Amplification of c-*myc* by Fluorescence *In Situ* Hybridization in a Population-Based Breast Cancer Tissue Array

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A total of 261 primary breast carcinomas were analyzed for amplification of the c-*myc* oncogene by fluorescence *in situ* hybridization performed on tumor tissue array samples. Results were compared with individual clinicopathologic and follow-up data. Thirty-eight (14.6%) of the tumors showed c-*myc* gene amplification (defined as two or more additional copies of c-*myc* gene in relation to the number of chromosome 8 centromere). The reproducibility of fluorescence *in situ* hybridization assay (defined by hybridization with two different *myc* probes) was good (kappa coefficient 0.402). Statistically significant associations were found between c-*myc* amplification and DNA aneuploidy (P = .0011), and progesterone receptor negativity (P = .0071), and c-*myc* amplification also tended to be associated with high histologic grade (P = .064), positive axillary nodal status (P = .080), and a high S-phase fraction (P = .052). c-*myc* amplification was not significantly associated with overall survival of patients with invasive cancer (P = .32). These data from a population-based tumor material suggest that c-*myc* amplification is a feature of aggressive breast cancers, but that it is unlikely to be a clinically useful prognostic factor.

KEY WORDS: c-*myc*, Multitissue array, Primary breast cancer.

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Structural aberrations of chromosome 8 are common in breast cancer, as well as in various other solid tumors (1, 2). The copy number alterations of chromosome 8 detected by comparative genomic hybridization typically consist of gains on the q-arm, either entire 8q or only its telomeric parts (3–5). These aberrations have commonly been interpreted to reflect amplification of the c-*myc* oncogene, which is located at 8q24.1.

The central role of c-*myc* gene in physiologic proliferation and malignant transformation of human cells has been thoroughly described (6–8). c-*myc* has a role in most cellular functions, including replication, growth, metabolism, differentiation and apoptosis (9–13). In addition to these functions, the protein product of c-*myc* has been shown in cultured breast cancer cells to mimic the functions of the known breast cancer promoter, estrogen (8, 14). Many *in vitro* studies have also shown that expression of c-*myc* correlates positively to treatment with estrogens (15, 16), but negatively to the growth inhibitory effect of tamoxifen *in vivo* in estrogen receptor (ER)-positive breast tumors (17). Also the putative breast tumor suppressor gene *BRCA1* has been shown to inhibit c-*myc*-mediated transcription and transformation (18). These findings implicate a specific role for c-*myc* gene in breast cancer pathogenesis.

Amplification of c-*myc* has been reported in breast cancer, as well as in other cancers in a large number of studies (19–21). Despite numerous studies the exact frequency of the amplification has remained obscure. The incidence of c-*myc* amplification ranges from 1 to 94% in different studies (22). Furthermore, unlike *e.g.*, for the *HER-2* oncogene amplification and its consequent protein overexpression, there is no clear consensus whether or not c-*myc* amplification is always associated with overexpression of its protein product (23). Wide variation has also been shown in the correlation

between the c-*myc* oncogene amplification and clinicopathologic variables. These results may largely reflect the methodologic difficulties in detecting c-*myc* amplification when using Southern blotting. In particular, when evaluating low degree of amplification, the choice of the reference gene is critical. Fluorescence *in situ* hybridization (FISH), which is currently considered as the most accurate method to analyze gene amplification in human tumors, has been applied to c-*myc* only in a few studies including clinical breast tumor samples (20, 24, 25). In this study we applied the newly established tumor tissue array technology (26) and studied c-*myc* amplification by FISH using two different *myc*-specific probes. Our aim was to further clarify the role of c-*myc* amplification as a prognostic marker in breast cancer.

MATERIALS AND METHODS

Preparation of Tumor Tissue Arrays

A total of 351 archival paraffin blocks from primary invasive breast cancers were collected for this study. According to the data files of the Finnish Cancer Registry, these tumors represent 84.2% of all primary invasive cancers operated during 1991–1992 in the Tampere University Hospital district (population ~400,000). The mean age of the patients was 61.6 years. The histologic types were available for 241 tumors including 198 (82%) ductal, 21 (9%) lobular, and 22 (9%) tumors of special histologic types. The previously collected clinicopathologic data (27) included patient age at the time of diagnosis, postoperative tumor size, axillary nodal status, and metastases, histologic grade, estrogen and progesterone receptor status (ER and PR, respectively), DNA ploidy, and S-phase fraction. The median follow-up time for the unrelapsed patients was 6.8 years (range 5.1 to 7.8 years), which enabled analysis of distant disease-free survival data 5 years from the diagnosis.

The tumors were routinely fixed (overnight in 10% buffered formalin) and processed into paraffin blocks according to the established protocols. Representative tumor regions were defined from hematoxylin and eosin-stained sections. Tumor tissue array blocks were made as follows: one tissue cylinder with a diameter of 0.6 mm was punched through selected tumor areas from each "donor" tissue block. Tissue cylinders were then inserted into "recipient" tissue array paraffin blocks using a specific custom-made instrument as described elsewhere (26). The 351 tumors formed a set of four tissue array blocks. Sections of 5 μm from the resulting multitumor tissue array blocks were then transferred to glass slides, and baked in a 60° C oven for 2 to 4 hours before the hybridization.

Fluorescence *In Situ* Hybridization

Locus-specific probes for chromosome 8 centromere (pJM128, ATCC, Rockville, MD) and c-*myc* gene (RMC08p001) were labeled with FITC (DuPont, Boston, MA) and digoxigenin, respectively, using nicktranslation. A second set of tissue array slides was hybridized with a commercial *myc* probe labeled with Spectrum Orange (LSI *c-myc*, Vysis, Inc, Downers Grove, IL), using the pJM128 as a reference probe.

Before hybridization, tumor array tissue sections were deparaffinized, pretreated in a microwave oven (10 minutes at 92° C in Tissue Pretreatment Buffer, Zymed Inc., South San Francisco, CA) and digested with proteinase K (0.25 mg/mL in 2 × standard saline citrate solution, SSC) for 20 minutes. Slides were rinsed with 2 × SSC, dehydrated with graded ethanols and air dried before hybridization. Ten μl of probe cocktail (probes for c-*myc* gene and the centromere of chromosome 8 with human placental DNA and Cot-1 DNA, Roche Biochemicals, Mannheim, Germany) was applied onto slides that were coverslipped and sealed with rubber cement. Denaturation was carried out at for 94° C for 3 minutes on a thermal plate. After an overnight hybridization at 37° C, the slides were stringency washed with two successive incubations in 0.5 × SSC and 4 × SSC. The digoxigenin-labeled *myc* probe was detected with anti-digoxigenin rhodamine (diluted 1:300). The slides were counterstained with 0.4 μM 4',6-diamino-2-phenylindole in antifade solution (Vectashield, Vector Laboratories, Burlingame, CA).

The analysis was performed using an Olympus epifluorescence microscope (with 60 × objective), equipped with a CCD camera (Photometrics, Tucson, AZ). At least 50 nonoverlapping nuclei in every tumor sample were scored to determine the number of test and reference probe hybridization signals. The scoring results were expressed as the actual copy numbers per cell in the majority of the cells in each sample. Amplification was defined, when at least two copies more of c-*myc* were detected in relation to the chromosome 8 centromere copy number in at least in 20% of the analyzed cells. Samples were considered as having c-*myc* amplification if either or both c-*myc* probes showed amplification. Alternatively, we also tested the criteria used by Schraml *et al.* (25).

Statistical Methods

Contingency tables of c-*myc* gene status and clinicopathologic variables were analyzed with Fisher's exact test. The χ^2 test for trend was used to compare c-*myc* with histologic grade. The analysis of disease-free survival was performed using Kaplan-

Maier survival analysis and log-rank test. All *P*-values are two-tailed.

RESULTS

In FISH analysis, 261 out of 351 samples were interpretable on with either c-*myc* probe (success rate 74.4%; 66.1% with RMC08p001 and 53.0% with Vysis' probe). Reasons for unsuccessful analyses included tissue damage, and a weak hybridization signal and high background equally with both *myc* probes. To confirm the results, we used two different *myc* probes in separate hybridizations. The results obtained with the two probes were highly concordant (kappa coefficient 0.402).

Out of the 351 samples, 90 (25.6%) could not be analyzed. Only tumors that consisted of at least 50 nuclei were scored. However, the most common reason for uninterpretable results was weak hybridization, which is not related to the tissue array technology. According to our experience, weak hybridizations occur in 10 to 15% of hybridizations on ordinary tumor tissue sections as well. Tissue array technique itself induced few additional technical problems: the small samples are sometimes detached from the slides during the procedure, and the pretreatments needed by each sample vary greatly. The same pretreatment gives perfect signals on one sample, and another may be either totally damaged or totally intact showing no signals at all. In 64 cases the reason was the same with both probes used: 21 tissue samples (6.0%) were not representative for the tumor, and 32 samples (9.1%) were detached from the slide during the FISH procedure. Cell damage due to the strong treatments required by the FISH procedure was responsible for eight lost samples (2.3%), and in three cases (0.9%) no signals were seen. In 25 cases (7.1%) the reasons for a failure to analyze c-*myc* copy number status varied between the two sets of hybridization: missing samples (18 cases), cell damage (19 cases) and presence of no signals (15 cases).

Amplifications of c-*myc* by FISH

c-*myc* was amplified in 38 (14.6%) out of the 261 informative cases. The distribution of extra copy numbers varied from 2 (12 cases) to more than 10 (3 cases, Fig. 1, A-B), the median being 4 additional copies relative to chromosome 8 centromere. The proportion of cells showing the amplification varied from 20 to 100%.

Association of c-*myc* with the Clinicopathologic Data and Patient Survival

Association of c-*myc* amplification with the clinicopathologic data is shown in Table 1. The statistically most significant correlation was found in aneuploidy ($P = .0011$, odds ratio 4.8). The inverse association of c-*myc* amplification with PR expression was also very significant ($P = .0071$, odds ratio 0.34 PR-negativity being associated with amplifications. The S-phase fraction size tended to be associated with c-*myc* amplification with a *P*-value of .052 (odds ratio 2.4), and presence of axillary nodal metastases with a *P*-value of .080 (odds ratio 2.0). The other parameters investigated (age, tumor size, presence of metastases, ER expression, and distant disease-free survival) were not significantly associated with c-*myc* amplification.

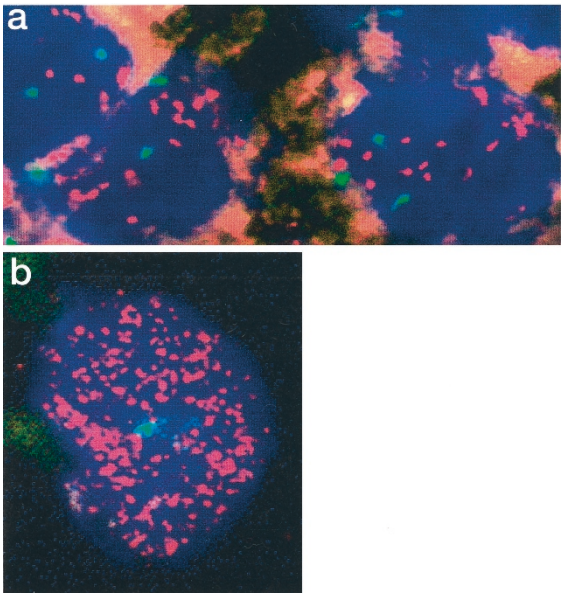

FIGURE 1. Centromere of chromosome 8 and c-*myc* in interphase nuclei of two primary breast tumor samples. Centromere is labeled with FITC (in green) and c-*myc* with Spectrum Orange (in red). **A**, Sample with 3 centromeres and 12 copies of c-*myc*. **B**, Highly amplified c-*myc* oncogene.

DISCUSSION

In the present study, we used the newly described tumor tissue array technology to examine c-*myc* oncogene amplification as a prognostic biomarker in breast cancer. The tissue array technology was found powerful and fast to perform once the tedious preparation of the multitissue array blocks was completed. FISH of 261 tumors could be done on four microscope slides only. The success rate of our FISH assay was 74.4%, which is 10 to 15% units lower than in our similar assays on ordinary tumor sections. According to our experience, the main reason for weak hybridizations is the difficulty to find pretreatment protocols that could uncover the DNA sequences of single copy genes. In our experience, the most efficient pretreatment is the combination of microwave oven boiling with mild protease digestion, as shown in our recent study using

TABLE 1. Comparison of c-myc Gene Status with Clinicopathological Variables in Primary Breast Tumors

Variable	c-myc		*P*-value[a]	Odds Ratio
	No Ampl. (*n*)	Amplified (*n*)		
All tumors	225	36		
Age				
ad 50	49	9		
> 50	174	29	n.s.	0.91 (0.40–2.0)
Tumor size				
ad 2 cm	133	19		
> 2 cm	73	18	n.s.	1.7 (0.85–3.5)
Histologic grade				
I	61	8		
II	86	13		
III	32	11	0.064[b]	
Axillary nodal status				
Negative	132	16		
Positive	70	17	0.080	2.0 (0.95–4.2)
Metastases				
Negative	207	36		
Positive	5	2	n.s.	2.3 (0.43–12.3)
ER				
Negative	50	13		
Positive	129	20	n.s.	0.60 (0.28–1.29)
PR				
Negative	72	22		
Positive	107	11	0.0071	0.34 (0.15–0.74)
SPF				
12	92	13		
Last	45	15	0.052	2.4 (1.04–5.38)
Ploidy				
Diploid	77	5		
Aneuploid	80	25	0.0011	4.8 (1.75–13.2)
DDFS[c]				
6	96%	100%		
12	95%	93%		
36	86%	70%		
60	79%	67%		
84	74%	67%	n.s.	

[a] Fisher's exact test.
[b] Chi-square test for trend.
[c] Distant Disease-Free Survival in different groups by months.
n.s., not significant.

chromogenic *in situ* hybridization of the *HER-2* oncogene (28). The other source for inaccuracy was the tumor array technology itself. A small fraction of the tissue samples on array slides repeatedly detached from the slides during the FISH protocol, which includes protease treatment and incubations at high temperatures. In spite of these technical shortcomings and a failure to analyze c-*myc* expression in a part of the original series, we believe that the present series is highly representative of breast cancer in general, because we were able to collect tumor tissue from nearly all breast cancer patients diagnosed the disease within a well-defined geographical area and a defined time period.

The c-*myc* oncogene was found to be amplified in 14.6% of the primary breast tumors. In a recent meta-analysis (22), c-*myc* gene was amplified threefold or more in breast cancer biopsies in 1 to 94% of the cases, with an average of 15.5%. The large variation has been explained by methodologic differences and at selection bias of the tumors studied, especially when small numbers have been studied. Our patient cohort chosen to be analyzed is based on the data files of the Finnish Cancer Registry, which has a coverage close to 100%. The majority of the cancers were diagnosed at an early stage (63% of tumors had size less than 2 cm and negative axillary lymph nodes) as in many modern series where a relatively large proportion of the cases are detected in mammography. Because c-*myc* is known to be amplified more frequently in more advanced stage cancers, this may be one reason for our relatively low percentage of amplifications detected as compared with some other recent studies. The method we used, two-color fluorescence *in situ* hybridization, is currently regarded as the most specific and sensitive method to detect gene amplification in human tumor samples (29). In this method, the amplification of c-*myc* can readily be detected as an excess of gene copies as compared with the number of chromosome 8 centromeres, detected in the same hybridization with another fluorescent color. However, despite the straightforwardness of the FISH assay itself, the criteria for scoring gene amplification has remained more or less arbitrary. In addition to classical bridge-fusion-

breakage type oncogene amplification, the presence of supernumerary copies of c-*myc* could be due to cytogenetic aberrations such as isochromosome formation, which are particularly common in chromosome 8 (1, 30). In these cases it remains uncertain whether or not the extra copies of c-*myc* lead to its functional oncogenic activation.

In general, c-*myc* amplification clearly correlated with adverse biological features of the tumors. DNA ploidy, low tumor histologic grade, presence of axillary nodal metastases, a high S-phase fraction size, and negative PR status tended to occur concomitantly with the amplification of c-*myc*. Statistically the most significant correlation was found with DNA aneuploidy of the tumor. These findings are in line with data suggesting that c-*myc* amplifications manifest in a later stage of tumor progression (22). The positive association with the S-phase fraction size and c-*myc* amplification fits with the wide experimental data supporting the central role of c-*myc* gene in DNA replication, and in tumor growth in general (6, 9, 11–13).

We found c-*myc* amplification to be more common in PR-negative tumors. This is also in concordance with the recent meta-analysis performed by Deming *et al.* (22), which suggested that PR-negativity is the only statistically significant association with c-*myc* amplification. For reasons not known, c-*myc* amplification is more strongly linked with progesterone rather than ER expression. The mechanisms by which progesterone affects differentiation, proliferation and other functions of breast tissue are complicated and still very poorly understood (31). The expression of c-*myc* mRNA is rapidly but transiently induced by progestin treatment, whereas relatively long-term treatment results in suppression of its expression (32, 33). The suppressive role of PR may explain at least partly the correlation between c-*myc* amplification and PR-negativity in breast tumors.

In our set of tumors, ER status was not significant in relation to c-*myc* amplification. According to the meta-analysis, there is great variability in the detected association between c-*myc* gene amplification and ER-status in different studies (22). The correlation between high estrogen levels and c-*myc* amplification and/or overexpression has nevertheless been found in *in vitro* and *in vivo* studies. For example, it has been shown that estrogen treatment induces the expression of c-*myc* mRNA directly (15, 16). Furthermore, ER-positive breast tumors excised from patients who have received tamoxifen treatment show a decreased level of c-*myc* mRNA as compared tumors from patients who have not been treated with tamoxifen (17). Because c-*myc* gene does not contain the canonical estrogen-responsive-element, the transcriptional activation of c-*myc* is induced through indirect mechanisms, possibly via estrogen-ER signaling.

c-Myc protein is also able to mimic estrogen inducing cyclinE/cdk2 activity in breast cancer cells in culture (14), and it induces also directly expression of *cyclin E* (8). Despite these data, it still remains unknown how estrogen-ER signaling regulates c-*myc* expression in human breast cancer, because several reports show that overexpression and/or amplification of c-*myc* occurs preferentially in ER-negative tumors.

These data from a population-based tumor material suggest that c-*myc* amplification is a feature of aggressive breast cancers, but that it is unlikely to be a clinically useful prognostic factor. The low percentage of c-*myc* amplification seen implicates that there may be other unknown mechanisms that connect c-*myc* with the breast cancer pathogenesis. It would be interesting to resolve other ways of c-*myc* oncogene activation, *e.g.*, specific translocations. c-*myc* oncogene is able to direct cells to different, partly opposite directions; proliferation, differentiation or apoptosis in physiologic situations, and the tumor formatting ability of the different isoforms of c-Myc protein product may also be of future interest. This challenges us to study further the possible mediating proteins and coexisting oncogenic factors required for the tumorigenic functions c-*myc*.

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