Correlation of Morphologic and Cytogenetic Parameters of Genetic Instability With Chromosomal Alterations in In Situ Carcinomas of the Breast

Horst Buerger, MD,1 Ellen C. Mommers, MD,2 Ruth Littmann,1 Raihanatou Diallo, MD,1 Christian Brinkschmidt, MD,1 Christopher Poremba, MD,1 Barbara Dockhorn-Dworniczak, MD,1 Paul J van Diest, MD, PhD,2 and Werner Böcker, MD1

Key Words: Ductal carcinoma in situ; DCIS; Lobular carcinoma in situ; LCIS; Comparative genomic hybridization; CGH; Morphologic features

Abstract

Classification of preinvasive breast disease could be better founded using biologic markers, thereby increasing reproducibility. We studied 57 breast ductal and lobular in situ carcinomas by means of comparative genomic hybridization and correlated these findings with quantitative features such as the mean nuclear area, mitotic index (MI), apoptotic index (AI), and the presence or absence of necrosis.

Loss of 8p and gains of 8q and 6q were associated, respectively, with a significantly higher MI and AI, whereas loss of 16q was associated with a lower MI and AI. A significantly higher number of alterations per case were seen in tumors with gains of 6q, 8q, and 17q and tumors with loss of 13q. Loss of 16q and gain of 17q correlated with the absence or presence of necrosis, respectively. Our data clearly demonstrate that distinct cytogenetic changes correlate with phenotypic changes, proliferation, and apoptosis. These data may be used to refine existing classification schemes.

Preinvasive breast lesions represent a spectrum of diseases with distinct patterns of morphologic, immunohistochemical, and genetic alterations. Different classification systems have been proposed that attempt to group these lesions in a morphologically, biologically, or clinically sensible way.1-3 There are, however, problems with reproducibility, and genetic data to substantiate these classifications are largely lacking. Furthermore, the stepwise progression in the evolution of breast cancer has been questioned in recent years, but emerging data on molecular and cytogenetic events associated with this progression have led us to propose an extended morphologic-cytogenetic progression model of preinvasive breast lesions with the loss of chromosomal material of 16q as an early genetic step in some subgroups of ductal carcinoma in situ (DCIS)/lobular carcinoma in situ (LCIS) and invasive breast cancer.4-8

Nevertheless, the relationships between distinct genetic alterations on the one hand and morphologic features (such as nuclear pleomorphism and necrosis), proliferation, and apoptosis on the other have remained unclear. This is important since classification schemes should preferably include relevant biologic and genetic features for biologic plausibility, and they should increase reproducibility. In addition, the correlation of the aforementioned parameters with cytogenetic aberrations provides insight into the functional effects of such changes. Against this background, we studied 57 cases of breast carcinoma in situ by means of comparative genomic hybridization (CGH) and correlated the findings with phenotypic changes (nuclear pleomorphism and necrosis) and proliferative and apoptotic activity. Our new data support the hypothesis of at least 2 genetic pathways in the evolution and progression of breast cancer, which stresses the importance of a stringent subclassification of preinvasive breast lesions.
Materials and Methods

We studied 57 cases of in situ breast cancer. These tumors comprised 51 cases of DCIS (10 well-, 19 intermediate, and 22 poorly differentiated DCIS) and 6 cases of LCIS. All cases were classified according to established protocols. All material had been fixed in neutral buffered formaldehyde and embedded in paraffin according to standard procedures.

CGH Analysis

The method of CGH analysis and the criteria for the evaluation of genetic alterations were as previously described.4,5,10

Mitotic and Apoptotic Index and Morphometric Analysis

The number of mitotic and apoptotic cells were counted microscopically in several representative ducts and lobules at x400 magnification using H&E-stained sections. Strict morphologic criteria were used to identify mitotic and apoptotic cells. Mitotic figures lacked a nuclear membrane, and hairy extensions of nuclear material had to be present.11 Apoptotic cells had retracted and often eosinophilic cytoplasm and condensation of nuclear chromatin at the nuclear membrane, throughout the nucleus, or in round nuclear fragments. These features concerned isolated cells not provoking inflammation.12

The total numbers of cells were counted in the same areas. The mitotic index (MI) and apoptotic index (AI) were expressed as the number of mitoses and apoptoses per 1,000 nuclei.

Measurement of the mean nuclear area (MNA) as a measure for nuclear atypicality was performed with the QPRODIT interactive digitizing video overlay system (Leica, Cambridge, England) at a final ×3,500 (objective, 100×) on-screen magnification using 4-µm H&E-stained sections. We traced 50 to 70 nuclei according to standard procedures, using systematic random sampling.13

Substantial amounts of necrotic neoplastic cells of ductal origin in the central lumina for any architectural pattern of DCIS were defined as necrosis.14

Statistical Tests

For statistical evaluation, the Student t test and the chi-square test were used. P values less than .05 were considered significant.

Results

Cytogenetic and Morphologic Parameters in Correlation With Histopathologic Grading

Parts of the cytogenetic results have been reported.4,7 As previously demonstrated, a statistically significant increased average rate of genetic alteration per case and of amplifications could be detected between well- and poorly differentiated DCIS (P < .05). This was accompanied by a significant increase in the MI, the MNA, and the AI TABLE 1.

Cytogenetic and Morphologic Parameters in Correlation With the Degree of Cytogenetic Progression

By subdividing all tumors by their number of genetic alterations per case, significant increases in the MI (P < .0001), the AI (P < .001), and the MNA (P < .01) were seen in the tumor group with 5 or fewer genetic alterations per case compared with cases with more than 10 genetic alterations per case (Table 1) FIGURE II.

Cytogenetic and Morphologic Parameters in “Genetically Early” In Situ Carcinomas

For tumors with 5 or fewer genetic alterations per case, irrespective of size, poorly differentiated DCIS showed an

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<td>Quantitative Cytogenetic Measurements</td>
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<td>Carcinoma in situ (No. of alterations per case)</td>
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<tr>
<td>5 or fewer</td>
<td>2.8 ± 1.3</td>
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<td>6-10</td>
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<td>&gt;10</td>
<td>13.7 ± 3.3</td>
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DCIS, ductal carcinoma in situ; LCIS, lobular carcinoma in situ.

* Data are given as mean ± SD unless otherwise indicated.
increased rate of loss of 8p, 11q, and 13q and gain of 17q and a statistically significant decrease of 16q loss \((P < .001)\) compared with well- and intermediately differentiated DCIS (with the inclusion of LCIS) **Figure 2**. This was accompanied by an increase of the MI \((P < .001)\) and the AI \((P < .05)\). Well- and intermediately differentiated DCIS and LCIS without 16q losses revealed an almost identical morphometric pattern to those with 16q losses within this special subgroup.

### Correlation of Distinct Cytogenetic Alterations With Morphologic Parameters and Signs of Genetic Instability

Four genetic alterations were correlated with an altered proliferative and apoptotic activity. Tumors with a gain of 6q and 8q and a loss of 8p revealed significantly higher AI and MI, respectively, compared with tumors without these alterations **Table 2**. In contrast, tumors with a loss of 16q showed a significantly lower mitotic activity compared with
tumors with an unaltered chromosome 16. A statistically significant increase in the MNA was not associated with any chromosomal alteration.

Alterations associated with absence or presence of necrosis in all precursor lesions were the loss of 16q and the gain of 17q with the inclusion of 17q12 ($P < .05$). However, this could not be demonstrated in intermediately differentiated DCIS with or without necrosis. In this subgroup, necrosis was associated with a statistically nonsignificant increase of the MI, AI, and MNA.

The average number of genetic alterations per case and the presence of amplifications were considered parameters of genetic instability. Tumors with gains of 6q, 8q, 17q with special emphasis on 17q12 ($P < .01$), and 20q and loss of 13q revealed a significantly higher number of genetic alterations per case and a higher rate of amplification in contrast to tumors without these changes.

### Discussion

Various factors involved in proliferative and apoptotic pathways in the pathogenesis of invasive breast cancer and its ultimate precursors, DCIS and LCIS, have been described.\textsuperscript{15-17} The interpretation of these results in the sense of tumor progression remains controversial, since these findings often are associated with the tumor grading or differentiation. Therefore, it is not clear whether they exclusively support a serial progression model in the evolution of breast cancer or they also allow the postulation of several parallel pathways.\textsuperscript{18}

The concept of a stepwise progression is supported by an increased proliferative activity in the suspected precursor lesions\textsuperscript{17} but also has been challenged by cytogenetic parameters.\textsuperscript{16} A cytogenetic alteration associated with growth properties and the grading in precursor lesions of invasive breast cancer definitely could enlighten this contradictory situation.

The demonstration of increased mitotic activity with an increased number of genetic alterations per case and a lower degree of tumor differentiation leave the question unanswered about whether the proliferative activity of a tumor is the cause or the consequence of genetic instability. This phenomenon also was seen in invasive breast cancer using microsatellite analysis.\textsuperscript{20} This problem could be handled by comparing one variable between cases matched for the other variable.

Regarding tumors with 5 or fewer genetic alterations per case, differences between well- and intermediately differentiated DCIS plus LCIS vs poorly differentiated DCIS are striking. One could expect a significant difference in the MNA as part of the chosen classification protocol.\textsuperscript{1} The highly statistically significant difference in the MI and AI is of special importance, since the only alteration significantly associated with this is the loss of 16q material. The overall importance of 16q losses also becomes evident taking into account all tumors regardless of their differentiation, tumors with 16q losses revealing a lower proliferation rate. The loss of 16q material might, therefore, account for a “slowed down” proliferation rate in a subgroup of well- and intermediately differentiated DCIS and LCIS in contrast to poorly differentiated DCIS. The shortest region of overlap is 16q22-24. Numerous candidate genes within this region, thought to be involved in breast cancer pathogenesis, like E-cadherin or the “basic breast conserved” gene, have been described.\textsuperscript{21,22} One factor so far not mentioned in detail could be E2F4, a p107/p130–binding and transcription factor with a central role in the cell cycle.\textsuperscript{23,24} At this stage of our knowledge, it remains speculative that the reduction of E2F4 in a gene dosage–dependent manner reduces the mitotic activity in this special subgroup of preinvasive breast tumors. Nevertheless, no other so-far-described gene in this region could explain the relationship between 16q losses and a lower mitotic rate as detected in tumors with a normal chromosome 16.
Because tumors without 16q losses within the subgroup of non–poorly differentiated tumors revealed an almost identical morphometric pattern to that of tumors with 16q losses, other factors responsible for this altered growth property have to be identified. For this subgroup, it cannot be excluded on a cytogenetic basis that they may progress toward poorly differentiated DCIS. Alternatively, submicroscopic mutations in the 16q22-24 region within this subgroup may have the same effect.

In contrast, the other genetic alterations associated with a significantly increased mitotic activity were seen mainly in intermediate and poorly differentiated DCIS. Among these, the loss of the whole 8p arm was seen with a frequency of 25% in the cases of poorly differentiated DCIS with 5 alterations or fewer, suggesting that these alterations cytogenetically represent early alterations. This is in agreement with microsatellite analysis studies that proposed a tumor suppressor gene within the region 8p12-23 as one of the most frequently deleted regions in breast cancer and its precursors. DNA array analysis identified FRP1/FRZB as one potential candidate gene within this region. Losses of 8p often are seen synchronous with 8q gains, one of the most common chromosomal alterations in breast cancer and associated with a worse prognosis. Whether this alteration primarily influences the growth potential at the time of tumor initiation needs to be questioned. The lack of 8q gains in the genetically early cases of DCIS and LCIS and invasive breast cancer characterizes this alteration more as a marker of tumor progression also associated with a high degree of genetic instability.

The same interpretation probably has to be given to 3q gains, an aberration exclusively found in tumors with more than 10 alterations per case on average. In the cervix, the step from severe cervical dysplasia toward invasive carcinoma was accompanied by the gain of 3q material, whereas in breast cancer, invasion does not seem to depend on a specific cytogenetic alteration. In concordance with the literature, the present study also demonstrated that an increased MI is balanced by a similar increase in AI. Therefore, it is noteworthy that almost all chromosomal alterations significantly associated with the MI are related to the AI. This is also true for the gain of 6q material as a highly significant correlate of mitotic and apoptotic activity. It has been hypothesized as a result of multiple microsatellite analysis that one or multiple tumor suppressor genes might be located on the long arm of chromosome 6. CGH studies associated the gain of chromosomal material of 6q with an intraductal and intralobular growth. An association with one of the postulated cytogenetic pathways is likely, since 6q gains were seen exclusively in poorly differentiated DCIS in the present study and in ductal invasive G3 carcinoma.

Necrosis in DCIS has been associated with the expression of p53 and c-erbB2. The finding of a significant correlation between 17q gains, including 17q12, the locus of erbB2, and the presence of necrosis and, in contrast, the absence of necrosis in tumors with 16q losses seems to support this. Nevertheless, these alterations correlate to a high degree with the nuclear grading per se, which again could be seen as further evidence for at least 2 different genetic pathways. Results for intermediate and poorly differentiated DCIS only, with or without necrosis, demonstrated that necrosis is not correlated with cytogenetic changes but with increased MI, AI, and MNA. It therefore seems justified, from this point of view, to include proliferation and nuclear grading parameters next to necrosis in classification systems of preneoplastic lesions of the breast, the nuclear grade as the most consistent one throughout a lesion. Nevertheless, it must be kept in mind that a dual classification system of DCIS with necrosis as a major determinant for the subclassification of one group does not open the possibility for a progression within DCIS. This has been demonstrated for well- and intermediate differentiated DCIS. It also becomes evident, with the inclusion of necrosis as one parameter, that intermediate differentiated DCIS, most of which are classified as non–high-grade DCIS, according to Holland et al, are themselves a rather heterogeneous group of tumors, some tumors genetically resembling low-grade and some high-grade DCIS according to the Van Nuys classification. It therefore seems reasonable to further develop concepts and parameters that would allow the definition of a 2-group classification system, because this seems to reflect the underlying genetic mechanisms.

By considering all data together, it becomes clear that the existing most commonly used classification systems for diagnosis of preneoplastic breast tumors have advantages and drawbacks. Revised classification systems with a higher reproducibility are needed that take into account new biologic data. The unraveling of the molecular disturbances behind the aforementioned cytogenetic alterations should provide the tools to achieve that aim.

From the 1Gerhard-Domagk-Institute of Pathology, University of Münster, Münster, Germany, and the 2Institute of Pathology, Free University Hospital, Amsterdam, the Netherlands.

Supported by grants 10-1140-Bö-I from the Deutsche Krebshilfe, Bonn, Germany; Bű 1 I 98 31 from the University of Münster, (IMF); and 95-930 from the Dutch Cancer Society, the Hague, the Netherlands.

Address reprint requests to Dr Böcker: Gerhard-Domagk-Institute of Pathology, Westfälische Wilhelmsuniversität Münster, Domagkstr.17, 48149 Münster, Germany.

Acknowledgments: We thank Ulrike Neubert and Lydia Grote for technical assistance in CGH analysis and Jane Brugghe and Mark Broeckaert for morphometric assessments.
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