) sections. As a cutoff value for *c-myc* amplification, the ratio of *c-myc* signals to centromere signals of chromosome; two is the most commonly used,[8,25,26] though the use of other cutoff values has also been reported.[20,27–29] For our study, we adopted the cutoff value of 1.5 because the association of *c-myc* amplification with pCR was found to be significant with a cutoff value of 1.5 but not with a cutoff value of 2.0 (data not shown). In addition, we were able to show by means of DNA microarray a significant ($P < 0.05$) increase in the expression of *c-myc* gene in breast tumours with *c-myc* amplification ($\geqslant 1.5$) compared to those without it (<1.5). Because of the limited number of patients enrolled in our study, the optimal *c-myc* amplification cutoff value for the prediction of response to chemotherapy remains to be established, since this value might be different from the optimal cutoff value for the prediction of recurrence.[8]

The limitation of the present study is that the number of patients analysed herein is too small to draw a definitive conclusion. We could demonstrate a significant association between *c-myc* amplification and pCR by the univariate analysis but not by the multivariate analysis, suggesting that *c-myc* is not an independent predictor of pCR. But it is too early to conclude that *c-myc* amplification is useless in the prediction of pCR since odds ratio of *c-myc* amplification was 2.77 in the multivariate analysis, though statistically not significant, and thus a future study including a larger number of patients might disclose its significant predictive value. Similarly, the presence of a significant association between *c-myc* amplification and pCR in ER-positive tumours but not in ER-negative tumours should be understood cautiously since the number of ER-negative tumours is too small. A further study is definitely needed for ER-negative tumours.

In conclusion, our present study suggests that *c-myc* amplification is significantly associated with a high pCR rate to P-FEC in breast cancer, especially in ER-positive tumours. Such an association might be explained, at least in part, by a higher proliferation of ER-positive tumours with *c-myc* amplification than those without such amplification. Our present observation needs to be confirmed by a future study covering a larger number of patients.

Conflict of interest statement

S. Noguchi received research grants from Pfizer and Bristol-Myers Squibb, and honoraria from Pfizer.

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