Prognostic Relevance of Gene Amplifications and Coamplifications in Breast Cancer

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ABSTRACT

Multiple different oncogenes have been described previously to be amplified in breast cancer including HER2, EGFR, MYC, CCND1, and MDM2. Gene amplification results in oncogene overexpression but may also serve as an indicator of genomic instability. As such, presence of one or several gene amplifications may have prognostic significance. To assess the prognostic importance of amplifications and coamplifications of HER2, EGFR, MYC, CCND1, and MDM2 in breast cancer, we analyzed a breast cancer tissue microarray containing samples from 2197 cancers with follow-up information. Fluorescence in situ hybridizations revealed amplifications of CCND1 in 20.1%, HER2 in 17.3%, MDM2 in 5.7%, MYC in 5.3%, and EGFR in 0.8% of the tumors. All gene amplifications were significantly associated with high grade. HER2 (P < 0.001) and MYC amplification (P < 0.001) were also linked to shortened survival. In case of HER2, this was independent of grade, pT, and pN categories. MYC amplification was almost 3 times more frequent in medullary cancer (15.9%), than in the histologic subtype with the second highest frequency (ductal; 5.6%; P = 0.0046). HER2 and MYC amplification were associated with estrogen receptor/progesterone receptor negativity (P < 0.001) whereas CCND1 amplification was linked to estrogen receptor/progesterone receptor positivity (P < 0.001). Coamplifications were more prevalent than expected based on the individual frequencies. Coamplifications of one or several other oncogenes occurred in 29.6% of tumors with only one of these amplifications. Furthermore, a gradual decrease of survival was observed with increasing number of amplifications. In conclusion, these data support a major prognostic impact of genomic instability as determined by a broad gene amplification survey in breast cancer.

INTRODUCTION

Gene amplification is an important mechanism for oncogene overexpression in malignant tumors. Gene amplification occurs frequently in breast cancer. HER2, EGFR, MYC, CCND1, MDM2, AIB1, FGFR1, S6K, TOP2A, EMS1, FGFI, AKT2, and PIP4K2 are genes for which amplification has been described in previous breast cancer studies (1–13). In addition, amplifications have been observed in various regions where the target gene has not been identified such as 1p, 1q, 3q, 4q, 6p, 6q, 8p, 10q, 14q, 15q, 16p, and 19q (14).

Because of the stability of the DNA, gene amplification is easier to measure than RNA or protein overexpression. Determination of gene amplifications would therefore be optimally suited for diagnostic applications. Indeed, amplification analysis is now increasingly being used for diagnostic analysis of the HER2 status instead of immunohistochemistry in clinical breast cancer samples. HER2, a transmembranous tyrosine kinase, which is overexpressed because of gene amplification in 15 to 20% of breast cancers in Western societies, represents the target protein for trastuzumab (Herceptin) therapy (15). Increasing evidence is suggesting that the HER2 status of a breast cancer may also be predictive for other therapies (16).

The potential clinical importance of amplifications of other oncogenes such as MYC, EGFR, MDM2, and CCND1 has been less extensively studied in breast cancer. MYC is located on 8q24 and codes for a G1-cyclin protein, which controls cell cycle progression during G1 phase. CCND1 has been found amplified in 10 to 27% of breast cancers (27, 28). CCND1 amplifications were found associated with estrogen receptor (ER) and progesterone receptor (PR) positivity (27, 29) as well as lobular histologic subtype (29). Studies on the prognostic significance have provided conflicting data including reports associating CCND1 amplification with good prognosis (27) and studies linking CCND1 amplifications with poor prognosis (26, 28). The MDM2 gene is located at 12q15. MDM2 protein down-regulates the tumor suppressor p53. MDM2 was found amplified in 4 to 7.7% of breast cancers (26, 30, 31). One study had suggested a worse prognosis in MDM2-amplified tumors as compared with nonamplified cancers (26). EGFR is located on 7q12 and codes for the EGFR, a transmembrane receptor protein with kinase activity. EGFR was found amplified in 0 to 14% of breast cancers (13, 32). The role of EGFR has regained interest in breast cancer because EGFR interacts with HER2, a therapeutic target protein and because of the availability of drugs that directly target EGFR.

Presence of gene amplification may not only be important because of the consecutive overexpression of the respective oncogene. Gene amplification may also serve as a surrogate parameter for increased genetic instability of a cancer and, as such, represent an indicator of poor patient prognosis. This may especially apply to tumors that have multiple amplifications. Indeed, a trend toward a worse prognosis in tumors with multiple amplifications was described recently in a study investigating 640 breast cancers by Southern blot for eight different oncogene amplifications (26).

In this project, we took advantage of a preexisting tissue microarray containing over 2,000 breast cancer samples for which clinical follow-up information was available. In an attempt to clarify the prognostic significance of gene amplifications and coamplifications in breast cancer, we analyzed the gene copy numbers of HER2, EGFR, MYC, CCND1, and MDM2 by fluorescence in situ hybridization (FISH). FISH is considered to be the most precise method for amplification detection (33). These data suggest strong prognostic signifi-
cancer of several individual amplifications and of the total number of amplifications in breast cancer.

**MATERIALS AND METHODS**

**Breast Cancer Tissue Microarray.** A total of 2197 formalin-fixed (buffered neutral aqueous 4% solution), paraffin-embedded tumors were available from the Institute of Pathology, Basel University Clinics, the Institute of Clinical Pathology in Basel, and the Triemli hospital in Zurich. The Ethics Committee of the Basel University Clinics approved the use of these specimens and the data in research. The median patient age was 62 (range 26–101) years. Raw survival data were either obtained from the cancer registry of Basel or collected from the patients attending physicians. The mean follow-up time was 68 months (range 1–176). The pathological stage, tumor diameter, and nodal status were obtained from the primary pathology reports. All slides from all tumors were reviewed by one of two pathologists (G. S. and J. T.) to define the histologic grade according to Elston and Ellis (Bloom, Richardson, Elston-Ellis grading [BRE]; ref. 34) and the histologic tumor type. The tissue microarray (TMA) composition is given in Table 1. Information on pT (n = 12), pN (n = 357), and BRE grade (n = 173) was missing in a fraction of arrayed tumors. TMA construction was as described previously (35). Briefly, we punched tissue cylinders with a diameter of 0.6 mm from representative tumor areas of a “donor” tissue block using a home made semiautomatic robotic slide system (Instrumedics Inc., Hackensack, NJ). An overview of an H&E-stained TMA section is shown in Fig. 1.

**Fluorescence In situ Hybridization.** A set of TMA sections was used for two-color FISH. For proteolytic slide pretreatment, a commercial kit was used (Paraffin pretreatment reagent kit, Vysis, Downers Grove, IL). Spectrum-Green-labeled gene-specific probes were used together with Spectrum-Green-labeled probes for the respective centromere region as a reference. All probes were provided by Vysis Inc. The probe combinations were HER2/ Centromere 17 (PathVysion), EGFR (LSI EGFR SpectrumOrange)/centromere 7 (CEP 7 SpectrumGreen), MDM2 (SpectrumOrange)/centromere 12 (CEP 12 SpectrumGreen), CCND1 (LSI Cyclin D1 SpectrumOrange)/centromere 11 (CEP 11 SpectrumGreen), and MYC (LSI c-myc SpectrumOrange)/centromere 8 (CEP 8Z2 SpectrumGreen). Before hybridization, TMA sections were deparaffinized, air dried, and dehydrated in 70, 85 and 100% ethanol followed by overnight hybridization at 37°C in a humidified chamber, slides were washed and counterstained with 0.2 μmol/L 4',6-diamidino-2-phenylindole in an antifade solution. For each tumor, the predominant gene and centromere copy numbers were estimated. A gene was considered amplified if the ratio of oncogene/centromere was ≥2.0.

**Immunohistochemistry.** Standard indirect immunoperoxidase procedures (ABC-Elite, Vector Laboratories, Burlingame, CA) in combination with monoclonal antibodies were used for detection of ER (NCL-L-ER-6F11, 1:1,000, Novocastra Labs, Newcastle upon Tyne, United Kingdom) and PR (NCL-L- PGR-312, 1:1,000, Novocastra Labs). Diaminobenzidine was used as a chromogen. Tumors with known positivity were used as positive controls. The primary antibody was omitted for negative controls. All slides were manually read by one pathologist (G. S.). Tumors were considered positive for ER and PR, if unequivocal nuclear positivity was seen in at least 10% of tumor cells (36).

**Statistics.** Contingency table analysis and χ² tests were used to study the relationship between molecular features and tumor phenotype. Survival curves were plotted according to Kaplan-Meier. A log-rank test was applied to examine the relationship between molecular or histologic data and raw survival. Cox proportional hazard model with stepwise selection of the covariates was used to determine the variables with greatest influence on patient survival.

**RESULTS**

**Technical Aspects.** The number of interpretable cases varied between the different FISH probes. Between 16% (MDM2) and 32% (MYC) of the analyses were noninformative because of the absence of tissue on the TMA, lack of unequivocal tumor cells in the arrayed sample, or insufficient hybridization. All TMA sections were only hybridized once. No attempts were made to increase the number of informative cases by additional experiments under different conditions because the absolute number of interpretable cases was considered large enough for the purpose of this study. Examples of amplified and nonamplified tumors are shown in Fig. 1.

**Gene Amplifications, Tumor Phenotype, and Prognosis.** The frequency of gene amplifications was 17.3% for HER2, 0.8% for EGFR, 5.3% for MYC, 20.1% for CCND1, and 5.7% for MDM2 among interpretable cases. The gene copy numbers per cell in amplified tumors ranged between 4 and 20 for EGFR, 4 and 100 for HER2, 4 and 50 for MDM2, 4 and 30 for CCND1, and 4 and 100 for MYC. Among amplified tumors, the fraction of cases with high-level amplification (>10 gene copies per tumor) was 31% for EGFR, 88% for
GENE AMPLIFICATION IN BREAST CANCER

Table 2. Gene amplifications and tumor phenotype

<table>
<thead>
<tr>
<th>GENE</th>
<th>HER2 amp (%)</th>
<th>HER2 P value</th>
<th>EGFR amp (%)</th>
<th>EGFR P value</th>
<th>MYC amp (%)</th>
<th>MYC P value</th>
<th>CCND1 amp (%)</th>
<th>CCND1 P value</th>
<th>MDM2 amp (%)</th>
<th>MDM2 P value</th>
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<td>All tumors*</td>
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<td>17.3</td>
<td>1797</td>
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<td>457</td>
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<td>450</td>
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<td>484</td>
<td>4.1</td>
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<td>13.5</td>
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<td>727</td>
<td>0.3</td>
<td>0.0219</td>
<td>606</td>
<td>3.1</td>
<td>&lt;0.001</td>
<td>721</td>
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<td>P value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<td>0.001</td>
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<td></td>
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<td></td>
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<tr>
<td>G3</td>
<td>567</td>
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<td>612</td>
<td>1.6</td>
<td>530</td>
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<td>0.0218</td>
<td>630</td>
<td>0.6</td>
<td>NS</td>
<td>538</td>
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<td>NS</td>
<td>620</td>
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<td>pT2</td>
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<td>0</td>
<td>842</td>
<td>0.9</td>
<td>94</td>
<td>8</td>
<td>108</td>
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<td>pT3</td>
<td>184</td>
<td>22.3</td>
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<td>206</td>
<td>1</td>
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<td>199</td>
<td>22.6</td>
<td>208</td>
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<td>pT4</td>
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<td>760</td>
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<td>644</td>
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<td>Medullary</td>
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<td>48</td>
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<td>52</td>
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<td>Tubular</td>
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<td>46</td>
<td>0</td>
<td>41</td>
<td>2.4</td>
<td>45</td>
<td>2.2</td>
<td>49</td>
<td>4.1</td>
</tr>
<tr>
<td>Others</td>
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<td>17.7</td>
<td>145</td>
<td>1.4</td>
<td>119</td>
<td>5</td>
<td>143</td>
<td>9.8</td>
<td>147</td>
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<tr>
<td>ER−</td>
<td>368</td>
<td>36.7</td>
<td>&lt;0.001</td>
<td>400</td>
<td>2.2</td>
<td>0.001</td>
<td>341</td>
<td>11.1</td>
<td>&lt;0.001</td>
<td>398</td>
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<td>ER+</td>
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<td></td>
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<td>1094</td>
<td>3.6</td>
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<td>PR−</td>
<td>963</td>
<td>21.8</td>
<td>&lt;0.001</td>
<td>1072</td>
<td>0.9</td>
<td>NS</td>
<td>920</td>
<td>7.2</td>
<td>&lt;0.001</td>
<td>1087</td>
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<tr>
<td>PR+</td>
<td>533</td>
<td>9.4</td>
<td>581</td>
<td>0.5</td>
<td>529</td>
<td>2.5</td>
<td>577</td>
<td>23.2</td>
<td>598</td>
<td>5.4</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant.

* Number of tumors that were successfully analyzed.
† for ductal versus lobular.
‡ for ductal versus medullary.

HER2, 45% for MDM2, 18% for CCND1, and 30% for MYC. All associations between gene amplifications and tumor phenotype or ER/PR status are shown in Table 2. All amplifications were strongly associated with high grade. The relationships of amplifications with local tumor extension (pT category) and nodal metastasis were much weaker or nonexistent. Only HER2 amplifications were weakly associated with advanced pT category. HER2 and MYC amplifications were weakly correlated to pN category. This was caused by a strong increase of the amplification frequency from pN1 to pN2 whereas the differences between pN0 and pN1 were less significant. The separate analysis of different tumor subtypes revealed strong associations of HER2 amplifications with ductal and of MYC amplifications with medullary subtype. MYC amplifications were almost 3 times more frequent in medullary cancers (15.9%) than in the subtype with the second highest frequency of MYC amplification (ductal cancer, 5.6%). The comparison with ER and PR status revealed a strong link of HER2 and MYC amplifications to ER/PR negativity whereas CCND1 amplification was linked to ER/PR positivity. Because CCND1 amplifications were linked to high tumor grade (P < 0.001) and high grade is linked to ER/PR negativity (P < 0.001), this latter result was caused by a particularly high prevalence of CCND1 amplifications in the small subgroup of 313 ER-positive grade 3 cancers (32.9%). In contrast CCND1 amplification was only found in 11.4% of 280 ER-negative grade 3 cancers (P < 0.001). EGFR amplifications were associated with ER-positive cancers whereas MDM2 amplifications were about equally frequent in receptor-positive and -negative cancers.

HER2 amplification was most strongly associated with poor patient prognosis. This was not only evident in the combined analysis of all tumors (P < 0.001, Fig. 2A) but also in separate analyses of nodal-positive (P < 0.001), nodal-negative (P = 0.0048), or ductal carcinomas (P < 0.001). MYC amplification was also associated with poor patient prognosis (P < 0.001, Fig. 2B). This could also be observed in the subgroups of ductal carcinomas (P < 0.001) and nodal-positive tumors (P < 0.001). Only a tendency toward poor prognosis could be observed in the analysis of the prognostic impact of CCND1 amplification in all cancers (P > 0.05, Fig. 2C).

Subgroup analyses of subtypes of ER-positive cancers revealed a slightly worse prognosis of amplified tumors as compared with nonamplified cancers (P < 0.05, Fig. 2D). No association with prognosis could be found for MDM2 amplifications (Fig. 2E) and EGFR amplifications (Fig. 2F). The latter was expected because of the few amplified cancers.

Coamplifications. A subset of 1,106 breast carcinomas with FISH data for all five genes was separately analyzed to determine the significance of coamplifications. Table 3 shows the observed coam-
plification frequencies together with the expected frequencies (based on the frequencies of the individual amplifications), the relative risk ratio (true frequency/expected frequency), and the P values of each association. These data show that all coamplifications occur at least slightly more frequently than to be expected based on the individual frequencies. This is consistent with the hypothesis that tumors, which are sufficiently genetically unstable to develop one gene amplification, have an increased probability to develop multiple amplifications.

For example, tumors with HER2 amplification had a ≥2.5-fold increased likelihood to also have MYC (P = 0.004) or EGFR amplifications. Tumors with MHC amplification had a 2.5-fold increased probability to also have MDM2 amplifications. In contrast, the probability of developing MDM2, EGFR, HER2, or MYC amplifications was only mildly increased in tumors with CCND1 amplifications. Accordingly, most associations between CCND1 amplifications and other associations were not significant. The likelihood of having at least one additional amplification was only about 30% in CCND1-amplified cancers. This was significantly less frequent than for example in cancers with MDM2, MYC, or EGFR amplification with other amplifications being detectable in >50% of cases (Fig. 3). The prognostic significance of coamplifications of two oncogenes is shown in Fig. 4. Breast cancer patients with HER2/MYC coamplification had a substantially worse outcome compared with patients harboring a single gene amplification (Fig. 4A). In contrast, the combined analysis of CCND1 and HER2 (Fig. 4B) or CCND1 and MYC (Fig. 4C) showed that prognostic relevance was mostly driven by the HER2 or MYC gene amplification whereas the impact of CCND1 amplification was minimal. Remarkably, there was also a strong association between patient survival and the number of amplifications (Fig. 5).

Multivariate Analysis. Among the examined gene amplifications and coamplifications, only HER2 amplification (P = 0.0031) had an influence on prognosis, which was independent from BRE grade (P < 0.001), pT (P = 0.0048), and pN (P < 0.001) categories in a multivariate analysis.

Table 3 Observed and expected coamplifications in 1,106 breast cancers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Observed</th>
<th>Expected</th>
<th>Risk Ratio</th>
<th>P value</th>
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<tbody>
<tr>
<td>HER2</td>
<td>1</td>
<td>2.6</td>
<td>0.45</td>
<td>0.001</td>
</tr>
<tr>
<td>MYC</td>
<td>1.2</td>
<td>1.4</td>
<td>0.57</td>
<td>0.06</td>
</tr>
<tr>
<td>CCND1</td>
<td>0.2</td>
<td>0.3</td>
<td>1.5</td>
<td>0.65</td>
</tr>
<tr>
<td>EGFR</td>
<td>0.07</td>
<td>0</td>
<td>0.32</td>
<td>nc</td>
</tr>
</tbody>
</table>

Abbreviations: exp, expected; obs, observed; RR, relative risk; nc, not calculable.

**DISCUSSION**

This project was designed to clarify the association between gene amplification and the prognosis of breast cancer patients. More than 1,700 tumors were analyzed successfully on TMAs. Most of our data obtained on TMAs looking at individual genes were consistent with data from previous literature. In addition, the many tumors allowed us to analyze the relationship between different amplifications and their combined importance.

Overall, the coamplification data are consistent with an “amplificator” subgroup of breast cancers with an increased level of genomic instability and high likelihood for amplification development. Histologically these tumors are mostly grade 3 cancers. Tumors having one detectable amplification in our study also had an increased likelihood of having amplifications of all other examined genes. More than 40% of cancers having detectable HER2 amplification, and >50% of tumors having MYC, CCND1, MDM2, or EGFR amplifications had more than one gene amplified. It is likely that this figure would be even higher after a more comprehensive survey involving more than the five genes selected for this study. The potential value of an extensive amplification survey in breast cancer is emphasized by the prognostic significance of the number of amplifications shown in this study. This result is in line with results from a previous study analyzing 640 tumors for 8 different amplicons (26). From all these data it seems that the number of amplifications may serve as a surrogate variable for the level of genomic instability of a tumor. Genomic instability has key importance for the development of new genetic alterations in tumor cells required for the acquisition of additional tumor properties needed for progression (e.g., ability to metastasize, expression of growth factors, and induction of angiogenesis). A more comprehensive analysis of the clinical and prognostic utility of DNA copy number changes could be facilitated by matrix or array comparative genomic hybridization technology allowing a simultaneous gene copy number analysis of hundreds or thousands of genes (37, 38).
Our large-scale analysis also provided strong data on the potential importance of individual genes. Obviously the need for additional information was least for HER2 as more than 2,000 publications had appeared previously on HER2 alterations in breast cancer. However, the confirmation of all previously established associations between HER2 amplification and tumor phenotype and prognosis provided strong additional evidence for the utility of the TMA approach analyzing just one piece of tissue measuring 0.6 mm in diameter and also showed the validity of the clinical data associated with our breast cancer TMA. In this study, HER2 amplification was strongly linked to high-grade tumors, ER/PR negativity, and unfavorable prognosis. Remarkably, the association with prognosis was independent of pN, pT, and BRE grade. This further emphasizes the value of HER2 amplification analysis in the routine work-up of newly diagnosed breast cancers. Previous studies that had failed to identify the prognostic role of HER2 alterations in breast cancer had either used immunohistochemistry (39–41) or less reliable methods for amplification detection like Southern blot (22, 26), dot blot (42), or PCR (43).

A prognostic importance of HER2 amplification was found in all previous studies that used FISH for their analyses (44–47). A significant association with high-grade tumors, ER/PR negativity, HER2 amplification, and poor prognosis was also found for MYC amplifications. Previous studies on the role of MYC amplifications in breast cancer had provided controversial results. For example, several studies including one study analyzing >400 tumors (48) had not found a prognostic relevance for MYC amplifications (4, 18, 49). Other studies had linked MYC amplifications to unfavorable tumor phenotype such as inflammatory carcinoma (50); high tumor grade (51); risk of recurrence (52); high cell proliferation, tumor size, and nodal status (22); estrogen receptor negativity (19, 29); and also to shortened disease-free and overall survival (23, 26). Interestingly, a striking association with medullary tumor phenotype was found for MYC amplification in this study. This is consistent with other evidence suggesting a peculiar biology of this rare breast cancer subtype. Medullary breast cancer has been linked to familial breast cancer (BRCA1; ref. 53), KIT overexpression (54), and (despite its high grade phenotype and high Ki67 labeling index; ref. 55), a relatively good prognosis (56).

MDM2 amplification was found in 5.7% of tumors and was unrelated to patient prognosis. Our frequency of positive cases is in line with previous studies finding MDM2 amplification in 4 to 7.7% of tumors (6, 30, 31). The prognostic relevance of MDM2 has not been clarified yet. One previous study, in which Southern blot was used for amplification analysis, had reported an association with poor survival in node-negative tumors (26). A similar subgroup analysis failed to identify significant associations in our study. Interestingly, MDM2 amplifications were linked to ER and PR positivity in a large previous study (29). This could not be confirmed in this study. It is remarkable, however, that MDM2 amplification was not associated with ER and PR negativity, despite its association with high tumor grade. This can only be explained by an increased likelihood of MDM2 amplification in ER-positive grade 3 tumors. Indeed, in a separate analysis of grade 3 tumors, MDM2 amplification was slightly more frequent in ER-positive (10.1%) than in ER-negative cancers (6%, P = 0.07). Previous data had linked CCND1 amplifications to a more benign phenotype, especially ER and PR positivity (27, 29, 57). The lack of a clear-cut association for CCND1 amplifications with prognosis was therefore not surprising. Most previous studies had also failed to show a significant association between CCND1 amplifications and poor prognosis (27, 29, 57) Interestingly, CCND1 amplification was still associated with high tumor grade, and there was a tendency of CCND1 amplification toward poor prognosis. This reached borderline significance if ER-positive tumors were analyzed. From these data it seems that among the ER-positive tumors, CCND1 amplification pinpoints toward a genetically instable subgroup with high-grade morphology and a relatively poor prognosis.

The results of this study are also relevant for the potentially rate-limiting question of the optimal number of tissue pieces needed per tumor on a TMA. Previous publications have emphasized that including 3 to 4 tissue cores per tumor into a TMA resulted in a good concordance between TMA and large section immunohistochemistry results (35). Some authors therefore recommended using multiple cores as a standard procedure for TMAs (35). However, studies
showing superiority of this more time- and tissue-consuming approach for discovering clinicopathologic associations are lacking. In contrast, in a combined TMA and large section analysis of >500 breast cancers, we found associations between positive p53 immunostaining and poor prognosis for four different TMAs (composed of one tissue sample per tumor each) but not for large sections (35). Because the large sections were considered p53 positive much more frequently than TMA spots, it must be assumed that many positive results on large sections represented either biologically irrelevant focal findings or local artificial staining. Because of the size of the tissue area to be stained, artificial staining results are a much greater problem on large sections than on TMAs. The strong associations found in this study between amplifications, tumor phenotype, and prognosis is strong additional documentation of the suitability of large TMAs containing just one tissue sample per tumor for efficient translational research (35).

In summary, the results of this study suggest a considerable prognostic relevance of gene amplification in breast cancer. This applies not only for HER2 or MYC analysis as individual parameters but most of all for a combination of multiple amplicons. Additional studies are needed to investigate whether a more comprehensive study of gene amplification across the genome would be able to further increase the prognostic value of amplification detection.

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Prognostic Relevance of Gene Amplifications and Coamplifications in Breast Cancer

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