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c-*myc* Gene Amplification in Selected Node-negative Breast Cancer Patients Correlates with High Rate of Early Relapse

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In breast cancers with histologically negative axillary nodes selected for high frequency of recurrence, the amplification of c-*myc*, *erb*B-2 and *int*-2 genes was found to concern, respectively 25% (16/65), 31% (25/81) and 14% (10/70) of tumours. Their relation with tumour progression expressed by relapse-free survival is reported. Using univariate analyses, c-*myc* amplified tumours showed significant association with early (30-month period after diagnosis) ($P = 0.0013$) and intermediate (50-month period after diagnosis) ($P = 0.0398$) risks of recurrence. In contrast, only a trend towards higher relapse was observed in *erb*B-2 amplified breast cancers with respect to later events (occurring over the first 30-month period). Multivariate analyses indicated that c-*myc* amplification is an independent prognostic factor stronger than oestrogen receptor status and tumour size to define a high risk subset in node-negative patients selected for high frequency of recurrence.
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INTRODUCTION

To date, involvement of axillary lymph nodes is the best prognostic indicator in breast cancer. It has been related to a considerable increase in early mortality. Nevertheless, high recurrence and early death rate are continuously observed among node-negative patients (30% relapse during the first 5 years after local-regional therapy). Several prognostic factors are used to describe high risk node negative subsets, mainly tumour size, oestrogen receptor level, tumour ploidy and mitotic index [1]. Nevertheless, these parameters do not match perfectly the disease evolution; this has resulted in the search for new prognostic parameters capable of distinguishing high risk patients in

order to select therapy [2–4]. Numerous reports have associated oncogene amplification and breast cancer progression [5–14]. In this study we examined the changes in disease-free survival of node-negative breast cancer patients selected for high frequency of recurrence according to intratumoural c-*myc*, *erb*B-2 and *int*-2 gene amplification.

PATIENTS AND METHODS

Oncogene amplification assay

Tissue specimens were snap frozen in liquid nitrogen at the time of mastectomy and maintained at −80°C until use. Intratumoural amplification of c-*myc*, *erb*B-2 and *int*-2 genes was estimated by Southern blot analysis as previously reported [15]. 10 μg of each genomic DNA sample were restricted with *Eco*RI or *Hind* III (Biolabs) before electrophoresis and further transfered to Hybond-N (Amersham). After autoradiography, the intensity of the bands was estimated by densitometric scanning of the autoradiograms (Shimadzu CS 930) to detect amplification. This was confirmed by serial dilutions of restricted DNA [15].

Three levels of controls were performed: (i) the amount of membrane-immobilised DNA actually available for hybridisation was estimated using a single copy probe (β-globin); (ii) amplification was distinguished from aneuploidy by further re-hybridisation of blots with single copy probes mapped to the chromosomes of interest; (iii) normal single copy signals were obtained from genomic DNA prepared from peripheral blood lymphocytes of healthy donors and analysed concomitantly. Tumour DNA samples showing signals with an intensity of two copies or more per haploid genome were considered to represent amplification. All experiments were repeated at least twice for each tumour DNA sample.

The following oncogene probes were used: c-*myc* (1.5 kb, *Cla* I-*Eco*RI fragment; [16]) mapped to chromosome band 8q24, provided by F. Cuzin, Nice, *erb*B-2 (0.440 kb, KpnI-XbaI fragment; [17] and 1 kb, *Hind* III fragment; [18]) mapped to chromosome band 17q21 [19] provided by T. Yamamoto, Tokyo; *int*-2 (0.9 kb, *Sac*I fragment), mapped to chromosome band 11q13 [20] provided by G. Peters and C. Dickson, London.

The control probes consisted of: c-*mos* (2.7 kb, *Eco*RI fragment; [21]), mapped to chromosome band 8q22; Human p53 (1.7 kb, *Stu*I-*Sph*I fragment; [22]), mapped to chromosome band 17p13 [23]; Human retinoic acid receptor (2 kb fragment; [24]) mapped to chromosome band 17q21 [25]; β-globin (1.4 kb *Eco*RI-*Hind* III fragment; [26]), mapped to chromosome band 11p15; All probes were labeled to high specific activity using the random-primer method [27].

Characteristics of patients

Tissue samples from primary breast cancers were retrospectively selected from our tumour library. They concern women with histologically proven node-negative breast cancers registered from February 1984 to December 1985. The end point was on 1 May 1989, the maximal duration for follow-up was 56.8 months and the median was 45.5 months. None of the patients received further treatment following the primary surgery.

At surgery, samples were collected in liquid nitrogen for hormone receptor examinations and the remaining tissue was stored at −80°C for oncogene evaluation. The rate of recurrence being very low among patients with node-negative tumours of good prognosis, they contribute little data to our evaluation. We therefore selected for increased relapse and among these 89 tumours, 26 events were scored. This corresponded with 92% and 59% of patients at risk after 30-month and 50-month periods, respectively.

Oestrogen (ER) and progesterone (PgR) receptors were assayed in our laboratory under EORTC control [28–31]. Specimens were considered ER and PgR positive if they contained at least 10 fmol of specific binding sites per milligram of cytosolic protein. The diameter of primary tumours was assessed by histological measurements and grading was achieved according to the Bloom and Richardson (SBR) classification [32]. To ensure the validity of the status of the nodes, selected cases had complete removal of the lower two levels of axillary nodes and pathological examination of at least 10 axillary nodes per patient.

Statistics

Experimental analyses were performed using Clinical Data Management System (Medlog, Information Analysis Corporation, Mountain View, California). All quantitative variables deduced from experimental evaluations were transformed into qualitative variables.

Clinical characteristics of the patients in relation to oncogene amplification were compared by the χ^2 test.

The monoparametric disease-free survival curves were drawn by the Kaplan–Meier method [33] to assess the effects of intratumoural c-*myc*, *erb*B-2 and *int*-2 gene amplification on disease-free survival. Statistical differences were evaluated with the log-rank test [34]. The events used as end points in the determination of disease-free survival concerned distant metastases. To provide information about oncogene amplification over time, cumulative *P* values were determined at 30 month (*P*) and 50 month (*P*).

Multivariate Cox model was run to look for independant parameters by entering variables according to the stepwise method [35]. Couples of qualitative variables consisting of (i) gene status, (ii) clinical, (iii) histopathological and (iv) biological data, were coded 1 if the answer was "yes" and 0 if the answer was "no". When patients were stratified within three subgroups—(age: < 40, 40–70, $\geqslant 70$ years), (size of tumour < 2, [2–5], $\geqslant 5$ cm) (histological SBR grade: I, II, III)—only lower and upper subsets were used to define suitable variables for analyses. Then, patients entering the intermediate group were coded 0 with respect to the low-value and the high-value groups.

RESULTS

Amplification of c-*myc*, *erb*B-2 and *int*-2 genes was investigated in selected node-negative breast cancers and concerned respectively 24.6% (16/65), 30.9% (25/81) and 14.3% (10/70) of the tumours.

The presence or the absence of amplification in patient DNAs was detected by internal comparison to normal indiyiduals. In all experiments, the amount of membrane immobilised DNA actually available for hybridisation was assessed using a single copy cDNA probe (β-globin and retinoic acid receptor) and

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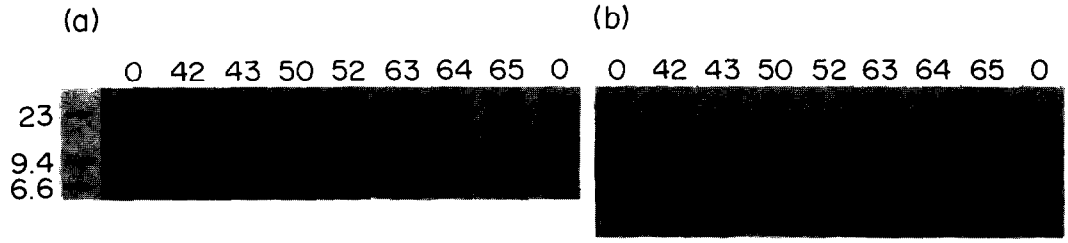

Fig. 1. Evaluation of oncogene amplification in tumours of patients with primary breast cancer. 10 μg of tumour sample (number at top) or placenta (0) DNA were digested with *Hind*III and analysed by Southern Blot; -*Hind*III fragments on left (in kb). (a) Filter on the left was hybridised with c-*myc* probe which detected at 12 kb fragment. (b) Filter hybridised with the c-*myc* probe on the left was washed and re-hybridised (filter on the right) with *erb*B-2 and β-globin probes; 14 kb band: *erb*B-2 gene detected with pKX044 probe; 8,2-kb band: major fragment detected with the β-globin probe.

normalisation of oncogene signals was performed according to the control. As aneuploidy frequently occurs in developing tumours, aneuploidy versus amplification was ruled out by further hybridisation of blots with single copy control probes mapped to the chromosomes of interest, i.e., c-*mos*, p53 and β-globin for c-*myc*, *erb*B-2 and *int*-2 genes, respectively. Low amplification levels (2–5 copies per haploid genome) were assessed by serial dilution analyses. Two-fold dilutions of DNA samples showing an intensity equal to the undiluted normal control DNA were considered to represent amplification. Representative hybridisation patterns are presented in Fig. 1. Because oncogene amplification evaluation was performed on subsets of the 89 previously selected tumours (c-*myc*, $n = 65$; *erb*B-2, $n = 81$ and *int*-2, $n = 70$), patient-related parameters are reported in Table 1. The characteristics among the three groups did not show any peculiar features and did not differ greatly from our local consecutive recruitment [15].

Table 2 presents univariate analyses in selected node-negative patients according to oncogene amplification. The disease aggravation associated with c-*myc* amplification was observed over the unamplified control. The mean early (30-months) disease-free survival was 68.7% as compared with 95.8% for the control ($P = 0.0013$). 50 months after surgery, the overall disease-free survival among patients with amplified tumours was 37% vs. 63.7% in the unamplified ones ($P = 0.0398$). Thus, the results indicated that amplification was effective in aggravating as early as 30 months disease-free survival among selected patients with axillary node-negative breast cancers. We also looked for association of *erb*B-2 and *int*-2 gene amplification with time to relapse. After 50 months, the disease recurred in 46% of patients with intratumoral *erb*B-2 amplification as compared with 38.2% of patients in the control group ($P = 0.0809$) indicating that a trend for relapse was occurring in patients with *erb*B-2 amplification. In contrast, no survival advantage was observed between patients according to *int*-2 gene status.

Individual node-negative patient's risk factors, such as tumour size, ER status and proliferative rate are commonly used to weight the risk of recurrence. Using univariate and multivariate analyses, we compared the disease-free survival according to those criteria (SBR grading being used to assess the proliferative index) to the one examining the effects of c-*myc* amplification (Table 3). Univariate analyses did not reveal differences between patient's behaviour whatever the tumour size after surgery. In contrast, low ER tumour content was found to be highly informative for relapse events occurring as early as 30 months after surgery ($P = 0.0163$ at 30 months, $P = 0.0005$ at 50 months). Finally, the histopathological SBR grading which takes into account the proliferative state of the tumour showed a positive association between low grade and survival after 30 months (not significant at 30 months, $P = 0.0334$ at 50 months). Multivariate analyses were performed to assess the independent predictive power of c-*myc* amplification with regards to the other parameters. Only patients with complete follow-up for all parameters were taken into account (51 of 65). Although ER status and SBR grade were correlated with the prognosis in the univariate analyses, their independent importance as prognostic factors was lower than that of c-*myc* amplification. c-*myc* gene alteration behaved as an independent variable highly informative

Table 1. Clinical, biological and histological parameters in selected node-negative breast tumours having oncogene amplification assay

Factor	c-*myc* $n = 65$	*erb*B-2 $n = 81$	*int*-2 $n = 70$
Age (%)			
<40	1 (1.5)	2 (2.5)	1 (1.4)
[40–70]	42 (64.6)	50 (61.7)	47 (67.1)
≥70	17 (26.2)	24 (29.6)	18 (25.7)
Unknown	5 (7.7)	5 (6.2)	4 (5.7)
Menopausal status (%)			
Pre-	15 (23.1)	15 (18.5)	15 (21.4)
Post-	47 (72.3)	63 (77.8)	53 (75.7)
Unknown	3 (4.6)	3 (3.7)	2 (2.9)
Histological grade (SBR) (%)			
I	16 (24.6)	18 (22.2)	15 (21.4)
II	26 (40.0)	33 (40.7)	30 (42.9)
III	14 (21.5)	19 (23.5)	17 (24.3)
Unknown	9 (13.8)	11 (13.6)	8 (11.4)
ER status* (%)			
<10	21 (32.3)	29 (35.8)	24 (34.3)
≥10	44 (67.7)	52 (64.2)	46 (65.7)
PgR status* (%)			
<10	26 (40.0)	38 (46.9)	31 (44.3)
≥10	39 (60.0)	43 (53.1)	39 (55.7)
Size of tumour† (%)			
<2	21 (32.3)	25 (30.9)	22 (31.4)
[2–5]	35 (53.8)	45 (55.6)	39 (55.7)
≥5	3 (4.6)	5 (6.2)	3 (4.3)
Unknown	6 (9.2)	6 (7.4)	6 (8.6)
Gene status (%)			
Amplified	16 (24.6)	25 (30.9)	10 (14.3)
Normal	49 (75.4)	56 (69.1)	60 (85.7)

*fmol/mg cytosolic protein, †in cm.

Table 2. Disease-free survival of selected node-negative breast cancer patients according to oncogene amplification

Factor	Patients at risk			*P*	
	0	30 months	50 months	30 months	50 months
c-*myc* status					
Amplified	16 (100)	11 (68.7)	1 (37)		
Normal	49 (100)	45 (95.8)	8 (63.7)	0.0013	0.0398
*erb*B-2 status					
Amplified	25 (100)	22 (88)	4 (54)		
Normal	56 (100)	50 (92.8)	11 (61.8)	NS	NS (0.08)
int-2 status					
Amplified	10 (100)	9 (90)	1 (50)		
Normal	60 (100)	53 (90)	9 (55.7)	NS	NS

NS = not significant.

Table 3. Univariate and multivariate analyses comparing disease-free survival (relapse) at 30-months and 50-months of selected node-negative patients

	Relapse (30-months)		Relapse (50-months)	
	Univariate	Multivariate	Univariate	Multivariate
c-*myc*	0.0013 [2.48 (0.842)]	0.0032	0.0398	0.0006 [1.68 (0.491)]
ER	0.0163	NS	0.0005	NS
Histological SBR grade	NS	NS	0.0334	NS
Tumour size	NS	NS	NS	NS

Results are shown as *P* [regression coefficient (SD)].

for relapse events occurring in selected node negative patients within the first 30 month period after surgery ($P = 0.0032$). Furthermore, these differences in disease-free survival were increasing after 4 years ($P = 0.0006$).

Then, in selected node-negative breast cancer, c-*myc* gene amplification behaved as an independent prognostic factor for early relapse-events occurring as early as 30 months after surgery.

DISCUSSION

Numerous clinical trials have established that axillary node status is the predominant factor for predicting recurrence and survival in breast cancer and there is an inverse correlation between the probability of disease-free survival and the number of involved lymph nodes. The continuous observation among node-negative patients of high recurrence and death rate within 5 years after diagnosis has lead to compare in randomised trials the benefit of receiving (or not) a short-term systemic chemo- or hormono therapy [2–4, 36–40]. The cost of receiving those systemic treatments has been evaluated. According to the limited benefits (adjuvant therapy will improve disease-free survival of only 4–15% of patients), the expense considerably outweighs the advantages of treating all node-negative women [1]. Therefore, there is a tremendous need for prognosis markers to elicit subsets of node-negative patients who will need adjuvant therapy to be cured of their disease. Recently, the thymidine labelling index has been used as a quantitative measure of tumour cells engaged in DNA synthesis. It was found that the higher the proliferative rate, the more likely the relapse occurred [41, 42]. Other techniques, such as flow cytometry permitted convenient measure of cells in S-phase as well as tumour ploidy allowing prediction early relapse in node-negative patients [43], and recently cathepsin D [44]. Oncogene alterations (mostly amplification) are thought to be faithful markers of the evolution of the disease. Slamon *et al.* [45] have shown a positive correlation between *erb*B-2 amplification and relapse in a cohort of 345 node-positive breast cancer patients. However, this association was no longer observed in node-negative tumours. Similar data were reported by Ro *et al.* [46] and Tsuda *et al.* [47] who additionally mentioned the independent predictive value of c-*myc* amplification in 164 breast tumours irrespective of their nodal status. In contrast, *int*-2/*hst*-1 gene examination did not elicit predictive significance.

In this report, we evaluated c-*myc*, *erb*B-2 and *int*-2 gene amplification to weigh the probability of relapse in patients with node-negative breast cancer. This was performed on a tumour sampling retrospectively selected for high frequency of recurrence, which has implications for the generability of the findings. Actually, no advantage can be gained by including patients with good prognosis because the rate of tumour recurrence is very low in such patients and they will contribute little data to the evaluation. In the selected population, disease aggravation associated with c-*myc* amplification was observed as early as 30 months after diagnosis and at a median follow-up of 50 months, the patients of who survived free of disease were 31% vs. 61% in the control group which possessed a single gene copy. Strikingly, a similar percentage (32%) was observed in the node-positive subset with c-*myc* amplification after 4 years whereas 55% were yet free of disease in the corresponding control group (unpublished data). Multivariate analyses established that c-*myc* amplification was an independent prognostic factor associated with early recurrence in selected node-negative breast tumours. Remarkably, patients with normal c-*myc* copy number were at better risk even though positive nodes were scored suggesting that node involvement might be more a consequence of tumour aggressiveness than a tumour feature.

The effects of adjuvant chemotherapy on the disease outcome of tumours with c-*myc* amplification is currently under investigation with special attention to node negative tumours.

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