

EXTENSIVE DUCTAL CARCINOMA *IN SITU* WITH SMALL FOCI OF INVASIVE DUCTAL CARCINOMA: EVIDENCE OF GENETIC RESEMBLANCE BY CGH

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Although ductal carcinoma *in situ* (DCIS) of the breast is accepted as a potential precursor lesion for invasive ductal cancer (IDC), the critical genetic events associated with the tumor progression remain unknown. Since some extensive DCIS may show a small focus of IDC, these cases seem to be particularly suitable to investigate the primary abnormalities that determine the progression from *in situ* to early invasive cancer. We combined laser-microdissection with degenerative oligonucleotide-primed PCR (DOP-PCR) and comparative genomic hybridization (CGH) to detect copy number changes in 7 cases of extensive (>4 cm) DCIS with 1 small adjacent invasive focus. In 3 of the cases, single lymph node metastases (LN) were already present and were also investigated. Analysis of DCIS, IDC and LN components in the same patients revealed several consistent chromosomal changes present at all 3 sites: 1q, 7q, 8q, 16, 17, 19, 20q, 21q and 22q, the most frequent losses on 4q, 11q and 13q. DNA gain on 3p and 12q were more frequently found in IDC than in DCIS, suggesting the presence of proto-oncogenes activated during the progression to invasive cancer on these regions. Using paired analysis, resemblance of alterations found in DCIS and IDC could be quantified (odds ratio 7.0, $p \leq 0.01$). Gains on 6p, 10q, 14q and 15q and losses on 9p were identified in DCIS and IDC but not in LN, which may, therefore, represent early events in the carcinogenic process. Additional losses were found in the LNs on 2q, 3q, 5q, 6q, 12q and 16q. CGH results on chromosome 1 and 20 were confirmed by FISH and on chromosomal region 9p by microsatellite analyses. Our findings strongly underline the precursor status of high-grade DCIS, in which most of the chromosomal changes identified in IDC are already present. However, although the early stages of breast cancer, *i.e.*, DCIS and the small foci of IDC were mainly characterized by DNA gains, the progression to metastatic tumor (LN) must have involved additional DNA losses on several regions. *Int. J. Cancer* 85:82–86, 2000.

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There is some evidence that DCIS may be a precursor lesion for malignant breast cancer, since it is often seen at sites adjacent to invasive breast cancer. Because of this finding and since DCIS lesions often recur as invasive ductal carcinoma (IDC), a linear progression relationship for these lesions is likely (James *et al.*, 1997). The paucity of information about genetic changes during the progression of DCIS, however, is due to the difficulties of studying the DCIS lesions that are surrounded by an abundance of stroma or phenotypically non-malignant epithelium.

Immunohistochemistry, fluorescence *in situ* hybridization (FISH) and tissue microdissection followed by specific molecular genetic assays have been applied to DCIS. For example, loss of heterozygosity (LOH) on chromosome 1 (Munn *et al.*, 1995), 8p, 13q, 16q and 17 (Chen *et al.*, 1996) and 6q (Chappell *et al.*, 1997) have been found in DCIS as well as overexpression of p53, erbB2 and c-myc (Murphy *et al.*, 1995) and aneuploidy for chromosomes 1 (Harrison *et al.*, 1995) and 17 (Murphy *et al.*, 1995). These studies, however, are biased toward regions of the genome previously implicated as important to tumor initiation or progression.

Comparative genomic hybridization (CGH) is a technique aimed at detecting amplified and/or deleted regions of DNA in tumors

(Kallioniemi *et al.*, 1992). It offers the opportunity to screen the entire genome for chromosomal imbalances. The technique has been applied to several sources of tumors, including cells microdissected from formalin-fixed, paraffin-embedded tissue sections (Kuukasjärvi *et al.*, 1997; Weber *et al.*, 1998; Aubele *et al.*, 1999; Zitzelsberger *et al.*, 1998). Using laser-microdissection, an increase in the purity of test samples can be achieved and contamination with non-tumorous cells can be avoided (Zitzelsberger *et al.*, 1998; Aubele *et al.*, 1999).

In the present study, we combined laser-microdissection, DOP-PCR and CGH to identify chromosomal aberrations in extensive DCIS (>4 cm) with small foci of IDC (0.4–1.5 cm) and small, single LN. Our aim was to identify chromosomal changes consistently found at all 3 sites, the characterization of early events already present in DCIS, as well as alterations additionally occurring at the transition to IDC and LN, respectively.

MATERIAL AND METHODS

Tumor samples

Formalin-fixed, paraffin-embedded tissue sections from 7 patients were investigated. Hematoxylin and eosin (H&E)-stained sections were classified according to Silverstein *et al.* (1995). Lesions in each case consisted of non-tumorous mammary gland, extensive (>4 cm) high-grade intraductal carcinomas of the comedo type (DCIS) and small (0.4–1.5 cm) foci of poorly differentiated (G3) invasive lesions (IDC). In 3 of the patients, small, single lymph node metastases (LN) were also investigated. Sequential 5 μ m sections were cut from the paraffin blocks. One section was mounted for laser-microdissection on a coverslip and H&E-stained. Further serial sections were used for FISH analysis.

Laser-microdissection, DOP-PCR and CGH

Laser-microdissection was performed to sample pure cell groups from the different lesions (Fig. 1) (Becker *et al.*, 1996; Aubele *et al.*, 1999). Each cell group consisted of approximately 300–500 cells of a defined histopathological entity, which were collected in a separate tube and further processed as described (Aubele *et al.*, 1999). For universal amplification of DNA, degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) was used (Zitzelsberger *et al.*, 1998; Aubele *et al.*, 1999). The tumor probe was labelled by biotin-16-dUTP (Boehringer Mannheim, Germany) using standard nick translation.

Grant sponsor: Wilhelm Sander-Stiftung, München, Germany; Grant number: (96 07 01).

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Received 17 May 1999; Revised 17 August 1999

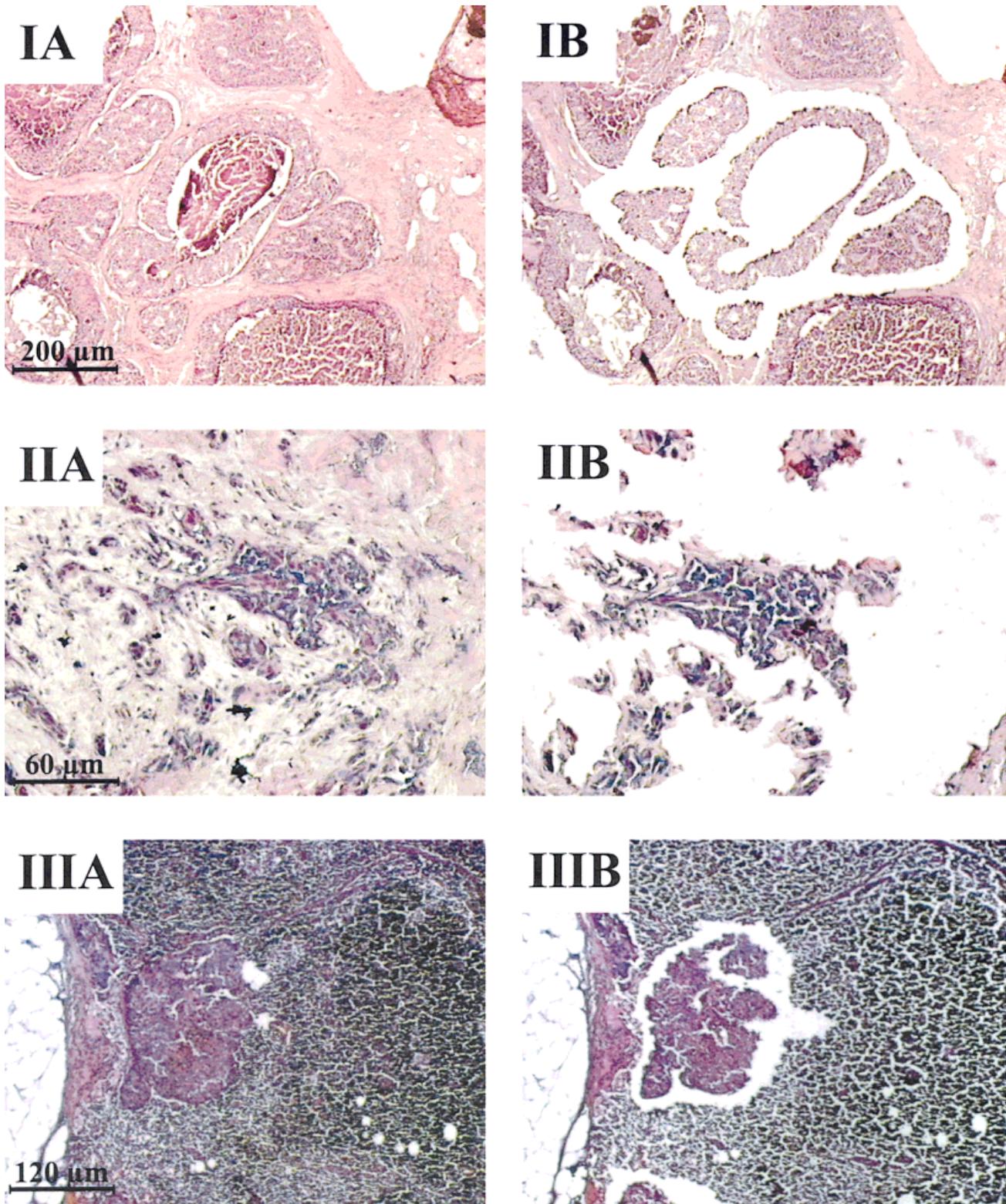


FIGURE 1 – Unmounted H&E-stained tissue sections of representative areas of intraductal carcinoma (I), infiltrating lesion (II) and lymph node metastases (III). All lesions are shown before (a) and after (b) UV-laser ablation of unwanted cells (e.g., lymphocytes, stromal cells).

CGH was performed according to a published protocol (Aubele *et al.*, 1999). Briefly, 200 ng of the DNA from the tissue sample and 200 ng SpectrumRed direct labelled normal female reference DNA

(Vysis, Downers Grove, IL) were hybridized together with 40 μ g Cot-1 DNA (Life Technologies, Grand Island, NY) on normal metaphase spreads. The tumor DNA was detected by Cy2-

conjugated streptavidin and biotinylated anti-streptavidin conjugates (Amersham Buchler, Braunschweig, Germany).

Image analysis

Image acquisition and processing was done as described previously (Zitzelsberger *et al.*, 1998; Aubele *et al.*, 1999). For CGH analysis, average red to green fluorescence ratios were calculated from 10 to 15 homologous chromosomes. They reflect copy number changes in the tumor genome and allow for a chromosomal band assignment of genetic imbalances. The CGH profiles were interpreted according to published criteria (Kallioniemi *et al.*, 1994; Zitzelsberger *et al.*, 1998). Since artifactual results had been occasionally observed on chromosomal band 1p34–p36 (Weber *et al.*, 1998), this region was excluded from the interpretation.

Control experiments

Several control experiments were carried out as described (Zitzelsberger *et al.*, 1998; Aubele *et al.*, 1999). Control PCR reactions were repeatedly performed without template and the DOP-PCR primer (negative control), and with template and a gene-specific primer (β -actin, positive control) to exclude possible DNA contaminations. For the positive control with template DNA, a clear band in a 3% agarose ethidium bromide gel of approximately 800 bp was observed, whereas no DNA smear was visible in the negative control (without template). The most important and revealing controls in our experiments were the investigation of non-tumorous ductal epithelial cells, which were laser microdissected from the same sections (n=7) and further processed likewise. In each of the non-tumorous samples, CGH profiles without alterations were achieved.

FISH and microsatellite analyses

For validation of CGH results, FISH was performed on 5- μ m-thick sections from 3 selected cases using DNA probes for the centromeric regions of chromosomes 1 (1q12, Spectrum Orange, Vysis, Stuttgart, Germany), 20 (D2071, biotin labelled, Oncor Appligene, Heidelberg, Germany) and the subchromosomal region 20q13 (20.13.2, Spectrum Orange). The hybridization was performed according to a published protocol (Aubele *et al.*, 1999). A total of 200 nuclei per histologically defined entity were evaluated. For reference, signal frequencies in normal tissue were also evaluated.

For validation of DNA losses on chromosomal region 9p, the following polymorphic microsatellite markers were selected for

LOH analyses: D9S970 (9p12–13), D9S1748 (9p21, p16) as the closest flanking marker to the p16 gene (Brenner and Aldaz, 1995) and IFNA.PCR2 (9p22). Primer sequences were obtained from the genome database (<http://gdbwww.gdb.org>). The same microdissected, DOP-PCR-amplified DNA probes (tumor and normal) used in CGH were analyzed for LOH with fluorescent-labelled primers (Aubele *et al.*, 1999).

Statistics

The one-dimensional data description was performed by calculating percentages and standard deviations of chromosomal changes. Paired analysis was done by constructing 2×2 tables comparing DCIS and IDC, IDC and LN, and DCIS and LN. To quantify resemblance, the parameters odds ratio (as a measure of resemblance) and the Jaccard coefficient (as a measure of agreement), which is suitable for asymmetric binary variables, were calculated. To overcome the problem of multiple testing, a global estimator for the parameters odds ratio was calculated. Here, independence between the different chromosomal regions was assumed.

RESULTS

We report CGH results from 7 cases of extensive (>4 cm) high-grade intraductal carcinomas (DCIS) with small foci of poorly differentiated invasive cancer as well as small, single lymph node metastases present in 3 of the cases. Patients' data, histological diagnosis and summary of the CGH results are given in Table I. Gains and/or losses of chromosome regions were detected in all lesions studied.

All 7 DCIS lesions had multiple genetic changes affecting 6–19 different chromosomal regions per tumor (mean 13.6 ± 5.4). Frequent aberrations identified in more than one-third of the lesions were gains on 1q, 6p, 7q, 8q, 10q, 14q, 15q, 16, 17, 19q, 20q, 21q and 22q and losses on 4q, 9p, 11q and 13q. The average number of chromosomal imbalances in the invasive lesions was slightly higher than in DCIS lesions (mean 15.0 ± 6.7). The chromosomal alterations identified in more than one-third of the IDC lesions were mainly identical to those detected in DCIS, except for gains of DNA on 3p and 12q, which were found more frequently in IDC lesions. The 3 small metastases showed 6–12 chromosomal changes (mean 9.7 ± 3.2). The DNA copy number changes common to all metastases were gains on 1q and 20q.

TABLE I – SUMMARY OF CASES, HISTOPATHOLOGICAL DIAGNOSIS AND CGH RESULTS IN DCIS, IDC AND LN

Patient number	Histological diagnosis	CGH results	1q+	1p36+	1p22–31–	2p24ter+	2q22–33–	3p22+	3q26–	4q22–26–	5q14–21–	6p21+	6q15–25–	7q35ter+	8q24+	9p13–21–	9q34+	10q25ter+	11p14ter+	11p12–14–	11q21–22–	11q24ter+	11q13+	12q15–	12q24+	13q11–22–	14q32+	15q26+	16+/16q+/16p+	16q11–13–	17q22ter+/17+	19q13+	20q13ter+	21q+	22q+					
			1	DCIS		+	+	+	+	+	–	–	+	+	+	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1	IDC		+	+	+	+	–	–	–	+	–	+	–	+	+	+	–	+	–	–	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	
2	DCIS		–	+	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
2	IDC		–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
3	DCIS		+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
3	IDC		+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
3	LN		+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
4	DCIS		+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
4	IDC		+	+	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	DCIS		+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	IDC		+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	LN		+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	DCIS		+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	IDC		+	+	–	–	+	+	–	+	+	+	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	LN		+	+	–	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	DCIS		+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	IDC		+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Chromosomal alterations more frequently found in advanced tumor stages (DCIS < IDC < LN) are in bold.

Comparison of lesions of different histopathological entities

Considering all chromosomal changes occurring in more than one-third of the lesions in one-dimensional, the aberrations in high-grade DCIS closely resemble those detected in the invasive tumor areas from the same patients. Alterations more frequently found in IDC were DNA gains on 3p and 12q. In both, DCIS and IDC gains on 6p, 10q, 14q and 15q and losses on 9p were found, which were not identified in the LN. The latter shared several chromosomal imbalances with DCIS and IDC lesions: gains on chromosomes 1, 7q, 8q, 16, 17, 19q, 20q, 21q and 22q and losses on 4q, 11q and 13q. Losses on 2q, 3q, 5q, 6q, 12q and 16q were more frequently found in LNs.

By means of paired analysis, resemblance of aberrations identified in DCIS and IDC lesions was analyzed by 2×2 tables. The calculated global estimator resulted in a 7 times higher chance (odds ratio 7.0, $p \leq 0.01$) for a given chromosomal change in IDC lesions when the same aberration was also present in the corresponding DCIS. A good agreement between chromosomal changes in DCIS and IDC was also indicated by an average Jaccard coefficient (0.51). Separate analyses of the 2×2 tables revealed significantly increased odds ratios ($p \leq 0.05$) between DCIS and IDC lesions for DNA gain on 7q and loss on 1p, and some tendency for DNA gains on 1q, 2p, 6p and 10q ($p \leq 0.15$). For comparison of aberrations in DCIS and LN and in IDC and LN, no significant statistical results can be expected because only 3 LNs were investigated. However, better agreement in the identified aberrations was found between IDC and LN (Jaccard coefficient 0.36) than between DCIS and LN (Jaccard coefficient 0.25).

Validation of CGH findings

The CGH results were confirmed by LOH analysis for DNA losses on 9p and by FISH analysis for DNA gains on chromosome 1 as well as 20q. LOH at 9p12–13, 9p21 and 9p22 was found in 9 samples also showing DNA losses within these regions by CGH.

In 3 selected cases, an increased copy number of chromosome 1 was found by FISH analysis, confirming our CGH results. The amplification of the chromosomal region 20q13 in DCIS, IDC and LN could also be confirmed by FISH analyses in the 3 cases analyzed, with a mean number of signals for the 20q13 region of 1 for normal tissue, 2.7–3.0 in DCIS lesions, 2.2–3.2 in IDC and 2.1 in LN.

DISCUSSION

According to the multistep model of carcinogenesis, tumors may develop and progress as a result of alterations in oncogene and tumor-suppressor gene loci (Chappell *et al.*, 1997). Some CGH findings suggest that progression from primary breast cancer to metastasis may be associated with the acquisition of further genetic changes (Nishizaki *et al.*, 1997). There is, however, no detailed molecular model of the critical genetic events in breast cancer (Chappell *et al.*, 1997). Thus, the role of DCIS in the progression pathway needs to be identified and makes it an important tumor to study (James *et al.*, 1997).

Several groups have reported the CGH analysis of primary invasive breast tumors (Kallioniemi *et al.*, 1994; Aubele *et al.*, 1999; Nishizaki *et al.*, 1997; Tirkkonen *et al.*, 1998). Their results confirm a complex pattern of gains and losses involving many chromosomes with common regions of DNA gains on 1q, 6p, 8q, 11q, 12q, 17q and 20q and losses, which were less frequently found, on 6q and 12q (Kallioniemi *et al.*, 1994; Aubele *et al.*, 1999). In addition, a distinct heterogeneity within an infiltrating lesion was demonstrated (Aubele *et al.*, 1999). In the present study, only 1 DNA sample per histopathological entity and patient was investigated so that intrasample heterogeneity could not be analyzed. The above findings are in good agreement with our results in invasive breast cancer in the present study. Few CGH studies pertain to CIS (Buerger *et al.*, 1999; James *et al.*, 1997; Kuukasjärvi *et al.*, 1997), focusing on different histological types and

grades. A wide variety of chromosomal imbalances were noted, which also correspond in large parts with our present results. They have suggested that alterations in DCIS almost resemble those previously detected and described in IDC (Buerger *et al.*, 1999; James *et al.*, 1997; Kuukasjärvi *et al.*, 1997). The latter, however, were based on only 2 IDC (Kuukasjärvi *et al.*, 1997) and on a comparison of alterations in DCIS with published data on chromosomal alterations in IDC (James *et al.*, 1997).

In our study, we selected only those cases with extensive high-grade DCIS and a small focus of invasive cancer (IDC) in the same patients, which suggests DCIS as possible precursor lesion of IDC in those cases. In addition, we used a laser-microdissection system that allowed isolation of a pure population of microscopically identified tumor cells. The goal was to identify chromosomal changes consistently present in all 3 lesions (DCIS, IDC, LN), the characterization of alterations present in DCIS only, representing possible early events, as well as alterations additionally occurring at the transition to IDC and LN, respectively. Separate analysis of synchronous samples from DCIS, IDC and LN revealed an increasing number of aberrations from DCIS to IDC but fewer alterations in LN, suggesting a possible clonal selection of chromosomal imbalances during tumor progression. An additional explanation, as discussed by Kuukasjärvi *et al.* (1997) for certain aberrations present in DCIS but not in IDC, may possibly be that a simple linear progression model and the hypothesis of a direct relationship between IDC and LN may not always apply. Furthermore, investigation of several hundreds of cells per sample in our study may not exclude any heterogeneity bias. The latter may also be a possible explanation for chromosomal changes observed in DCIS and LN but not in IDC. Additionally, occurring alterations at the transition to the next histopathological stage were identified, although this could be done only one-dimensional due to the small sample size. Resemblance of aberrations in DCIS and IDC lesions was demonstrated by means of paired analyses.

Multiple chromosomal imbalances were identified in all 3 histopathological entities (gains on 1q, 7q, 8q, 16, 17, 19q, 20q, 21q and 22q; losses on 4q, 11q and 13q). The additional alterations to DCIS identified here in the IDC were gains on 3p and 12q, where no putative oncogenes are identified so far (<http://gdbwww.gdb.org>). LN metastases showed further losses on 2q, 3q, 5q, 6q, 12q and 16q. Interestingly, several genes involved in metastasizing processes have been located on chromosome 16q, *e.g.*, MMP2 (matrix metalloproteinase 2) or NME2 (protein expressed in non-metastatic cells-3) (<http://gdbwww.gdb.org>).

An increased copy number at 20q13, which emerged as one of the most common genetic aberrations in breast cancer after CGH analysis (Kallioniemi *et al.*, 1994; Aubele *et al.*, 1999; Tanner *et al.*, 1994; Moore *et al.*, 1999), was not identified in the CIS lesions studied by Kuukasjärvi *et al.* (1997) and only in a minority of CIS lesions by Buerger *et al.* (1999). We found this alteration in almost all samples from high-grade DCIS (5/7), as well as in 7/7 IDC and in 3/3 LN, and we could confirm these findings by FISH analysis using centromere 20- and 20q13-specific DNA probes. The finding of the 20q amplification in high-grade DCIS without invasive components by Moore *et al.* (1999) may possibly suggest that this region harbour genes more responsible in early developments than in further progression to invasive cancer. The 20q13 region is thought to harbour a novel oncogene termed AIB (amplified in breast cancer-1) (Anzick *et al.*, 1997).

Amplifications of chromosome 1 and 17 are often found in breast cancer (Murphy *et al.*, 1995; Harrison *et al.*, 1995; Coene *et al.*, 1997). Our CGH findings of gain on chromosome 1q in DCIS are in agreement with the findings of Kuukasjärvi *et al.* (1997) and James *et al.* (1997) and were confirmed in our study by additional FISH analyses. Frequent alterations of chromosome 1 in DCIS of the breast have also been shown by LOH analyses (Munn *et al.*, 1995) and by interphase cytogenetics using a centromere-specific DNA probe (Harrison *et al.*, 1995). This holds true also for amplification on 17q21, harbouring *erbB2* (Murphy *et al.*, 1995) as well as for

polysomy 17 in DCIS and IDC, which resulted from CGH studies (James *et al.*, 1997; Kuukasjärvi *et al.*, 1997; Tirkkonen *et al.*, 1998; Nishizaki *et al.*, 1997) and from FISH analyses (Murphy *et al.*, 1995; Coene *et al.*, 1997). In our study, 6/7 DCIS and 7/7 IDC as well as 2/3 LN revealed gain on 8q with a smallest common region on 8q24, harbouring the *MYC* proto-oncogene locus.

Most frequent losses in DCIS studied here were localized on 4q, 9p, 11q and 13q. Loss on 13q was detected in 6/7 DCIS and in 7/7 IDC. Deletions at 13q11–22 as well as loss on 4q have been reported to occur frequently in breast cancer (Nishizaki *et al.*, 1997). Losses on 4q, which was also identified in a higher percentage of DCIS and IDC in our study (3/7 samples each), has previously only been identified in invasive breast cancer (Nishizaki *et al.*, 1997; Tirkkonen *et al.*, 1998) but not in CIS lesions (James *et al.*, 1997; Kuukasjärvi *et al.*, 1997). Losses detected on 9p by CGH as well as LOH in a higher percentage here in DCIS and IDC include the locus 9p21 of tumor-suppressor gene *CDKN2A* (p16, *INK4A*).

We report here CGH results from extensive DCIS and small foci of IDC in the same patients. We did show that chromosomal alterations in DCIS lesions are complex and that the pattern of changes in general resemble that seen in IDC ($p < 0.01$). We identified consistent chromosomal changes in all 3 entities. Alterations in addition to those in DCIS were identified in IDC (gains on 3p and 12q) and in LN (losses on 2q, 3q, 5q, 6q, 12q and 16q), possibly harbouring potential oncogenes and/or tumor-suppressor genes not yet identified. Our data indicate the chromosomal regions associated with tumor progression, thus providing a basis for searching for the relevant genes in specific tumor stages.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the technical assistance of Mrs. D. Angermaier, Mrs. I. de Grazia and Mrs. S. Schulte-Oberberg.

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