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Amplification of Growth Regulatory Genes in Intraductal Breast Cancer Is Associated with Higher Nuclear Grade but Not with the Progression to Invasiveness

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SUMMARY: Ductal carcinoma in situ (DCIS), as an identifiable progenitor lesion of invasive breast cancer, represents a morphologically, biologically, and prognostically heterogeneous disease. It is not clear which molecular mechanisms are involved in progression to infiltrative growth. In this study, 83 DCIS classified according to the Van Nuys grading scheme were examined for amplification of growth regulatory genes that have been found to be amplified in invasive breast cancer (*c-erbB2*, *topoisomerase IIα*, *c-myc*, and *cyclinD1* genes). Exact quantification of gene amplification was enabled by a combination of laser microdissection of paraffin-embedded tissue with real-time PCR. In DCIS, gene amplifications of all tested genes were found. The most frequently amplified gene was *c-erbB2* found in 21 of 83 (25%) cases. Amplification of the other genes under investigation was observed in 4% to 6% of cases, high-grade DCIS being predominantly affected. High-grade DCIS differed significantly from low- and intermediate-grade DCIS in frequency and level of *c-erbB2* amplification. In addition, high-grade DCIS revealed an accumulation of genetic aberrations. Amplification status in pure in situ lesions did not differ from intraductal carcinoma with an infiltrative component**,** indicating that although associated with a higher nuclear grade gene amplification might not represent an independent prognostic marker of disease progression. (*Lab Invest 2001, 81:565–571*).

Ductal carcinoma in situ (DCIS) is not a uniform entity, but a heterogeneous disease revealing a spectrum of morphologic differentiations (Tavassoli, 1992). The morphologic heterogeneity may correspond to diverse underlying genetic defects that, however, have not been identified so far (van de Vijver, 1998). Considering the phenotypic diversity, various morphologic classification schemes for DCIS have been proposed as an attempt to estimate its biologic potential as a precursor to invasive carcinoma and to predict the risk of local recurrence after breast conserving therapy (Holland et al, 1994; Silverstein et al, 1995).

A recently proposed grading system, designated the Van Nuys (VN) classification, is a simplified, clinically relevant system defining prognostic groups with differing likelihood of local recurrence after excision (Silverstein et al, 1995). This easily applicable classification facilitates categorization of DCIS by grouping it into three grades based on nuclear size and pleomorphism and the presence or absence of necrosis.

The novel grading schemes of intraductal carcinoma have proven their usefulness in several studies (Silverstein et al, 1995). However they share with histopathologic grading protocols in other organs the difficulties concerning standardization and independence of subjective influences such as the individual experience of the histopathologists (Bethwaite et al, 1998; Sloane et al, 1998). To improve the reproducibility of grading, in particular of borderline cases that are difficult to classify, more must be known about the underlying genetic defects that give rise to the various forms of intraductal carcinoma.

The diversity of genetic alterations found in intraductal and invasive breast cancer so far appears to support the hypothesis of different genetic pathways in the clonal evolution of breast cancer (Buerger et al, 1999a, 1999b). In contrast to epithelia at other sites, where a progression from low through intermediate to severe dysplasia and finally invasive carcinoma is generally accepted, a multistep carcinogenesis model for invasive mammary carcinoma through successive precursor lesions has not yet been proven. Recent studies suggest that the clinical behavior of invasive carcinoma may be determined at a preinvasive stage (Gupta et al, 1997).

Among the different genetic mechanisms acting in carcinogenesis, that is, the activation of oncogenes and the inactivation of suppressor genes, oncogene amplification seems to play an important role, especially in the pathogenesis of invasive breast cancer concerning about 20 amplified chromosome regions in

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comparative genomic hybridization analyses (Courjal et al, 1997; Knuutila et al, 1998). The genes most frequently affected by amplification in breast cancer are the genes for c-erbB2, topoisomerase IIα, c-myc, and cyclinD1.

To investigate whether gene amplification is associated with the grading or progression to invasiveness of intraductal carcinoma, we determined the frequency and degree of gene amplification in low- to high-grade DCIS as defined by the VN classification. Laser-based microdissection of paraffin-embedded DCIS specimens was applied to obtain pure tumor cell groups (Lehmann et al, 2000). The subsequent quantitative PCR analysis with the TaqMan detection system (Applied Biosystems, Foster City, California) allowed the objective and reproducible quantification even of low-level amplifications in minute tissue samples (Bieche et al, 1998; Heid et al, 1996). The combination of these two novel technologies provides an excellent tool for studies of genetic alterations in neoplastic preinvasive breast lesions, which are often of limited size or available only in paraffin-embedded tissue (Lehmann et al, 2000).

Results

Gene Amplifications and DCIS

The results of the PCR analyses of all DCIS specimens are summarized in Table 1. Among the 83 cases included in this study, 30 (36%) DCIS specimens displayed gene amplifications.

c-erbB2 amplification was observed in 21 of 83 (25%) cases, representing the most frequent copy number increase of all oncogenes tested. Six tumors revealed low-level amplification (2-fold to <5-fold), whereas 11 cases presented intermediate-level amplification (≥5-fold to <10-fold). The remaining four cases showed high-level *c-erbB2* amplifications (up to 25-fold).

Topoisomerase IIα amplification was found in 3 of 83 (4%) cases. Copy number increase did not exceed 4-fold, corresponding to low-level amplification.

CyclinD1 amplification occurred in 5 (6%) tumors with a copy number increase ranging from 2-fold to 6-fold, corresponding to low- and intermediate-level amplification, respectively.

c-myc amplification was exhibited in 5 (6%) cases, with copy number increase ranging from 3-fold to 4-fold.

Gene Amplifications and VN Classification

DCIS VN1. Eleven cases were classified as low-grade DCIS, VN1. In this group no copy number aberrations of the tested genes were detected.

DCIS VN2. Twelve cases were classified as VN2. Two of these specimens revealed low-level gene amplifications: in one case *c-erbB2* amplification (4-fold) was detected, another case showed *c-myc* amplification (4-fold).

DCIS VN3. Sixty cases were classified as high-grade DCIS, VN3. Altogether, 28 cases showed copy number increase of the tested genes. The most frequent amplification was *c-erbB2* amplification, which was found in 20 (33%) cases. Statistical analysis revealed a highly significant association between high-grade DCIS VN3 and frequency of *c-erbB2* amplification by exact linear trend test ($p = 0.006$), as well

Table 1. Frequency of Gene Amplifications in 83 Cases of Ductal Carcinoma In Situ Correlated with Van Nuy's (VN) Grading and the Absence (pTis) or Presence (pT1–pT2) of Invasive Tumor Components

			c-erbB2	*topo IIα*	*cyclinD1*	*c-myc*
pTNM	Grading	*n*	+/−[a]	+/−	+/−	+/−
pTis	VN1	6	0/6	0/6	0/6	0/6
	VN2	4	1/3 (25%)	0/4	0/4	0/4
	VN3	27	11/16 (40%)	2/25 (7%)	3/24 (11%)	1/26 (4%)
	pap	2	0/2	0/2	0/2	0/2
	cribri	9	0/9	0/9	2/7 (22%)	0/9
	micpap	2	1/1	0/2	0/2	0/2
	solid	8	4/4 (50%)	0/8	0/8	0/8
	mixed	9	4/5 (44%)	2/7 (22%)	0/9	0/9
	comedo	7	3/4 (33%)	0/7	1/6 (14%)	1/6 (14%)
pT1–2	VN1	5	0/5	0/5	0/5	0/5
	VN2	8	0/8	0/8	0/8	1/7 (12%)
	VN3	33	9/24 (27%)	1/32 (3%)	2/31 (6%)	3/30 (9%)
	pap	2	1/1	0/2	0/2	0/2
	cribri	5	0/5	0/5	0/5	0/5
	micpap	1	1/0	0/1	0/1	0/1
	solid	8	4/4 (50%)	1/7 (12%)	1/7 (12%)	1/7 (12%)
	mixed	25	2/23 (8%)	0/25	1/24 (4%)	2/23 (8%)
	comedo	5	1/4 (20%)	0/5	0/5	1/4 (20%)

pap, papillary; *cribri*, cribriform; *micpap*, micropapillary; *mixed*, mixed growth pattern.
[a] +/−, amplified/not amplified.

as *c-erbB2* amplification level by Kruskal-Wallis test ($p = 0.02$).

There were three cases of *topoisomerase IIα* amplifications. Although in two of these cases, a combined *c-erbB2/topoisomerase IIα* coamplification was found, a single case displayed an isolated *topoisomerase IIα* copy number increase. In five cases, a copy number increase of the *cyclinD1* gene was detected. Four DCIS specimens in this group exhibited *c-myc* amplification. One case in this group revealed increased copy numbers of three target genes at different loci, that is, amplification of the *c-erbB2*, *c-myc,* and *cyclinD1* genes. The distribution of gene amplifications exhibited highly significant correlation to grading according to VN classification by exact linear trend test ($p = 0.003$).

DCIS Associated with Invasive Components vs Pure DCIS

In contrast to pure intraductal carcinoma (pTis), in which 12 of 37 (32%) cases exhibited *c-erbB2* amplifications, in DCIS associated with an invasive component (pT1-2), 9 of 46 (20%) cases showed a lower frequency of *c-erbB2* amplification. The comparison of these rates reached no statistically significant difference. Accordingly, no significant differences were observed with the other genes under study.

Discussion

Laser-based microdissection enables molecular studies even on small lesions without contamination by non-neoplastic stroma cells, thus preserving the cytologic and tissue architecture necessary to confirm histopathologic criteria. The subsequent quantitative PCR analysis of the selected cell groups using the TaqMan detection system allows the accurate detection of low-level amplifications even in minute tissue samples (Lehmann et al, 2000).

As target genes we selected four genes found to be frequently amplified in invasive breast cancer (Cuny et al, 2000). *c-erbB2* at locus 17q21–22 encodes a membrane-bound tyrosine kinase receptor (Kallioniemi et al, 1992). In proximate vicinity to *c-erbB2*, the *topoisomerase IIα* gene at 17q12–21 encodes a key enzyme in DNA replication (Kreipe et al, 1993b), which is the molecular target for the topoisomerase IIα-inhibitors that represent important anticancer drugs (Jarvinen et al, 2000). The oncogene *c-myc* at 8q24 is a key regulator of cell cycle progression from G1 to S phase (Kreipe et al, 1993a). The cell cycle regulatory gene *cyclinD1*, sited on locus 11q13, is essential for G1 phase progression and is implicated in the pathogenesis of several human malignancies, including breast cancer (Barnes and Gillett, 1998; Tanner et al, 1998; Vos et al, 1999a).

Investigating the amplification status of these genes in DCIS might elucidate at which stage of evolution of invasive carcinoma these common amplifications occur. Current classifications of DCIS are based on morphologic criteria to define well-, intermediately, and poorly differentiated lesions that have been validated by clinical follow-up and biologic marker studies (Kanthan et al, 2000). Knowledge about molecular alterations in the different types of DCIS so far has been achieved mostly by loss of heterozygosity (LOH) (Fujii et al, 1996) and comparative genomic hybridization (Buerger et al, 1999a; Kuukasjarvi et al, 1997; Moore et al, 1999) studies as suitable screening methods. These studies detected widespread chromosomal abnormalities, particularly in high-grade DCIS. In contrast, this study focuses on amplification of growth regulatory genes in DCIS and relates them to the grading scheme of the VN classification.

We found that oncogene amplification does occur in DCIS. Consequently, it might represent an early step in tumorigenesis. The most common gene amplification detected concerned *c-erbB2*, which appeared in 21 of the 83 examined cases. These data suggest that *c-erbB2* amplification may play an important role in growth factor–independent growth. In keeping with previous studies performed on invasive breast carcinomata (Jarvinen et al, 2000), *topoisomerase IIα* amplifications were found mostly combined with *c-erbB2* amplifications, which might be attributable to extensive amplicons constitute both neighboring gene loci. The comparably low-frequency of *c-myc* amplifications in only 6% (5 of 83) of DCIS specimens compared with results of studies (Kreipe et al, 1993a) performed on invasive carcinoma implies that *c-myc* amplification might not represent an early genetic alteration in carcinogenesis. Vos and colleagues did not find *c-myc* amplification in DCIS by Southern analysis (Vos et al, 1999b), indicating the higher sensitivity of the method used in this study. Considering the almost exclusive occurrence of this oncogene amplification in high-grade DCIS lesions, *c-myc* amplification might increase cell growth and promote tumor progression in later stages of carcinogenesis. In this study, *cyclinD1* amplification was detected in 6% (5 of 83) of the examined cases. Previous studies on DCIS using Southern blot (Vos et al, 1999a) and FISH (Simpson et al, 1997) obtained amplification frequencies of 10% (3 of 32) to 18% (16 of 88), which differ only slightly from our results. Similar to *c-myc*, the rather low percentage of *cyclinD1*-amplified cases of DCIS compared with the figures published in invasive tumors (Courjal et al, 1996; Dickson et al, 1995) might be due to a later occurrence during tumor evolution.

The distribution of gene amplifications shows significant differences between the three groups of VN classification. Among the four target genes, the frequency and the level of *c-erbB2* amplification differ significantly in Group VN3 compared with low- or intermediate-grade DCIS of groups VN1 and VN2. Moreover, high-grade lesions of Group VN3 show an accumulation of genetic alterations; coamplifications or the combined occurrence of amplifications and deletions at different genes were seen. Our findings, combined with a number of previous studies that used different approaches, particularly comparative genomic hybridization (Buerger et al, 1999a; Kuukasjarvi et al, 1997; Moore et al, 1999), indicate that high-grade DCIS harbors extensive genetic alterations.

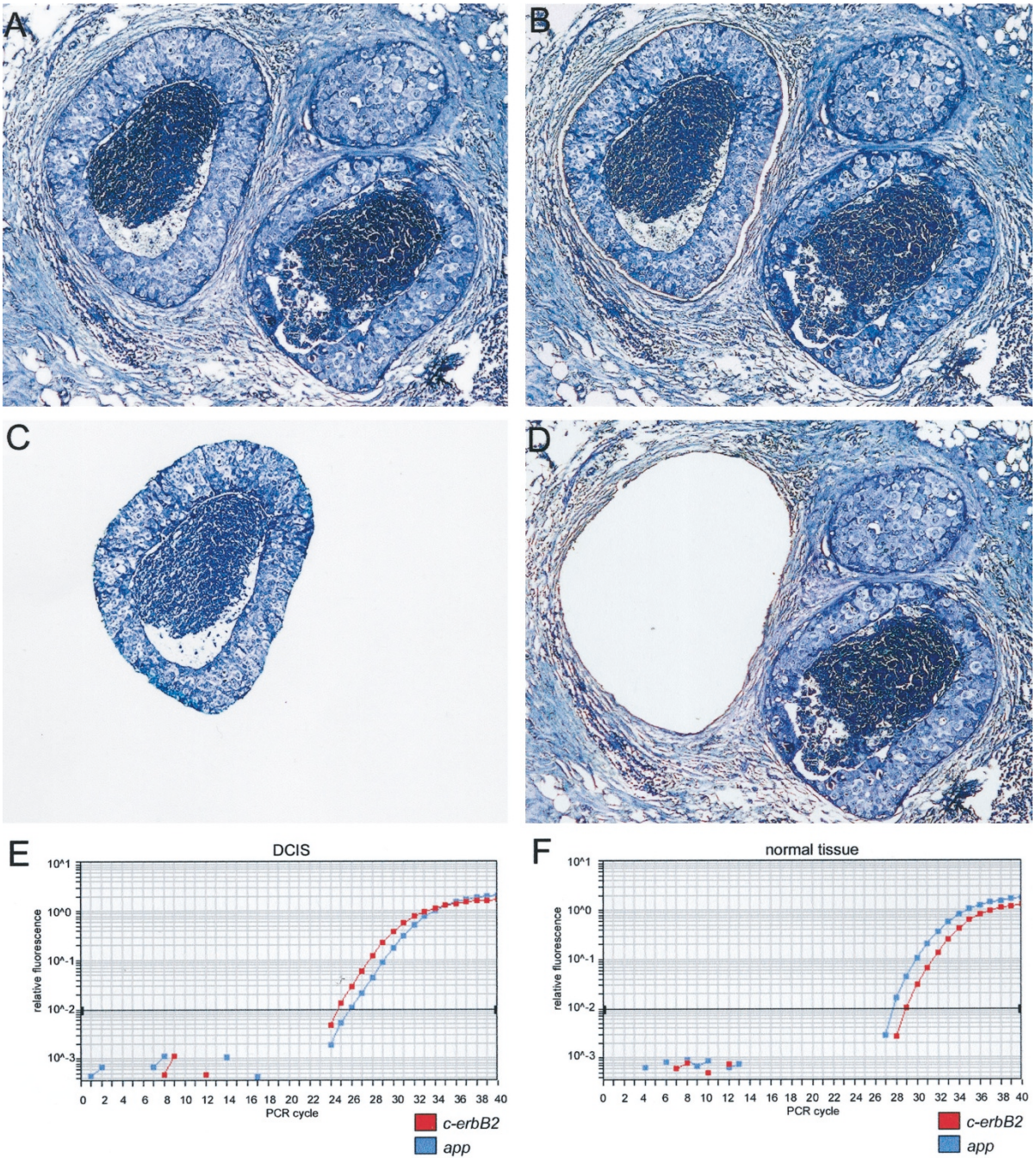

Figure 1.

Laser microdissection and subsequent quantitative PCR analysis of intraductal carcinoma cells (Case 9). A to D, Dissection and isolation of intraductal tumor cells (methylene blue staining, original magnification, ×100). E and F, Amplification of the *c-erbB2* gene in DCIS (E) leads to shifting of the amplification plot (red line) to the left compared with adjacent microdissected normal tissue (F) (intermediate level of amplification: 5.2 ± 0.8). *App* gene (blue line) serves as internal reference gene.

In conclusion, this study reveals that gene amplifications are present from the early preinvasive stages of breast cancer development and tend to occur in high-grade lesions as classified by the VN grading scheme. When intraductal carcinomata with progression to invasiveness were compared with pure in situ lesions, no significant differences of the amplification status of the genes under study were found. Future studies that directly compare invasive and noninvasive components of breast cancer are required to elucidate whether amplification of *c-myc* and *cyclinD1* evolve during the progression to invasiveness. Oncogene amplification in intraductal cancer does not appear to be an independent indicator of disease progression.

Materials and Methods

Patient Material and Histopathologic Diagnosis

A total of 83 cases of DCIS were retrieved from the surgical pathology files of the archive of the Hannover Medical School. One representative formalin-fixed,

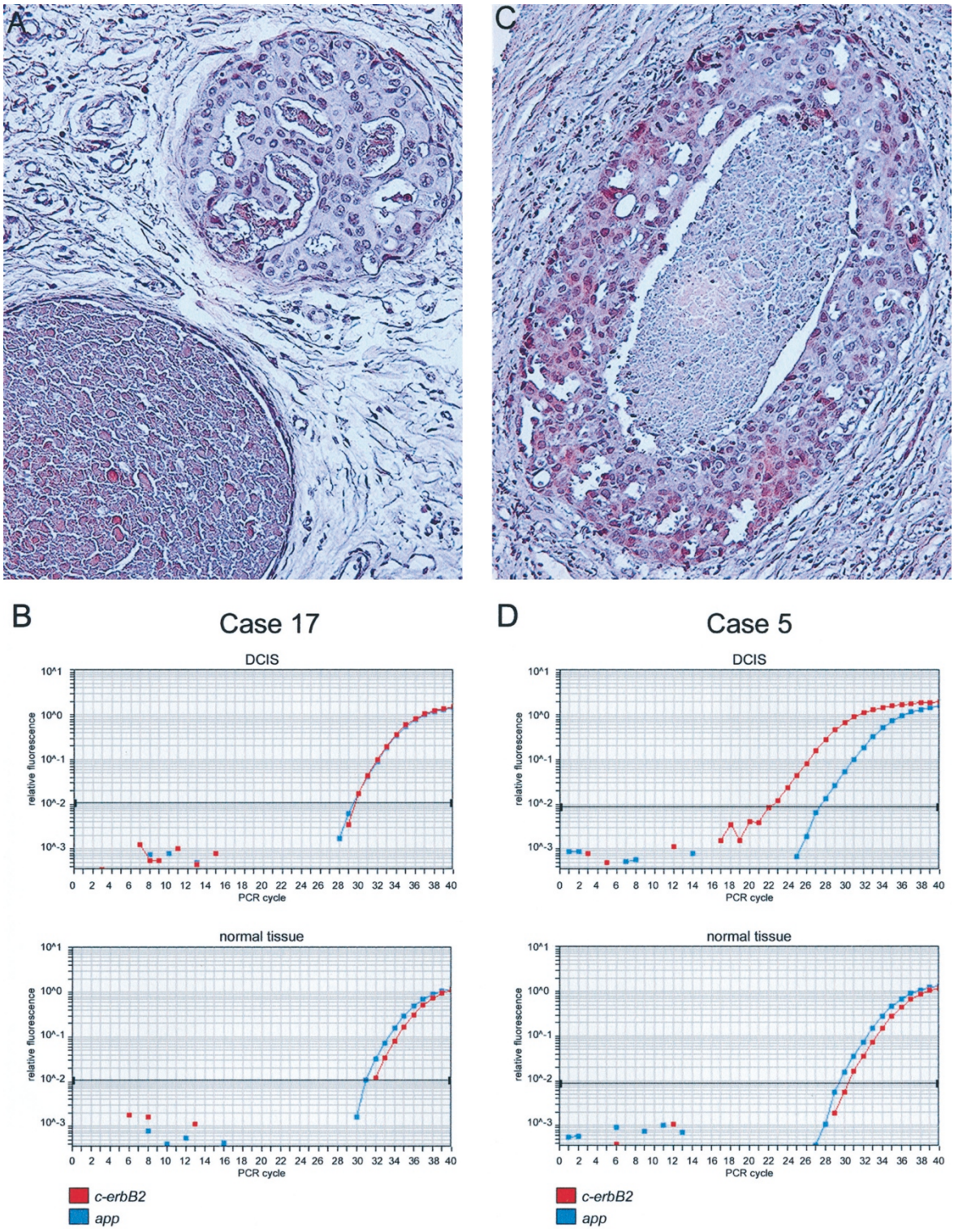

Figure 2.

Quantification of *c-erbB2* amplification in two specimens of DCIS VN3. A and C, The histologic appearance (hematoxylin and eosin stained, original magnification, ×200); and B and D, the corresponding amplification plots. A and B, Low-level amplification in Case 17 with a small shift of the *c-erbB2* amplification plot (red line) of about one PCR cycle to the left compared with adjacent normal tissue. *App* gene (blue line) serves as an internal reference gene. C and D, High-level amplification in Case 5 with a much greater shifting range of the *c-erbB2* amplification plot of about five PCR cycles to the left. *App* gene (blue line) serves as an internal reference gene.

paraffin-embedded block was chosen for study from each case after review of hematoxylin and eosin (HE)–stained slides. Thirty-seven cases were diagnosed as pure DCIS without concomitant or subsequent invasive carcinoma development, corresponding to a tumor node metastasis (TNM) stage of pTis. Another 46 cases revealed intraductal and adjacent invasive tumor components, correspond-

ing to a TNM-stage of pT1–2 (for case selection see Table 1).

According to the three-tiered system of the VN classification (Silverstein et al, 1995), DCIS was categorized into high (VN3), intermediate (VN2), and low grade (VN1). Each case was independently examined by two pathologists (HK and FL). Conflicting cases with discrepant grades were reviewed and discussed on a multiheaded microscope to achieve consensus. Apart from the evaluation of nuclear grade and the presence or absence of necrosis according to the VN classification, growth pattern and other histologic criteria, and adjacent pathologic lesions, such as proliferating mastopathy, were recorded.

Eleven cases (13%) were classified as low grade, VN1, 12 cases (14%) as intermediate grade, VN2, and 60 cases (72%) as high grade, VN3 (Table 1).

Microdissection and Real-Time PCR

Microdissection and real-time PCR were performed essentially as previously described (Bieche et al, 1998; Heid et al, 1996; Lehmann et al, 2000) (Fig. 1).

The primer and probe sequences and the magnesium concentration are as follows (in all cases, the first oligonucleotide is the forward primer, the second is the TaqMan probe, and the third is the reverse PCR primer): *c-erbB2*: 5'-AGCCTCTGCATTTAGGGATTCTC; 6FAM5'-TGAGAACGGCTGCAGGCAACCC-3'TAMRA, and 5'-CTAGCGCCGGGACGC (4.5 mmol/l $MgCl_2$); *topoisomerase IIα*: 5'-GCCAGAATCTGTTCGCTTCAAC, 6FAM5'-AAGCAGCCAGGCTGCCTGTCCAG-3'TAMRA, and 5'-AGGAAACTGAGTGCCGGCTT (5 mmol/l $MgCl_2$); *c-myc*: 5'-CCACGTCTCCACACATCAGC, 6FAM5'-AACTACGCAGCGCCTCCCTCCAC-3'TAMRA, and 5'-TTGGCAGCAGGATAGTCCTTC (6 mmol/l $MgCl_2$); *cyclinD1*: 5'-GGTCTGTGCATTTCTGGTTGC, 6FAM5'-CGGCGCTTCCCAGCACCAA-3'TAMRA, and 5'-GCTGGAAACATGCCGGTTAC (4 mmol/l $MgCl_2$); app: 5'-TCAGGTTGACGCCGCTGT, 6FAM5'-ACCCCAGAGGAGCGCCACCTG-3'TAMRA, and 5'-TTCGTAGCCGTTCTGCTGC (3.5 mmol/l $MgCl_2$).

Evaluation of Results

The relative gene copy number was evaluated on the basis of the threshold cycles (C_T-values) of the gene of interest and of an internal reference gene. The *app* gene served as an internal reference gene, sited on locus 4q11–q13, for which no amplifications in breast cancer have been reported. By subtraction of the C_T-value of the reference gene from the C_T-values of the test gene in the same tumor sample, the $\Delta C_{T[DCIS]}$-values for each of the four target genes were determined. These $\Delta C_{T[DCIS]}$-values for each test gene were then compared with a reference range derived from $\Delta C_{T[normal\ tissue]}$-values. To determine this reference range, normal non-neoplastic tissue samples (mostly stroma cells and lymphocytes) were examined after microdissection from 60 slides of DCIS cases included in this study that had adjacent non-neoplastic tissue on the selected paraffin block.

In consideration of PCR variabilities caused by fixation artifacts or sample impurities, the reference range for each test- or reference-gene pair in normal tissue was determined as follows:

Reference range $= [\bar{x}\ (\Delta C_{T[normal\ tissue]}) \pm SD] \pm 1$, where $\bar{x}$ is mean and SD is standard deviation.

In general, a reduction of the relative C_T-value by one cycle corresponds to a level of amplification of 1.8 $\pm$ 0.1 (Lehmann et al, 2000).

The level of amplification was graded as follows: low-level amplification (copy number increase of 2-fold to $<$ 5-fold), intermediate-level amplification ($\geq$5-fold to $<$ 10-fold), and high-level amplification ($\geq$10-fold) (Fig. 2).

Statistical Analysis

Statistical analyses for comparing amplification frequency/level and grades according to VN classification, as well as TNM-stage, were performed by linear trend test (exact), Fisher's test (exact), and Kruskal-Wallis test.

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