Marked Intratumoral Heterogeneity of *c-myc* and *CyclinD1* But Not of *c-erbB2* Amplification in Breast Cancer

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SUMMARY: Intratumoral heterogeneity mirrors subclonal diversity and might affect treatment response. To investigate molecular heterogeneity of primary breast cancer specimens, we determined the amplification status of growth regulatory genes (*c-erbB2*, *topoisomerase II α* , *c-myc*, and *cyclinD1*) in macroscopically and microscopically separate areas of individual tumors ($n = 21$). Using laser-assisted microdissection and quantitative PCR, we found marked intratumoral heterogeneity with different patterns for each gene. Molecular heterogeneity in amplification pattern could be demonstrated between both macroscopically (0.5 to several centimeters) and microscopically (10 to several hundred micrometers) distant tumor areas. *C-erbB2* amplification proved to be the most stable amplification in individual tumors, with heterogeneity occurring in only 36% of amplified cases. By contrast, amplification of *c-myc* and *cyclinD1* revealed varying patterns in the vast majority of amplified cases (100% and 83%). The constancy of *c-erbB2* amplification underlines its presumed importance in breast cancer biology. We conclude that the molecular heterogeneity of breast cancer as evidenced in this study requires thorough and representative sampling of different tumor areas when the biologic significance of somatic mutations is considered. Patterns of heterogeneity can be used to trace the clonal evolution within different compartments of an individual tumor. (*Lab Invest* 2002, 82:1419–1426).

Intratumoral heterogeneity within individual breast cancers has been evidenced by studies on allelic loss (Fujii et al, 1996; Shen et al, 2000). The coexistence of several cell clones with differing biologic characteristics within a malignant breast tumor is supposed to arise during tumor progression (Fujii et al, 1996) as a consequence of growing genetic instability (Shen et al, 2000). Tumor heterogeneity might contribute to overcome host defense against invasion and metastasis and to develop resistance to anticancer drugs (Kuukasjarvi et al, 1997). In breast cancer, a frequent mechanism of oncogene activation is provided by gene amplification. Affected are growth regulatory genes such as *c-erbB2*, *topoisomerase II α* (*topoll α*), *c-myc*, and *cyclinD1* (Bieche and Lidereau, 1995). Among these, *c-erbB2* (*HER2/neu*) has been shown to have particular diagnostic, prognostic, and therapeutic relevance (Cooke et al, 2001). There are concerns that molecular heterogeneity for *c-erbB2* amplification within a tumor, or between primary and metastatic cancer foci, may affect treatment response to the human anti-HER2/neu-antibody Trastuzumab

because of selection of resistant subclones lacking this alteration (Thor, 2001). Similarly, an intratumoral heterogeneity for *topoll α* amplification with partial amplification loss leads to altered chemosensitivity to *topoll α* inhibitors like Doxorubicin (Jarvinen et al, 2000).

However, because of practical restrictions and technical difficulties, studies focusing on intratumoral heterogeneity of gene amplification in breast cancer are sparse (Jarvinen et al, 2000; Lonn et al, 1994; Pertschuk et al, 1999; Szollosi et al, 1995). For diagnostic purposes in clinical management, it is customary to analyze one representative sample of an individual breast tumor, and comprehensive sampling from surgical specimens for research studies is usually difficult. Moreover, breast carcinomas often induce marked desmoplastic or inflammatory stromal reactions, which might lead to contamination with non-neoplastic cells (Slamon et al, 1989). As a consequence, for exact analysis of intratumoral heterogeneity in breast cancer, precise microdissection techniques in combination with sensitive PCR-based methods on small cell groups of archival tissue are required to yield the necessary answers.

Therefore, we combined laser-assisted microdissection and quantitative TaqMan PCR in this study to analyze amplification of growth regulatory genes in 21 archival breast cancer specimens. To get an impression of intratumoral heterogeneity, we performed multiple sampling from macroscopically and microscopically distant tumor areas.

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Results

Analysis of Macroscopically Distant Tumor Areas in Different Paraffin Blocks

In a first approach to examine a possible intratumoral heterogeneity, we compared macroscopically distant tumor areas in different paraffin blocks dissected from individual specimens. In detail, we chose 2 to 6 tumor blocks each from a total of 10 breast cancer specimens, depending on the size of the primary tumor (Table 1). From each block, all available intraductal and invasive tumor lesions were collected by laser microdissection and pooled for subsequent PCR analysis. This approach enabled determination of regional amplification heterogeneity in distances of 0.5 centimeter up to several centimeters. The PCR results of macroscopically dissected tumor areas in different blocks from individual specimens are summarized in Table 1.

Among the 10 cases studied by macrodissection, 6 tumors altogether revealed intratumoral heterogeneity regarding the amplification pattern of all genes under investigation. In detail, five of seven tumors with *c-erbB2* amplification presented increased *c-erbB2* copy numbers throughout all samplings, whereas the remaining two amplified cases revealed heterogeneous *c-erbB2* amplification patterns with loss of amplification in the invasive sample of one block (Case 3) or isolated amplification in only one of the intraductal samples (Case 7). None of the cases in this group exhibited *topoll α* amplification. In contrast, all three *c-myc*-amplified cases (Cases 1, 4, and 5) revealed intratumoral heterogeneity for *c-myc* amplification in association with a homogeneously positive *c-erbB2* amplification status. The four tumors with *cyclinD1* amplification showed homogeneous (Cases 2 and 9) and heterogeneous (Cases 5 and 8) amplification patterns in two cases each.

Analysis of Microscopically Distant Tumor Areas Within One Paraffin Block

To investigate intratumoral heterogeneity on a microscopical level, we compared distinct tumor lesions

within one single paraffin block. One representative block was selected for further analysis in 13 cases (Table 2). In each case, we dissected six intraductally growing tumor cell groups from the same block into separate samples for subsequent PCR analysis. Additionally, to examine a possible heterogeneity within one focus, we collected two samples of each selected lesion by separately sampling the corresponding cell groups from consecutive serial step sections (see "Materials and Methods"). The aims of this approach were to investigate microheterogeneity on both the interlesional and the intralesional level between distinguishable tumor foci.

The results of the PCR analyses of microdissected intraductal tumor cell samples within 1 single block from 13 individual breast cancer specimens are summarized in Table 2. Eleven of 13 tumors investigated for microheterogeneity revealed varying amplification patterns in multiple samples of distinct tumor cell populations. In accordance with the macroheterogeneity described above, considerable variability between the different genes under study was evident.

Homogeneity of *c-erbB2* amplification status was found in four amplified cases and in another six nonamplified cases. Three cases revealed a heterogeneous *c-erbB2* amplification pattern. Four *c-erbB2*-amplified tumors revealed a heterogeneous coamplification of *topoll α* . In contrast to *c-erbB2*, all nine tumors with *c-myc* amplification revealed intratumoral heterogeneity, with only one to three positive foci of intraductal cancer. Similarly, all eight tumors with *cyclinD1* amplification revealed intratumoral heterogeneity of intraductally growing tumor cells.

Differences Between In Situ and Invasive Tumor Components

As shown in Table 1, heterogeneity of amplification status with regard to in situ and invasive tumor component could be observed. Most frequently this phenomenon occurred with *c-myc* in 3 of 10 cases. Differences in the copy numbers of *cyclinD1* between both tumor components were found in two cases. Only 1 of 10 cases revealed heterogeneous *c-erbB2*

Table 1. Amplification Status of Intraductal and Invasive Samples from Macroscopically Distant Tumor Areas (blocks)

Case no.	Blocks n	Results of intraductal tumor samples				Blocks n	Results of invasive tumor samples			
		<i>c-erbB2</i>	<i>topollα</i>	<i>c-myc</i>	<i>cyclinD1</i>		<i>c-erbB2</i>	<i>topollα</i>	<i>c-myc</i>	<i>cyclinD1</i>
1	4	■■■■	□□□□	■■■□	□□□□	1	■	□	□	□
2	2	■■	□□	□□	■■	2	■■	□□	□□	■■
3	4	■■■■	□□□□	□□□□	□□□□	3	■■□	□□□	□□□	□□□
4	4	■■■■	□□□□	■■■□	□□□□	1	■	□	□	□
5	2	■■	□□	■□	□□	2	■■	□□	□□	■□
6	2	■■	□□	□□	□□	2	■■	□□	□□	□□
7	2	■□	□□	□□	□□	2	□□	□□	□□	□□
8	4	□□□□	□□□□	□□□□	■■■□	6	□□□□□□	□□□□□□	□□□□□□	□□□□□□
9	3	□□□	□□□	□□□	■■■	3	□□□	□□□	□□□	■■■
10	2	□□	□□	□□	□□	2	□□	□□	□□	□□

■, amplified; □, not amplified; each square represents one block.

Table 2. Amplification Status of Microscopically Distant Intraductal Foci Within One Representative Block of Breast Cancer Specimens

Case no.	Results of six distinct intraductal tumor lesions in two separate samples each			
	<i>c-erbB2</i>	<i>topoIIα</i>	<i>c-myc</i>	<i>cyclinD1</i>
6	■ ■ ■ ■ ■ ■ ■ ■	□ □ ■ □ □ □ □ □	□ □ □ ■ ■ □ □ ■	□ □ □ □ □ □ □ □
7	■ ■ □ ■ ■ ■ ■ ■	■ ■ □ □ ■ ■ □ □	□ □ □ □ ■ □ □ □	■ ■ □ ■ ■ ■ □ □
11	■ ■ ■ ■ ■ ■ ■ ■	□ □ □ □ □ □ □ □	■ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □
12	■ ■ ■ ■ ■ ■ ■ ■	□ ■ ■ ■ ■ ■ ■ ■	■ ■ □ □ □ □ □ □	□ □ ■ ■ □ □ □ □
13	■ ■ ■ ■ ■ ■ ■ ■	■ ■ ■ ■ □ □ ■ ■	■ ■ □ □ □ □ □ □	□ □ □ □ □ □ □ □
14	□ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □	■ □ □ □ □ □ □ □	□ □ □ □ □ □ ■ □
15	■ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □	□ ■ □ □ □ □ □ □
16	□ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □
17	□ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □
18	□ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □	□ ■ □ □ ■ ■ □ □	□ □ □ □ ■ □ □ □
19	□ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □	■ □ □ ■ □ □ ■ □	□ □ □ □ ■ □ □ □
20	□ ■ □ □ □ □ ■ ■	□ □ □ □ □ □ □ □	□ □ □ ■ ■ □ □ □	■ ■ ■ ■ □ ■ ■ ■
21	□ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □	□ □ □ □ □ □ □ □	□ □ □ ■ □ □ □ ■

■, amplified; □, not amplified; paired squares indicate the results of the two samples obtained from each focus.

amplification status between both growth components (Case 7). In this case, a loss of amplification was noticed in the invasive component but also already in one of two intraductal samples.

In addition, for further determination of heterogeneity between intraductal and invasive tumor components, we separated invasive and noninvasive cell populations from the same block in three cases (Cases 7, 11, and 18). In previous analyses of representative intraductal and invasive tumor components, these tumors had shown different *c-erbB2* amplification patterns, with constant amplification in intraductal and invasive components in Case 11, loss of amplification in the invasive component in Case 7, and lack of amplification in both components in Case 18. In detailed analysis, Case 11 revealed a homogeneously positive *c-erbB2* amplification status in all tumor cell samples and Case 18 a homogeneously negative *c-erbB2* amplification status throughout all samples. In contrast, Case 7 presented marked intratumoral heterogeneity for all genes under study in detailed analysis (Fig. 1). For quantitative analysis of *c-erbB2* amplification in both study groups see Figure 2.

Discussion

In this study, we determined intratumoral heterogeneity for gene amplifications in 21 ductal infiltrating breast cancer specimens using a combination of laser-assisted microdissection and kinetic PCR. In an attempt to examine the expansions of potential divergent subclones from centimeter to micrometer dimensions, we macrodissected and microdissected 21 breast cancer specimens.

Macrodissection showed a constancy of *c-erbB2* amplification status in the majority of amplified cases. By contrast, striking variability in *c-myc* and *cyclinD1* amplification status was evident between spatially distant samples of individual tumors. When pooling various intraductal or invasive tumor cell foci from one

macrodissected tumor area, we could not rule out that homogeneity of *c-erbB2* amplification might be a result of leveling out a hidden microheterogeneity.

Consequently, our second approach was aimed at determination of microheterogeneity between tumor foci within one block. In microdissected tumor foci, we also observed frequent homogeneity of *c-erbB2* amplification status. However, in three of seven amplified cases, microdissection revealed intratumoral heterogeneity of *c-erbB2* status on the interlesional and intralesional levels (Table 2). To a far greater extent, this intratumoral heterogeneity was also observed for all other genes under study, when neighboring foci were microdissected and individually analyzed.

Altogether, in both approaches, we found *c-erbB2* amplification to be by far more stable than amplification of the other genes under study. This constancy of *c-erbB2* genotype might reflect the substantial growth advantage that subclones carrying this amplification gain through increased cell motility (De Corte et al, 1994) and accelerated growth factor-independent proliferation (Lane et al, 2000). In expression studies, *c-erbB2* status was found to be concordant in about 95% of cases even between primary and metastatic lesions (Menard et al, 2001), which also points to conservation of *c-erbB2* status during further disease progression.

Our results slightly differ from those of a previous study that reported an extensive intratumoral heterogeneity for *c-erbB2* status in intra-assay and interassay comparisons using fluorescence in situ hybridization (FISH), slot-blot assay, and immunohistochemistry when analyzing several blocks of primary breast tumors (Pertschuk et al, 1999). One important reason for this discordance may be that Pertschuk and coworkers did not apply microdissection. Contamination of samples with non-neoplastic stroma cells may lead to false-negative results in slot-blot assay. Lonn et al (1994) reported a more frequent intratumoral heterogeneity for *c-erbB2* amplification using a semiquantitative PCR in fine-

Figure 1.

Amplification patterns of Cases 7, 11, and 18. A, Case 7 with intratumoral heterogeneity for all genes under study in intraductal lesions I and III to VI shows complete lack of gene amplification in one single intraductal (II) as well as all invasive foci (1–3). For topographic and histologic appearance see Figure 4. B, Case 11 reveals a constant *c-erbB2* amplification in all intraductal (I–VI) and invasive (1–3) tumor lesions. Note the peak amplification with amplification factor of 40.6 (corresponding to about 82 gene copies) in lesion I versus intermediate- to high-level amplification in the other lesions ranging from 8.8-fold in invasive focus 3 to 18.9-fold in intraductal focus III. C, Case 18 with homogeneous lack of *c-erbB2* amplification in all intraductal (I–VI) and invasive (1–3) lesions reveals intratumoral heterogeneity for *c-myc* and *cyclinD1*. Note the inconsistent pattern of *c-myc* amplification in intraductal lesions I and IV and in invasive lesion 3. *CyclinD1* amplification was found in intraductal lesion IV as well as all invasive lesions (1–3). For topographic and histologic appearance see Figure 3.

needle aspirates from two to four lesions (metastases/local recurrences) in women treated with chemotherapy. Because this study was focused on effects of therapy on

late stage disease, no primary breast carcinomas were included.

In quantitative analysis of *c-erbB2* amplification, we found moderate variability of amplification levels between different samples of an individual tumor, which was grossly confined within the (arbitrary) limits of low-, intermediate-, and high-amplification levels (Fig. 2). Remarkably, individual samples of highly amplified tumors revealed peak values with amplification factors up to 40.6. In keeping with this finding, FISH studies reported the existence of subclones carrying extremely high copy numbers in uncultured *c-erbB2*-amplified primary breast cancers (Kallioniemi et al, 1992).

All four cases with *topollα* amplifications were coamplified for *c-erbB2*, supporting the concept of an extensive amplicon comprising both closely neighboring gene loci (Smith et al, 1993). This consistent coamplification is particularly evident in Case 7 with heterogeneous *c-erbB2* amplification pattern. However, the complex *c-erbB2/topollα* coamplification patterns in all *topollα*-amplified cases points to the coexistence of subclones either harboring or lacking *topollα* amplifications. In keeping with this finding, Jarvinen et al (1999) previously reported an intratumoral heterogeneity in *topollα* status in 8 (14%) of 59 high-level *c-erbB2*-amplified primary breast tumors using FISH.

In contrast to *c-erbB2*, we found an extensive intratumoral heterogeneity for *c-myc* and *cyclinD1* amplification. Initial observations reported that *c-myc* overexpression precedes its gene amplification (Mai, 1994) and plays a role in amplification of multiple other genes as well as in other chromosomal instability events (Mai et al, 1996). These studies imply that mechanisms other than gene amplification are likely to be responsible for *c-myc* regulation in breast cancer (Liao and Dickson, 2000). Similarly, the incoherence of *cyclinD1* amplification and overexpression in breast cancer (Buckley et al, 1993) also indicates that other, more complex mechanisms besides amplification may be decisive for *cyclinD1* regulation. With regard to these findings, the remarkable intratumoral heterogeneity for *c-myc* and *cyclinD1* amplification might rather reflect an inconsistent epiphenomenon as a consequence of growing genetic instability in the clonal evolution of breast cancer.

For determination of heterogeneity between intraductal and invasive tumor components, we additionally examined invasively growing cell populations in three cases. In two of these cases, we could demonstrate clonal divergence between the intraductal and the invasive tumor components. However, single intraductal tumor lesions resembled the amplification pattern of the invasive subclone, indicating their clonal relationship (Fig. 1). It can be speculated, therefore, that these single intraductal lesions represent either the intraductal precursor or an intraductal spread of the invasive tumor component.

In conclusion, we found a considerable intratumoral heterogeneity for amplification of growth regulatory genes in primary breast cancers, with higher incidence in detailed analyses of interlesional and intralesional

Figure 2.

Quantitative analysis of *c-erbB2* amplification in macrodissected tumor areas in multiple blocks (A) versus microdissected tumor lesions within one block (B). A, Quantitative results of all amplified block samples of Cases 1 to 7 are shown. In semiquantitative analysis, measurements of different blocks from individual tumors grossly confine to ranges of low (amplification factor 2–5), intermediate (>5–10), and high (>10) level amplification. (* indicates heterogeneous cases.) B, Quantitative results of all lesions revealing *c-erbB2* amplifications in Case Group 2 are shown. Note the peak value of individual lesions from high-amplified cases (Case 11 and 12) and the quantitative intratumoral heterogeneity of Case 7, which presented low-level amplification in the block sample (A) because of a mixture of foci with varying degrees or complete lack of amplification. (* indicates heterogeneous cases.)

heterogeneity. Among the genes under study, *c-erbB2* represented the most stable characteristic of individual tumors, whereas *cyclinD1* and *c-myc* in particular showed extensive intratumoral heterogeneity with inconsistent amplification patterns.

Materials and Methods

Case Selection

All 21 ductal infiltrating breast cancer specimens included in this study were selected because of availability of sufficient archival tumor tissue for analysis of intratumoral heterogeneity. Selected cases were divided into two groups. Group 1 included 10 cases (Table 1). From each of these surgical specimens, two to six areas were macroscopically dissected out of the tumor mass after formalin fixation and embedded into

paraffin blocks. From each block, intraductal or invasive tumor cell populations were microdissected and pooled into one sample for further analysis.

Group 2 comprised 13 cases presenting several clearly distinguishable tumor cell populations on one representative paraffin block (Table 2). Two tumors (Cases 6 and 7) were included in both study groups. For all 13 breast cancer specimens in this group, one single representative tissue block was chosen after review of hematoxylin- and eosin- (HE) stained slides, and 20 consecutive serial sections (3–5 μ m) were cut for further microdissection. On corresponding documentary HE-stained slides, separate tumor lesions were chosen for microdissection and labeled with numbers I to VI for intraductal and 1 to 3 for invasive tumor components on a survey photomicrograph (Figs. 3 and 4). In three cases, we additionally exam-

Case 18

Figure 3.

Laser microdissection in Case 18. A, Photomicrograph of membrane-mounted, uncovered serial section after isolation of distinct intraductal (I–VI) and invasive (1–3) tumor cell groups; methylene blue, original magnification $\times 1.6$. B, Isolated cell groups after needle-picking; methylene blue, original magnification, $\times 200$ (I, II, V, 1–3), $\times 100$ (III, IV), $\times 50$ (VI).

ined three distinct invasive cell populations for comparison of preinvasive and invasive tumor components (Figs. 1, 3, and 4). For three-dimensional analysis of the selected lesions, one PCR sample was collected from the first 10 consecutive serial sections (layer 1–10) and another one from the following 10 serial sections (layer 11–20).

Laser-Assisted Microdissection and Kinetic TaqMan PCR

The specific methods of tissue handling and preparation for laser-assisted microdissection using the P.A.L.M. Laser-MicroBeam System (P.A.L.M. Robot

Microbeam; P.A.L.M., Bernried, Germany) and subsequent real-time PCR using the TaqMan detection system (ABI PRISM 7700 Sequence Detection System; Applied Biosystems, Weiterstadt, Germany) for amplification analysis in archival tissue have been thoroughly described previously (Glöckner et al, 2001, 2000; Lehmann et al, 2000).

For microdissection of distinct tumor cell populations within one representative tissue block, we modified the isolation of the target lesions using needle picking instead of laser pressure catapulting of the selected cell groups (Fig. 3).

A

Case 7

B

Figure 4.

Intratumoral heterogeneity for *c-erbB2* amplification in Case 7. A, Photomicrograph of documentary HE section with intraductal (I–VI) and invasive (1–3) tumor cell groups marked for microdissection on corresponding membrane-mounted slides; HE, original magnification $\times 1.6$. B, Histologic appearance of selected cell groups (HE, original magnification, $\times 200$ for I, III, IV, 1–3; $\times 100$ for II, V, VI) and corresponding PCR plots. Amplification plots of *c-erbB2* (open rhombs) and *app* gene (closed squares) demonstrate marked intratumoral heterogeneity for *c-erbB2* amplification status. Note the high-level amplification in lesions I and IV with a large left-shift of the *c-erbB2* curve in relation to the *app* curve of about five cycles versus low- or intermediate-level amplification in lesions III, V, and VI with smaller left-shifting ranges. Foci II and 1 to 3 lack amplification and reveal a pattern similar to normal tissue (not shown). *App* gene serves as internal reference gene.

Primer and probe sequences have already been published previously (Glockner et al, 2001). In each PCR run using a 96-well reaction plate, one corre-

sponding non-neoplastic tissue sample (lymphocytes, stroma) was included in the sample set for negative control. All reactions were run in duplicate. For evalu-

ation of PCR results, an internal relative calibration method using the *app* gene as an internal reference gene was performed as previously described (Fig. 4) (Glöckner et al, 2001; Lehmann et al, 2000).

For qualitative analysis, a reference range for each test gene was statistically determined from measurements of at least 60 normal tissue samples as follows: reference range = mean (ΔC_T [normal tissue]) $\pm$ 2 SD.

For quantitative analysis, level of amplification was graded as follows: low-level amplification with copy number increase ranging from 2- to 5-fold, intermediate-level amplification from >5 to 10-fold, and high-level amplification above 10-fold (Fig. 2). As previously described, a reduction of the relative C_T value by one cycle corresponds to a level of amplification of 1.8 ± 0.1 (Lehmann et al, 2000).

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