Local Recurrence after Breast-Conserving Therapy in Relation to Gene Expression Patterns in a Large Series of Patients

Bas Kreike,1,2 Hans Halfwerk,2,3 Nicola Armstrong,4 Peter Bult,6 John A. Foekens,7 Sanne C. Veltkamp,8 Dimitry S.A. Nuyten,1,3 Harry Bartelink,1 and Marc J. van de Vijver2,5

Abstract

Purpose: The majority of patients with early-stage breast cancer are treated with breast-conserving therapy (BCT). Several clinical risk factors are associated with local recurrence (LR) after BCT but are unable to explain all instances of LR after BCT. Here, gene expression microarrays are used to identify novel risk factors for LR after BCT.

Experimental Design: Gene expression profiles of 56 primary invasive breast carcinomas from patients who developed a LR after BCT were compared with profiles of 109 tumors from patients who did not develop a LR after BCT. Both unsupervised and supervised methods of classification were used to separate patients into groups corresponding to disease outcome. In addition, for 15 patients, the gene expression profile in the recurrence was compared with that of the primary tumor.

Results: The two main clusters found by hierarchical cluster analysis of all 165 primary invasive breast carcinomas revealed no association with LR. Predefined gene sets (molecular subtypes and “chromosomal instability” signature) are associated with LR (P = 0.0002 and 0.003, respectively). Significant analysis of microarrays revealed an association between LR and cell proliferation, not captured by histologic grading. Class prediction analysis constructed a gene classifier, which was successfully validated, cross-platform, on an independent data set of 161 patients (log-rank P = 0.041). In multivariate analysis, young age was the only independent predictor of LR.

Conclusions: We have constructed and cross-platform validated a gene expression profile predictive for LR after BCT, which is characterized by genes involved in cell proliferation but not a surrogate for high histologic grade.

Local treatment for early-stage breast cancer can consist of mastectomy or breast-conserving therapy (BCT). Several randomized controlled trials have shown no difference in survival rates after BCT or mastectomy for stage I and II breast cancer (1–3). Unfortunately, BCT is associated with a higher rate of local recurrence (LR) compared with mastectomy (1–3). A LR rate of <1% per annum is generally considered as clinically acceptable for T1-2 N0-1 breast cancers. The choice for the primary treatment modality is therefore often based on risk factors for LR.

Several of these risk factors for LR after BCT have been identified: involvement of the surgical margins by invasive carcinoma [hazard ratio (HR), 2.8-3.9] and/or extensive ductal carcinoma in situ (DCIS; HR, 2.5-4.2), vascular invasion (HR, 2.0-2.9), young age (HR, 2.4-9.2), and tumor multicentricity (HR, 1.8-3.3; ref. 4–20). The addition of a radiation boost after whole-breast irradiation and adjuvant systemic therapy reduces LR rates by 40% to 60%, especially in younger patients (9, 11, 16, 17, 19, 20).

The importance of preventing LR is illustrated by the updated analyses of the Early Breast Cancer Trialists’ Collaborative Group showing that LR is associated with poorer survival (21) and by Voogd et al. (22) who suggest that early detection of LR could improve survival. Thus, robust risk factor identification for LR is of great clinical significance.

It can be argued that LR is only a result of tumor cells left behind in the breast after surgery. However, margin status (and other clinical risk factors) does not predict all LRs after BCT. We hypothesize that there is also a biological mechanism involved in this process, and here, we use gene expression microarray technology to profile a large set of primary tumors with the aim of elucidating this mechanism. It has been shown that gene
expression profiling of primary breast carcinomas using microarray technology is a potent tool for predicting distant metastases and overall survival in breast cancer (23–27). Recently, we have attempted to use this technique to develop a gene expression profile predictive for LR (28, 29). It has been shown that there is not a large difference between the overall gene expression profiles of primary tumors that recur locally and those that do not (28). We previously identified a group of genes associated with LR; this gene set was based on an in vitro model of wound response that proved to be predictive for metastasis development (30). After training a classifier for LR with this gene set, we were also able to predict LR in a validation set (29); however, this research was done on a limited set of patients. Recently, Nimeus-Malmstrom et al. (31) also showed that a selection of genes is associated with LR after BCT; unfortunately, they did not do a validation in an independent series of tumors to confirm this association. These studies show that there is a possible gene expression profile associated with LR after BCT and that further research is needed before gene expression profiles can be applied in clinical practice for better patient tailored therapy.

Here, we extend our initial study group to 165 primary breast carcinomas from patients at high risk for LR who underwent BCT. We report our results comparing gene expression profiles of primary tumors that recurred locally with those that did not recur. In addition, for a small subset of patients, we have compared the gene expression pattern of primary tumors developing a LR with the gene expression profile of their recurrences.

Materials and Methods

Patient selection. In a collaboration between four hospitals in the Netherlands (Netherlands Cancer Institute, Amsterdam; Radboud University Nijmegen Medical Centre, Nijmegen; Erasmus Medical Center, Rotterdam; and Ziekenhuis Amstellaand, Amstelveen), we collected fresh-frozen tumor material of breast cancer patients. We selected tumors from patients, treated by BCT between January 1984 and December 2000, who were under 51 y of age at the time of treatment and had no prior malignancies (excluding nonmelanoma skin cancer and dysplasia of the uterine cervix). All tumors were smaller than 5 cm in diameter at pathologic examination (pT1 or pT2). We selected patients who developed a LR within 10 y after BCT or who continued to be LR-free for 10 y or more after BCT. Of note, only tumors developing a LR were selected from the Erasmus Medical Center (n = 26).

BCT consisted of breast-conserving surgery (segmentectomy or wide local excision) followed by whole-breast irradiation with a median dose of 50 Gy (range, 45.5–54 Gy). A radiation boost to the tumor bed was delivered in 152 patients. The boost was applied by means of iridium-192 implantation or external beam irradiation (either photons or electrons) with a median dose of 15 Gy (range, 13–26 Gy).

All patients underwent axillary lymph node dissection or sentinel node procedure; 91 patients had no lymph node metastases, 68 patients had tumor-involved lymph nodes, and for 6 patients these data were not available.

The majority of patients receiving systemic adjuvant therapy (n = 52) were given a schedule containing cyclophosphamide, methotrexate, and fluorouracil (CMF) chemotherapy; 11 patients were given antracycline-based chemotherapy. Three patients were treated by endocrine therapy only and eight patients received a combination of chemotherapy followed by endocrine treatment.

The medical ethical committee of the Netherlands Cancer Institute approved this study, and wherever possible, we adhered to reporting Recommendations for Tumor Marker Prognostic Studies (32).

Histopathologic characterization of tumors. One pathologist (M.J.v.d. V) reviewed histopathologic characteristics for all tumors. The features scored included tumor diameter, histologic type, grade, shape, amount of vascular invasion, type of carcinoma in situ component, amount of DCIS present, resection margin status, and presence of tumor-involved lymph nodes. The histopathologic review was done, when possible, on the original diagnostic slides. For three cases, the original slides could not be retrieved, and the review was done on slides taken from the fresh-frozen tumor material used for microarray analysis. Margin status was determined by combining the information from the pathology report with the histopathologic analysis. Margin status was assessed for the invasive component and for the in situ component separately. Margins were assessed as free if there was at least 1 mm of unaffected breast tissue between the tumor and the resection margin (the exact distance of tumor to the resection margin was not available for many patients). Focal micropapillary involvement was defined as invasive tumor extending in the resection margin focally. If the resection margin could not be assessed with certainty, this was recorded as doubtful.

Estrogen receptor (ER) and HER2 status, assessed by immunohistochemistry, was retrieved from the pathology report for 65 cases. ER and HER2 status of all tumors was assessed using the gene expression data as described previously (28). In brief, we identified the probes representing ERα and HER2 on our microarray platform (two probes per gene). The averaged expression ratio of the probes for each gene was calculated and rank ordered from low to high; we then plotted the known immunohistochemical data of 65 tumors against the ranked gene expression ratios and derived a cutoff for ER status at ~1.2 and for HER2 at ~0.4. For the analyses in this study, the microarray-derived ER and HER2 status was used.

Freezing of tumor samples, RNA isolation, and microarray analysis. Tissue samples were snap frozen in liquid nitrogen within 1 h after surgery. From these frozen tissue blocks, sections were cut for RNA isolation. The first and last sections were used to assess the percentage of tumor cells by H&E staining; only tumors containing on average >50% tumor cells were included. Total RNA was isolated with RNAzol B (Campro Scientific) and dissolved in RNase-free water. The RNA was treated with DNase; 2 μg RNA was amplified and 1 μg aRNA was used for hybridization on the microarray; detailed information on protocols can be found online.9

We used the Human Genome Oligo Set version 3.0 arrays containing 34,580 probes representing 24,650 genes and 37,123 gene transcripts. These arrays were obtained from the central microarray facility at the Netherlands Cancer Institute; detailed information on these arrays can be found online.9 Tumor samples were cohybridized with reference RNA isolated from a reference pool consisting of >100 breast cancer samples. For all tumors, hybridization was repeated after reverse color labeling. Fluorescent intensities were normalized and corrected for biases as previously described by Hannemann et al. (33), and confidence levels were computed according to the error model (33). Gene expression data are publicly available at ArrayExpress.10

Data analysis of 165 primary tumors. A subset of probes on the microarray was selected based on the following criteria: expression data should be available for at least 98% of all experiments and the expression level

http://microarrays.nki.nl/  
10 Accession number E-NCMF-24 (http://www.ebi.ac.uk/arrayexpress/).
should be significantly different from the reference expression in at least 28 experiments with a P value of <0.01. This filtering reduced the total number of probes from 34,580 to 7,708.

Average-linkage hierarchical clustering of uncentered Pearson correlation similarity matrices was applied using Cluster and results were visualized with TreeView (34).

We first did a cluster analysis of the 165 primary tumors with all 7,708 filtered probes. The two-sided Fisher's exact test was used to compare the two dominant cluster branches with the distribution of 10 tumor characteristics (LR status, age, tumor size, nodal status, margin, tumor type, tumor shape, grade, DCIS component, and vascular invasion).

Next, the “Intrinsic/UNC” gene set previously described by Hu et al. (24) to define basal-like, luminal A-like, luminal B-like, ERBB2-like, normal-like, and IFN-like tumor classes was mapped to our microarray platform (293 of 306 unique genes identified). The genes were then clustered over all primary tumor samples. Again, the two-sided Fisher's exact test was used to compare the two dominant cluster branches with the distribution of 10 tumor characteristics as described above. In addition, we calculated the correlation coefficient between the gene expression profiles of the 165 tumors and the class centroids of the molecular subtypes as provided by Hu et al. (24). We tested for nonrandom distribution of the 10 tumor characteristics (see above) among these five classes using the Pearson χ² test. Tumors were scored as unclassifiable when the correlation coefficient to any of the molecular subtypes was <0.1.

Third, we assessed the predictive value for LR of eight additional gene expression signatures that were constructed to predict disease outcome in studies by others. For this purpose, we used five gene signatures predictive for distant metastasis-free survival: 70-gene prognosis profile by van 't Veer et al. (23), 76-gene prognosis profile by Wang et al. (27), 512-gene wound response signature by Chang et al. (30), 253-gene hypoxia signature by Chi et al. (35), and the 70-gene chromosomal instability signature by Carter et al. (36). In addition, we used 3-gene signatures with a LR-related background: 52-gene radiosensitivity signature by Khodarev et al. (37), 45-gene proliferation signature by Whitfield et al. (38), and the 81 genes associated with LR after BCT by Nimeneus-Malmstrom et al. (31). For each gene profile, the corresponding probes on our platform were identified. The two-sided Fisher's exact test was applied comparing the two dominant cluster branches and the distribution of primary tumors with and without LR in these branches.

We also assessed the performance of our previously constructed gene classifier for LR (29). We calculated the correlation coefficient of our samples to the LR centroids of the activated and quiescent wound response signature (29) and used the preset cutoff of 0.32 for LR risk class assignment.

Significant analysis of microarrays (SAM) software (39) was used to identify differentially expressed genes between the LR and no-LR samples. We used the settings in the software for two-class unpaired data. A threshold was chosen, which reflects the lowest median false discovery rate as estimated after repeated (1,000 times) permuting the labels and counting the number of genes that were called significant at each threshold. Overrepresentation of genes representing specific Gene Ontology categories in the gene list derived by the SAM analysis was identified using EASE software (40).

Class prediction was done using BRB-ArrayTools version 3.4.0 Beta_2 developed by Dr. Richard Simon and Amy Peng Lam.¹¹ A linear discriminant analysis classifier was trained with 1,000 permutations and a 10-fold cross-validation. This classifier was validated on an independent data set (26), which is a consecutive series of early-stage breast cancer patients treated with BCT (n = 161). Because this series (26) partly overlaps with the current series (n = 67), we first removed all these overlapping patients from our data set before training the classifier. This was done to retain the consecutive character of the validation series (26). This series (26) was constructed using a different microarray platform. We matched the two platforms and constructed the classifier on the overlapping probes (n = 13,404). Significance of the classifier was assessed using the log-rank test. The independent predictive power of the validated classifier in relation to known clinical risk factors for LR after BCT was tested using Cox regression analyses. First, a Cox model was built with only the known clinical risk factors for LR (age, surgical margin status, presence of extensive DCIS component, angioinvasion, and histologic grade). Second, the gene classifier was introduced into the model. Age was used as a linear variable and the other variables as ordinal or nominal variables.

**Data analysis of 15 tumor pairs.** For the analysis of the paired samples (primary tumor and recurrence in the same patient), we used a similar approach to the one described above. We first filtered the 35K-gene set for significantly regulated genes by selecting those probes with expression data available for all experiments and whose expression levels were significantly different from the reference expression in at least six experiments with a P value of <0.01. This filter reduced the total number of probes from 34,580 to 6,769. We then did a hierarchical cluster analysis using this gene set to assess the differences and similarities in gene expression between primary tumors and their recurrences. Six additional probe sets, extracted from the literature, were also used to cluster the samples. These probe sets were defined in studies using *in vitro* and *in vivo* models to compare gene expression profiles before and after exposure to radiation (41–46). The cluster analyses were tested for significance using the two-sided Fisher's exact test comparing the two dominant cluster branches and the distribution of primary tumors and recurrences in these branches.

**Statistical analysis.** The Bonferroni adjustment for multiple testing was applied to the P values in all analyses comparing the two dominant cluster branches or molecular subtype class assignment with the distribution of the 10 tumor characteristics. Statistical analyses were done using Statistical Package for the Social Sciences software, version 12 (SPSS).

### Results

We investigated, using microarray analysis, the relationship of gene expression profiles of primary invasive breast carcinomas with LR after BCT in a series of 165 patients. For this purpose, we studied tumors from 56 patients who developed a LR (after a median interval of 3.0 years; range, 0.2–9.4 years) and 109 tumors from patients who remained free of LR for at least 10 years (median time interval, 13.9 years, range, 10.2–22.7 years). To avoid bias, we did not match the patients' groups for known risk factors because these risk factors may be surrogates for gene expression patterns. In addition, we were able to study the gene expression profiles of primary invasive breast carcinoma in relation to their recurrences in 15 of the 56 patients. In total, we generated 180 unique gene expression profiles from tumors of 165 patients.

**Clinical and pathologic characteristics.** Table 1 displays the clinical and pathologic characteristics of the patients and tumors. The 165 primary breast carcinomas include 89.7% infiltrating ductal carcinomas; 80% of the tumors had microscopically free margins; 46.1% of the tumors were histologic grade 3; an extensive DCIS component was present in 15.2%. The majority of the clinical and histologic parameters are equally distributed between both groups, but a significantly larger proportion of poorly differentiated tumors (P = 0.002) and tumors with an extensive DCIS component (P = 0.016) are present in the group that developed a LR. However, the association of an extensive DCIS component with LR is not significant after adjusting for multiple testing. The use of adjuvant systemic therapy is not significantly associated with LR status; however, there is a difference in the use of CMF versus anthracyclin-based therapy when the groups with and without LR are compared (Table 1).

Table 1. Clinical and pathologic characteristics of 165 primary tumors

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients with no LR, n = 109 (%)</th>
<th>Patients with LR as first event, n = 56 (%)</th>
<th>Total, N = 165 (%)</th>
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<tr>
<td>Age category (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤40</td>
<td>30 (27.5)</td>
<td>18 (32.1)</td>
<td>48 (29.1)</td>
</tr>
<tr>
<td>&gt;40</td>
<td>79 (72.5)</td>
<td>38 (67.9)</td>
<td>117 (70.9)</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-2</td>
<td>76 (69.7)</td>
<td>35 (62.5)</td>
<td>111 (67.3)</td>
</tr>
<tr>
<td>&gt;2</td>
<td>33 (30.3)</td>
<td>19 (33.9)</td>
<td>52 (31.5)</td>
</tr>
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<td>Unknown</td>
<td>0 (0.0)</td>
<td>2 (3.6)</td>
<td>2 (1.2)</td>
</tr>
<tr>
<td>No. tumor-positive lymph nodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>61 (56.0)</td>
<td>31 (55.2)</td>
<td>92 (55.8)</td>
</tr>
<tr>
<td>1-3</td>
<td>42 (38.5)</td>
<td>15 (26.8)</td>
<td>57 (34.5)</td>
</tr>
<tr>
<td>&gt;3</td>
<td>6 (5.5)</td>
<td>5 (8.9)</td>
<td>11 (6.7)</td>
</tr>
<tr>
<td>Unknown</td>
<td>0 (0.0)</td>
<td>5 (8.9)</td>
<td>5 (3.0)</td>
</tr>
<tr>
<td>Complete excision of invasive component</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>91 (83.4)</td>
<td>41 (73.2)</td>
<td>132 (80.0)</td>
</tr>
<tr>
<td>No</td>
<td>11 (10.1)</td>
<td>9 (16.1)</td>
<td>20 (12.1)</td>
</tr>
<tr>
<td>Doubtful</td>
<td>7 (6.4)</td>
<td>3 (5.4)</td>
<td>10 (6.1)</td>
</tr>
<tr>
<td>Unknown</td>
<td>0 (0.0)</td>
<td>3 (5.4)</td>
<td>3 (1.8)</td>
</tr>
<tr>
<td>Complete excision of in situ component</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>103 (94.5)</td>
<td>50 (89.3)</td>
<td>153 (90.0)</td>
</tr>
<tr>
<td>No</td>
<td>4 (3.7)</td>
<td>3 (5.4)</td>
<td>7 (12.1)</td>
</tr>
<tr>
<td>Doubtful</td>
<td>2 (1.8)</td>
<td>3 (5.4)</td>
<td>5 (6.1)</td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infiltrating ductal carcinoma</td>
<td>98 (89.9)</td>
<td>50 (89.3)</td>
<td>148 (89.7)</td>
</tr>
<tr>
<td>Infiltrating lobular carcinoma</td>
<td>6 (5.5)</td>
<td>3 (5.4)</td>
<td>9 (5.5)</td>
</tr>
<tr>
<td>Ducto-lobular carcinoma</td>
<td>2 (1.8)</td>
<td>3 (5.4)</td>
<td>5 (3.0)</td>
</tr>
<tr>
<td>Others</td>
<td>3 (2.8)</td>
<td>0 (0.0)</td>
<td>3 (1.8)</td>
</tr>
<tr>
<td>Tumor shape</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multinodular</td>
<td>6 (5.5)</td>
<td>2 (3.6)</td>
<td>8 (4.8)</td>
</tr>
<tr>
<td>Stellate</td>
<td>66 (60.6)</td>
<td>36 (64.3)</td>
<td>102 (61.8)</td>
</tr>
<tr>
<td>Sharply demarcated/lobulated</td>
<td>24 (22.0)</td>
<td>13 (23.2)</td>
<td>37 (22.4)</td>
</tr>
<tr>
<td>Unknown</td>
<td>13 (11.9)</td>
<td>5 (8.9)</td>
<td>18 (10.9)</td>
</tr>
<tr>
<td>Grade (P = 0.002)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good</td>
<td>27 (24.8)</td>
<td>2 (3.6)</td>
<td>29 (17.6)</td>
</tr>
<tr>
<td>Moderate</td>
<td>39 (35.8)</td>
<td>21 (37.5)</td>
<td>60 (36.4)</td>
</tr>
<tr>
<td>Poor</td>
<td>43 (39.4)</td>
<td>33 (58.9)</td>
<td>76 (46.1)</td>
</tr>
<tr>
<td>Quantity DCIS around tumor (P = 0.016)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>58 (53.2)</td>
<td>21 (37.5)</td>
<td>79 (47.9)</td>
</tr>
<tr>
<td>Minimal</td>
<td>17 (15.6)</td>
<td>10 (17.9)</td>
<td>27 (16.4)</td>
</tr>
<tr>
<td>Moderate</td>
<td>22 (20.2)</td>
<td>8 (14.3)</td>
<td>30 (18.2)</td>
</tr>
<tr>
<td>Extensive</td>
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<td>15 (26.8)</td>
<td>25 (15.2)</td>
</tr>
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<td>2 (3.6)</td>
<td>4 (2.4)</td>
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<tr>
<td>Lobular carcinoma in situ component</td>
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<td></td>
<td></td>
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<tr>
<td>None</td>
<td>104 (95.4)</td>
<td>49 (87.5)</td>
<td>153 (92.7)</td>
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<tr>
<td>Present</td>
<td>4 (3.7)</td>
<td>4 (7.1)</td>
<td>8 (4.8)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (0.9)</td>
<td>3 (5.4)</td>
<td>4 (2.4)</td>
</tr>
<tr>
<td>Vascular invasion</td>
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<td></td>
<td></td>
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<tr>
<td>None</td>
<td>76 (69.7)</td>
<td>39 (69.6)</td>
<td>115 (69.7)</td>
</tr>
<tr>
<td>Present</td>
<td>30 (27.5)</td>
<td>14 (25.0)</td>
<td>44 (26.7)</td>
</tr>
<tr>
<td>Unknown</td>
<td>3 (2.8)</td>
<td>3 (5.4)</td>
<td>6 (3.6)</td>
</tr>
<tr>
<td>ER based on microarray</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER negative (log2 ratio &gt;−1.2)</td>
<td>32 (29.4)</td>
<td>23 (41.1)</td>
<td>55 (33.3)</td>
</tr>
<tr>
<td>ER positive (log2 ratio &gt;−1.2)</td>
<td>77 (70.6)</td>
<td>33 (58.9)</td>
<td>110 (66.7)</td>
</tr>
<tr>
<td>HER2 based on microarray</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2 negative (log2 ratio &lt;−0.4)</td>
<td>95 (87.2)</td>
<td>43 (76.8)</td>
<td>138 (83.6)</td>
</tr>
<tr>
<td>HER2 positive (log2 ratio &gt;−0.4)</td>
<td>14 (12.8)</td>
<td>13 (23.2)</td>
<td>27 (16.4)</td>
</tr>
<tr>
<td>Total radiation dose to tumor bed (Gy)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;67</td>
<td>73 (67.0)</td>
<td>45 (80.4)</td>
<td>118 (71.5)</td>
</tr>
<tr>
<td>&gt;66</td>
<td>36 (33.0)</td>
<td>10 (17.9)</td>
<td>46 (27.9)</td>
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<td>0 (0.0)</td>
<td>1 (1.8)</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td>Adjuvant systemic therapy (P = 0.001)*</td>
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<tr>
<td>No</td>
<td>62 (56.9)</td>
<td>36 (64.3)</td>
<td>98 (59.4)</td>
</tr>
<tr>
<td>CMF</td>
<td>37 (33.9)</td>
<td>7 (12.5)</td>
<td>44 (26.7)</td>
</tr>
<tr>
<td>Anthracylin based</td>
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<td>9 (16.1)</td>
<td>11 (6.7)</td>
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<td>Endocrine therapy</td>
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<td>2 (3.6)</td>
<td>3 (1.8)</td>
</tr>
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<td>CMF + endocrine therapy</td>
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<td>2 (3.6)</td>
<td>8 (4.8)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (0.9)</td>
<td>0 (0.0)</td>
<td>1 (0.6)</td>
</tr>
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</table>

*χ² analysis.
Fig. 1. Hierarchical cluster analysis of 165 primary breast carcinomas and 7,708 significantly regulated genes. On the right-hand side of the heat map, specific gene clusters are indicated.
For 132 patients, the resection margin was found to be free of tumor. Of 20 patients with tumor-involved resection margins, 14 patients (3 in the LR group and 11 in the control group) had focal involvement of the resection margin by invasive carcinoma, and in 6 patients, the extent of margin involvement could not be retrieved.

Of the seven patients with involvement of the surgical resection margin with DCIS, three patients had an extensive in situ component around the tumor and two of these patients developed a LR. Focal involvement of both an invasive component and in situ component in one patient, who did not develop a LR. More than focal involvement of the surgical resection margin was only seen for an in situ component in one patient, who did not develop a LR. In total, 38 patients, with either positive or doubtful surgical resection margin, received a radiotherapy boost, of whom 25 were given a high boost dose attributing a total dose of >66 Gy to the tumor bed.

Table 1 shows that there is an equal distribution of ER and HER2 receptor status (based on gene expression data) between the tumors with and without LR.

**Overall gene expression profiles of 165 primary tumors.** Hierarchical cluster analysis of all 165 tumors resulted in the formation of two main tumor clusters that can be further subdivided into five subclusters (Fig. 1). None of the 10 tumor characteristics investigated (LR status, age, tumor size, nodal status, margin, tumor type, tumor shape, grade, DCIS component, and vascular invasion) were associated with the two dominant groups after adjusting for multiple testing.

Next, we assessed the association of the gene expression profile of the primary tumors with that of the molecular subtypes defined by Perou et al. and Sorlie et al. (24, 25, 47) using the updated “intrinsic gene” list as described by Hu et al. (24). We did a hierarchical cluster analysis with the 165 primary breast carcinomas and the 293 intrinsic genes present on our platform. This resulted in the formation of five main tumor clusters representing the well-known molecular subtypes (basal like, ERBB2 like, luminal A like, luminal B like, and IFN like; data not shown; ref. 24). In addition, we tested class assignment based on the predetermined centroids of the five molecular subtypes as provided by Hu et al. (24). For this purpose, we calculated the correlation coefficient between the gene expression profiles of the 165 tumors and the centroids of the 5 molecular subtypes. We tested for non-random distribution of the 10 tumor characteristics among these 5 classes. Using this approach, LR status (P = 0.00016) and histologic grade (P < 6 × 10^{-13}) were the only two factors still significant after adjusting for multiple testing. Tumors of patients who developed a LR were significantly more frequently classified as luminal B–like or ERBB2-like tumors (basal-like subtype tumors developed a LR in 36% of this case-control series, luminal A–like tumors in 21%, normal epithelial-like tumors in 10%, versus ERBB2-like tumors in 63% and luminal B–like in 68%; Table 2).

We assessed how eight additional gene expression signatures (23, 27, 30, 31, 35–38), which were constructed to predict disease outcome, performed in differentiating between tumors with and without LR (Table 3). There was a nonrandom distribution, after adjusting for multiple testing, of tumors developing LR between the two dominant clusters using the 70-gene prognosis signature (P = 0.005; ref. 23) and the chromosomal instability signature (P = 0.003; ref. 36).

Finally, in a previous study, we retrained the wound response signature by Chang et al. (30) to predict LR. Because this was a relatively small study, we wished to repeat the analysis in this larger series. The correlation coefficient between the gene expression ratios and the LR centroids of the activated wound response signature were calculated for all tumors. There was a significant difference in mean correlation coefficient between the two classes (mean correlation coefficient: LR class = 0.038; no-LR class = 0.077; P = 0.034). However, the correlations with the activated

**Table 2. Association of tumor molecular subtype assignment based on the nearest centroid principle of 165 primary breast carcinomas with LR status (P = 0.00016)**

<table>
<thead>
<tr>
<th>Tumor molecular subtype</th>
<th>Patients with no LR, n = 109 (%)</th>
<th>Patients with LR, n = 56 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal epithelial like</td>
<td>9 (90.0)</td>
<td>1 (10)</td>
</tr>
<tr>
<td>Luminal A like</td>
<td>61 (79.2)</td>
<td>16 (20.8)</td>
</tr>
<tr>
<td>Basal like</td>
<td>21 (63.6)</td>
<td>12 (36.4)</td>
</tr>
<tr>
<td>ERBB2 like</td>
<td>6 (37.5)</td>
<td>10 (62.5)</td>
</tr>
<tr>
<td>Luminal B like</td>
<td>6 (31.6)</td>
<td>13 (68.4)</td>
</tr>
<tr>
<td>Unclassifiable</td>
<td>6 (60.0)</td>
<td>4 (40)</td>
</tr>
</tbody>
</table>

**Table 3. Predictive value for LR of eight additional prognosis profiles**

<table>
<thead>
<tr>
<th>Prognosis profile</th>
<th>No. LR tumors/total of tumors in dominant cluster 1 (%)</th>
<th>No. LR tumors/total of tumors in dominant cluster 2 (%)</th>
<th>P</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>70-gene signature (23)</td>
<td>23/93 (25)</td>
<td>33/72 (46)</td>
<td>0.005</td>
<td>59%</td>
<td>64%</td>
</tr>
<tr>
<td>76-gene signature (27)</td>
<td>18/76 (24)</td>
<td>38/89 (43)</td>
<td>0.013</td>
<td>68%</td>
<td>53%</td>
</tr>
<tr>
<td>Wound response signature (30)</td>
<td>14/59 (24)</td>
<td>42/106 (40)</td>
<td>0.041</td>
<td>75%</td>
<td>41%</td>
</tr>
<tr>
<td>Hypoxia signature (35)</td>
<td>24/55 (44)</td>
<td>32/110 (29)</td>
<td>0.081</td>
<td>57%</td>
<td>28%</td>
</tr>
<tr>
<td>Radiosensitivity signature (37)</td>
<td>29/79 (37)</td>
<td>27/86 (31)</td>
<td>0.51</td>
<td>52%</td>
<td>54%</td>
</tr>
<tr>
<td>Chromosomal instability signature (36)</td>
<td>18/80 (23)</td>
<td>38/85 (45)</td>
<td>0.003</td>
<td>68%</td>
<td>57%</td>
</tr>
<tr>
<td>Proliferation signature (38)</td>
<td>23/92 (25)</td>
<td>33/73 (45)</td>
<td>0.008</td>
<td>59%</td>
<td>63%</td>
</tr>
<tr>
<td>LR after BCT signature (31)</td>
<td>25/71 (35)</td>
<td>31/94 (33)</td>
<td>0.87</td>
<td>55%</td>
<td>42%</td>
</tr>
</tbody>
</table>
wound response signature are very low and the preset cutoff for class assignment (0.32) is not reached. Furthermore, the higher correlation coefficients (activated wound response) are associated with the no-LR class, contradictory to the original findings (29).

Supervised analysis. We first identified differentially expressed genes between tumors developing a LR and those that do not. A two-class SAM analysis for unpaired data was used. Using the threshold with the lowest median false discovery rate of 0.66%, a list of 104 genes was generated (Supplementary Data). To identify if the 104-gene list is enriched for genes representing particular Gene Ontology categories compared with the entire list of 7,708 genes, the EASE software was used. We found an overrepresentation of genes involved with cell proliferation (Supplementary Data). This finding could be explained by the significant difference in the distribution of histologic grade between tumors with and without LR (Table 1); hence, we repeated the analysis after excluding all grade 1 tumors (grade 2 and 3 tumors were well balanced between the two groups; \( P = 0.38 \)). This resulted in a list of 129 genes with a slightly higher median false discovery rate of 4.2%. There were 63 genes in common with the list derived from all tumors. The 129-gene list was also enriched for genes representing Gene Ontology categories related to cell proliferation.

To build a classifier for LR, BRB-ArrayTools was used. First, the overlapping samples in our series with the validation series (26) were removed \( (n = 67) \). We trained a linear discriminant analysis classifier on 98 tumors using 1,000 permutations and 10-fold cross-validation (48). This resulted in a 111-gene classifier with a positive predictive value of 0.68 and 0.58 for correct classification of a no-LR tumor and a LR tumor, respectively. Using this classifier, we predicted the LR status for 161 tumors in the validation series (26). With a median follow-up of 10.6 years, 22 of the 161 patients developed a LR. The validation showed a significant difference in LR-free survival after BCT between tumors classified as LR-free versus those predicted to develop LR (log-rank \( P = 0.041 \); Fig. 2). Seventeen of the 22 tumors developing a LR were correctly classified (sensitivity, 77%) and 60 of 139 no-LR tumors were correctly classified (specificity, 43%).

To test if the 111-gene classifier is independently associated with LR in the presence of known clinical variables, we did a multivariate Cox regression analysis. First, we built a Cox regression model with the known clinical risk factors for LR (age, surgical margin status, presence of an extensive DCIS component, angioinvasion, and histologic grade). In this model, only age had independent predictive value for LR after BCT (HR, 0.88; 95% confidence interval, 0.82-0.95; \( P = 0.001 \)). We then added the 111-gene classifier to the model and found that the classifier was not significant \( (P = 0.21) \); again, age was the only independent predictive variable for LR (HR, 0.89; 95% confidence interval, 0.83-0.96; \( P = 0.002 \)).

Gene expression in 15 pairs of primary tumors and LRs. Using unsupervised hierarchical cluster analysis on 6,769 probes, we compared the overall gene expression pattern of 15 primary tumors and 15 patient-matched LRs. The hierarchical cluster analysis shows close clustering of recurrences with their primary tumors in almost all cases (12 of 15; Fig. 3). This indicates that the overall gene expression profile of a LR is more similar to its primary tumor than to other LRs. This is interesting because all LRs, in compression with all primary tumors, have been irradiated up to a dose of at least 50 Gy. We repeated the clustering of these 30 samples using 6 additional probe sets extracted from the literature. The probe sets...
were defined in studies using in vitro and in vivo models where
gene expression profiles were compared before and after expo-
sure to radiation (41–46). None of the additional probe sets
was able to significantly separate the recurrences from the primary
tumors (P range, 0.14-1.0).

Discussion

We have done gene expression profiling of 165 primary inva-
sive breast carcinomas that were treated with BCT; we also pro-
filed 15 patient-matched locally recurred tumors. A case-control
approach was used to achieve an optimal comparison of primary
tumors that develop a LR with those that remain free of LR. For
this reason, tumors were denoted as controls only if they re-
mained free of LR for at least 10 years after treatment.

In a previous study, we tried to elucidate the underlying mech-
anism of LR after BCT using gene expression profiling (28) and
found that there was no significant difference in the overall gene
expression pattern between primary tumors with and without
LR. We have now analyzed a larger number of patients using
more advanced microarray techniques. In the present study, we
show that there is a difference in gene expression between prima-
ry tumors with and without LR after BCT. Genes associated with
cell proliferation, not captured by histologic grading, are ex-
pressed at higher levels in tumors from patients who developed
a LR after BCT. We were able to construct a classifier predictive for
LR after BCT and validated this profile on an independent data
set of 161 consecutive breast cancer patients treated by BCT. It is
of note that the predictive classifier for LR could be validated on a
series constructed using a different microarray platform (26).
However, with large numbers of false positives and few false neg-
atives, a positive gene signature prediction for LR is in itself poor
at predicting LR (positive predictive value, 18%) and needs fu-
ture research to be optimized in larger series. However, 77% of
all LRs are predicted. Furthermore, a no-LR prediction is very
good at reassuring that a patient has a low risk for LR (negative
predictive value, 92%).

As young patients are at increased risk for LR, this study only
involved patients younger than 51 years. Despite this preselection,
age was again the only independent predictor of LR in mul-
vivariate analysis, which is in line with the results of the European
Organization for Research and Treatment of Cancer boost-no-
boost trial, where patients under the age of 40 years had a signif-
icant higher risk than patients between 40 and 50 years (20).

We found a significant relation between the molecular breast
carcinoma subclasses [as described by Hu et al. (24)] and LR.
Class assignment based on the correlation coefficient with the
centroids of the 5 subclasses indicate that there are 10 tumors with
a gene expression profile resembling the normal breast-
like subtype, of which 9 had no LR. The majority of luminal
A–like tumors also remained free from LR, in contrast to luminal
B–like and ERBB2-like tumors.

In addition, several other known gene expression signatures
that were constructed to classify tumors into prognostic groups
(23, 27, 30, 31, 35–38) were assessed. The chromosomal insta-
bility signature by Carter et al. (36) showed the most significant
separation of tumors with and without LR. This agrees with our
own classifier because the chromosomal instability signature is a
reflection of proliferation. Of note, gene expression signatures
that are also associated with sensitivity to radiotherapy [Khodar-
ev et al. (37) and Chi et al. (35)] did not predict for LR. We also
analyzed the signatures described in Table 3 for their association
with the development of distant metastases (Supplementary
Data). The 70-gene prognosis signature and the chromosomal in-
stability signature were associated with the development of distant
metastases; however, after Bonferroni adjustment for multiple
testing, these differences were not statistically significant; the other
signatures were not associated with the development of distant
metastases in this patient series. In addition, we assessed the ability
to predict distant metastases using the 111-gene LR classifier. This
showed a significant separation of tumors developing distant me-
tastases versus tumors not developing distant metastases (Supple-
mentary Data).

The 81-gene list by Nimeus-Malmstrom et al. (31) did not pre-
dict LR in our study, although this signature was identified using comparable selection criteria for patients (LR after BCT).
This study is one of many microarray studies done in the past that
did not use an independent validation set to confirm the con-
structed gene signature. From a statistical point of view, it is rel-
atively easy to construct a gene signature using few samples and a
large number of variables (genes); one will always find a corre-
lation purely based on change and only a robust classifier will hold
up in a validation series (49).

This may also be one of the reasons that we were unable to
confirm our previously constructed LR classifier (29) based on
the wound response signature (30). Although this signature
was validated on an independent cohort of patients; the overall
sample size for both the training and validation series was small
and especially the number of events was low (nine LRs in training
series and eight LRs in validation series; ref. 29). Another reason
might be the loss of essential genes (n = 56) in the classifier after
matching the microarray platforms.

The paired analysis showed close clustering of the primary tu-
mor and its recurrence, although the tumor cells out of which the
recurrence grew were irradiated up to a dose of at least 50 Gy. This
is in contrast with studies showing changed gene expression in
cell lines and tissue of patients peri radiotherapy (41–46). The
main difference between these studies and our study is the time
interval. The postirradiation expression profile in these studies is
generated relatively shortly after the end of the irradiation
(hours-days) in contrast to years (1.01–9.34) for a LR. This may
indicate that the overall gene expression profile of an irradiated
tumor changes back to the profile before irradiation after the
acute radiation induced effects have settled. We tried to identify
a set of genes that is differently expressed between the primary
tumors and the recurrences using two-class SAM analysis for
paired data. This resulted in very high false discovery rates, indi-
cating that such a set of genes could not reliably be detected (data
not shown).

The close clustering of the primary tumor and its recurrence
agrees with the study of Bollt et al. (50). They compared the sin-
gle-nucleotide polymorphism profile of primary breast carcino-
mas and ipsilateral recurrences in 22 patients treated with BCT
and showed that, in the majority of the cases, the overall sin-
gle-nucleotide polymorphism profile of the primary tumor and
its recurrence are more related to each other than to other
primaries or recurrences.

There are several potential confounding factors that may have
influenced the results of our study. The sample size of our study is
relatively small, which makes it unfeasible to analyze important
subgroups, such as patients that received the same boost of ra-
diotherapy or patients subdivided based on margin status. Some
tumors recorded as LR may have been secondary primary tumors developing in the same breast. In our previous study (28), for 11 cases, we compared the primary tumor and LR using loss of heterozygosity analysis and could thus show that all 11 cases were true recurrences. This indicates that the way we selected for LR cases actually does well in identifying true LRs. Furthermore, although margin involvement is an important risk factor for LR, not for all patients in our study the surgical margins were free of tumor. We believe that this has not confounded our study because involved margins were present in both the cases and controls. In conclusion, we have constructed a cross-platform validated gene expression profile predictive for LR after BCT. In relation to already established clinical risk factors for LR, it has no independent additive value; however, it may help in elucidating the underlying mechanism of LR after BCT as genes involved in cell proliferation characterize this profile. Furthermore, analyses of primary tumors and paired LRs show a preservation of the overall gene expression pattern in the LR even after radiotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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