

DNA Sequence Losses on Chromosomes 11p and 18q Are Associated with Clinical Outcome in Lymph Node-negative Ductal Breast Cancer¹

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ABSTRACT

Purpose: Histological and other markers alone cannot predict the risk of disease progression in node-negative breast cancer. Several genomic aberrations have been linked to clinical outcome in breast cancer.

Experimental Design: In this study, comparative genomic hybridization was applied to screen for specific DNA copy number gains and losses in 20 pT₁/pT₂ node-negative invasive ductal carcinomas with no disease recurrence with at least 8 years of follow-up and in 20 pT₁/pT₂ node-negative tumors with distant disease recurrence.

Results: The number of genomic aberrations (copy number gains and losses) per tumor was significantly higher in tumors with disease recurrence ($P < 0.05$). The number of genomic aberrations was associated with histological grade ($P < 0.02$). Within the group of tumors with disease recurrence, the total number of genetic aberrations per tumor ($P < 0.02$) and the number of DNA sequence losses per tumor ($P < 0.01$) were significantly associated with poor survival. Of the individual loci involved, only losses at chromosomes 11p ($P < 0.002$) and 18q ($P < 0.004$) were associated with poor survival in the recurrence group. Histological grade and loss of 18q were independent prognostic variables in multivariate analysis.

Conclusions: This genome-wide analysis by comparative genomic hybridization suggests that node-negative ductal breast cancers with a high number of genomic aberrations have an increased risk of disease recurrence. The number of DNA sequence losses, particularly losses of chromosomes 11p and 18q, were associated with poor prognosis.

Genes on chromosomes 11p and 18q may play a role in the progression of ductal breast carcinoma.

INTRODUCTION

Breast cancer is the most common malignancy among women throughout the developed world. Decreases in the incidence of breast cancer have not been achieved, but there is a downward trend in age-adjusted breast cancer mortality rates (1). Classification and prognosis of breast cancer have been based on histopathological parameters such as lymph node status, tumor grade, and size. Of these, lymph node status is a strong indicator of high risk for progression. However, within the group of lymph node-negative patients, 20–30% die of the disease, necessitating additional prognostic markers to select high-risk patients within this subgroup (2). Because approximately half of breast cancers are diagnosed without axillary lymph node metastasis, the development of prognostic parameters for women at high risk of relapse would enable the identification of individuals who would benefit most from adjuvant systemic therapy. On the other hand, those at low risk could be spared the morbidity associated with adjuvant systemic therapy (3).

Previous cytogenetic studies have detected several structural and numerical chromosomal abnormalities in primary breast cancer. CGH³ allows the detection of DNA copy number changes across the genome and is applicable to all tumors regardless of their mitotic activity or the complexity of chromosomal changes (4). CGH enables the detection of DNA sequence copy number changes if the affected region spans >10 Mb (5, 6). LOH analyses may detect smaller aberrations in specific regions than CGH (7), but LOH analyses cannot distinguish DNA losses from amplifications and cannot be applied on a genome-wide scale. Therefore, CGH has opened interesting possibilities for the understanding of oncogenesis of solid tumors because copy number changes are important indicators of cancer-related genes in these chromosomal regions. CGH has been applied in several breast cancer studies (8–12) and considerable molecular and chromosomal data are now available (13, 14). However, little is known about the relationship of chromosomal aberrations to the clinical behavior of the tumor and therefore to prognosis. Isola *et al.* (10) showed in a pilot study that DNA copy number changes are associated with clinical outcome in lymph node-negative breast cancer.

The aims of this study were to test the ability of CGH to predict the patient outcome in archived pT₁ and pT₂ node-negative invasive ductal breast carcinoma tissue specimens. CGH was also used to identify specific chromosomal loci asso-

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³ The abbreviations used are: CGH, comparative genomic hybridization; LOH, loss of heterozygosity; TGF- β , transforming growth factor β .

ciated with poor prognosis. The tumors were stratified according to the recurrence status, the histological grade, and the tumor size.

MATERIALS AND METHODS

Patients. The surgical pathology reports (Institute of Pathology, University of Basel, Basel, Switzerland) of patients who underwent surgery for invasive ductal breast cancer were reviewed. Breast tumor samples were taken from lumpectomy or mastectomy specimens. The tumor status was confirmed by histopathological analysis. Forty consecutive specimens with invasive ductal carcinoma were retrospectively selected. Twenty patients were free of disease for at least 8 years, and 20 patients developed distant metastasis. All patients had palpable disease. The invasive ductal carcinomas were staged according to the fifth edition of the Union International Contre le Cancer (15) and American Joint Committee on Cancer TNM system (16). All histological sections were reviewed by one pathologist (J. T.). Tumor size, histological grade (17), and lymph node status based on at least 10 nodes were assessed. The median tumor diameter for all node-negative invasive ductal breast cancers was 2 cm (range, 1.0–5.0 cm). Twenty-one tumors were pT₁ and 19 were pT₂ according to the 1997 TNM staging. A complete axillary lymph node dissection was performed in all patients, and the median number of removed lymph nodes was 18 (range, 10–33). All removed lymph nodes were evaluated by the pathologist.

Local treatment was lumpectomy with postoperative irradiation of the breast or mastectomy. No systemic adjuvant treatment was given in these patients (start of treatment between 1982 and 1990) because systemic adjuvant treatment for node-negative breast cancer was not recommended at that time (18).

Follow-up information, information about metastasis, and causes of death were obtained from the Department of Gynecology and/or from the Basel Cancer Registry. Survival time was available for all patients. Survival time was calculated from the time of biopsy diagnosis to death of patients with distant metastases.

Tissue Preparation. Tissue probes of 40 invasive ductal carcinomas and the axillary lymph nodes were fixed in 4% buffered formalin, embedded in paraffin, and routinely stained for histological diagnosis. Specimens were trimmed to enrich for tumor cells by excising tumor tissue from the paraffin block. The excised tumor tissue was re-embedded in a paraffin block. Five- μ m tissue sections were cut from these tumor blocks. The first and the last sections were stained with H&E to verify the presence of at least 75% tumor cells in the sample. Genomic DNA was isolated by proteinase K extraction according to standard protocols.

DNA Preparation. CGH analysis was performed as described (4, 19). Briefly, 20- μ m-thick sections were deparaffinized and suspended in DNA extraction buffer containing 0.5 mg/ml proteinase K. Additional proteinase K was added 24 and 48 h later, for a total incubation time of 72 h. Two μ g of tumor DNA were nick translated by a commercial kit (BioNick kit; Life Technologies, Inc., Gaithersburg, MD) with SpectrumGreen Direct-Labeled Total Human Genomic dUTPs (Vysis, Inc., Downers Grove, IL) for direct labeling of tumor DNA.

SpectrumRed-labeled normal reference DNA (Vysis) was used for cohybridization.

CGH and Digital Image Analysis. The hybridization mixture consisted of 200 ng of SpectrumGreen-labeled tumor DNA, 200 ng of SpectrumRed-labeled normal reference DNA, and 20 μ g of human Cot-1 DNA (Life Technologies, Inc.) dissolved in 10 μ l of hybridization buffer [50% formamide-10% dextran sulfate-2 \times SSC (pH 7.0)]. Hybridization was performed over 3 days at 37°C to normal metaphase spreads (Vysis). Posthybridization washes were performed as described previously. Digital images were collected from six to seven metaphases by a Photometrics cooled CCD camera (Microimager 1400; Xillix Technologies, Vancouver, British Columbia, Canada) and a Sun workstation. The Vysis software program was used to calculate average green-to-red ratio profiles for each chromosome. At least four observations per autosome and at least two observations per sex chromosome were included in each analysis, according to previous recommendations by Kallioniemi *et al.* (6).

Controls and Threshold Definitions. CGH experiments included a tumor cell line (SpectrumGreen-labeled MPE-600 DNA; Vysis) with known aberrations (positive control) and a hybridization of two differentially labeled sex-mismatched normal DNAs to each other (negative control). Sex-mismatched normal controls were also used to test the ability of each metaphase batch to allow for a linear relationship between fluorescence intensities and DNA sequence copy numbers. Metaphases were used only if the color ratio of sex-mismatched normal DNAs was ≤ 0.66 at the X chromosome. The thresholds used for definition of DNA sequence copy number gains and losses were based on the results of CGH analyses of formalin-fixed normal tissues. Gains of DNA sequences were defined as chromosomal regions where both the mean green to red fluorescence ratio and its SD were > 1.20 , whereas losses were defined as regions where both the mean and its SD were < 0.80 . Overrepresentations were considered amplifications when the fluorescence ratio values in a subregion of a chromosome arm exceeded 1.5. In negative control hybridizations, the mean green-to-red ratio occasionally exceeded the fixed 1.2 cutoff level at the following chromosomal regions: 1p32-pter, 16p, 19, and 22. These known GC-rich regions were therefore excluded from all analyses.

Immunohistochemistry. A monoclonal mouse antibody to keratin (Cocktail CK22; Biomedica, Foster City, CA) was used for the detection of micrometastases in axillary lymph nodes. The incubation was performed according to the standard procedure of the ABC method and the protocol instructions provided by Biomedica. Sections were counterstained with hematoxylin, dehydrated, and finally, mounted with Crystall/Mount (Biomedica).

Statistics. Results are given as mean values and SD. Relationships between categorical features and counts were evaluated by the nonparametric method of the Mann-Whitney *U* test. Multiple comparisons were performed by Fisher's test. Contingency table analysis was used to analyze the correlations among ductal carcinomas with and without distant disease recurrence, histological grade, and TNM stage. Survival was defined as the time between primary treatment and death with distant metastases. Patients who survived were censored at the time of last follow-up. Survival analysis was completed using

the Kaplan-Meier method with a log-rank test. The median values of the numbers of DNA aberrations, *i.e.*, DNA sequence losses or gains, were used as cutoff points to define patients with high and low numbers of corresponding aberrations. A Cox proportional hazards analysis was used to test for independent prognostic information. Statistical analyses were performed by use of the StatView 4.5 Software program (Abacus Concepts).

RESULTS

Tumors. There was no difference in the median tumor diameter or in the median number of removed lymph nodes between tumors with and without distant recurrence. Immunohistochemically, no difference was observed in the detection rate of micrometastases in the axillary lymph nodes. In tumors with and without distant disease recurrence, micrometastases were detected in only two cases per group (Table 1).

The set of 40 invasive ductal carcinomas was stratified by histological grade (30 grade I and II tumors; 10 grade III tumors). Tumors without recurrence were mostly grade I and II tumors (18 grade I and II *versus* 2 grade III). Tumors with recurrence included 12 grade I and II tumors and 8 grade III tumors. The relationship between recurrence status and histological grade was significant (χ^2 test, $P < 0.02$).

Overview of Genomic Changes in Node-negative Breast Carcinoma. Genetic aberrations were detected by CGH in all tumors. The chromosomal aberrations are summarized in Fig. 1. DNA aberrations occurred across the entire genome. Copy number gains were most frequently identified on chromosomes 17q, 1q, and 20q, whereas copy number losses were most frequently detected on chromosomes 13q, 4q, 6q, and 9p (Table 2). The frequency of copy number changes varied across the genome with chromosomes showing a high frequency of aberrations, as mentioned above, and chromosomes with infrequent changes, such as 5p, 6p, 7p, 10p, and 12p. There was a difference in the number of gains and losses per tumor when tumors with and without relapse were compared (Table 3). In tumors without disease recurrence, the total number of aberrations was 9.3 ± 5.2 , the frequency of copy number gains was 2.6 ± 1.9 , and the frequency of copy number losses was 6.7 ± 3.9 . In tumors with disease recurrence, the total number of aberrations (13.3 ± 5.3), the number of losses (9.5 ± 4.4), and the number of gains (3.8 ± 1.8) were significantly higher (Fisher's test, $P < 0.05$) than in tumors without disease recurrence. When tumors were grouped according to histological grade (grade I/II *versus* grade III), the number of genomic aberrations was significantly lower in grade I/II ($P < 0.01$) than in grade III tumors (Table 3). When individual chromosomal loci were tested, there were significantly more copy number losses on chromosome 9q in grade III tumors (χ^2 test, $P < 0.03$). Grade I and II tumors showed no losses on 9q.

Clinical Outcome and CGH. Only one patient died in the group of tumors without disease recurrence. The death was unrelated to breast cancer disease. In the group of patients with distant disease recurrence, 18 patients died of disease during clinical follow-up. Survival analysis (Kaplan-Meier estimate) in cases with disease recurrence revealed a poor overall survival in patients whose tumors showed a high number of genetic aberrations. The total number of genetic aberrations per tumor was

Table 1 Pathological parameters of 40 node-negative invasive ductal carcinomas

Numbers are median values.

Parameter	Without progression (n = 20)	With progression (n = 20)
Tumor diameter, mm (range)	21.0 (10.0–35.0)	20.0 (10.0–50.0)
Grade III tumors (%)	10	40
No. of lymph nodes per axilla (range)	17 (10–24)	20 (11–33)
No. of axillae with micrometastases	2	2

significantly associated with poor survival (Fig. 2A) in the recurrence group ($P < 0.02$). When copy number losses and gains were analyzed separately, only losses (Fig. 2B) were significantly associated with survival ($P < 0.01$). To define patients with high and low numbers of corresponding aberrations, the median value of 11 for the total number of aberrations and the median value of 9 for the number of losses were used as cutoff points.

Of the individual loci involved, losses on the chromosomes 18q ($P < 0.004$; Fig. 3A) and 11p ($P < 0.002$; Fig. 3B) were significantly associated with poor survival. DNA sequence losses on chromosomes 18q and 11p were detected in only four and two tumors, respectively, without disease progression. However, there were no significant differences between the losses on chromosome loci 18q and 11p in tumors with and without disease recurrence. Cox proportional hazards analysis with the parameters histological grade, tumor diameter, loss of 11p, and loss of 18q indicated that histological grade and loss of 18q were independent prognostic variables (Table 4).

DISCUSSION

This study reports a significant association between the risk of disease progression and a high number of genomic aberrations in node-negative invasive ductal breast carcinoma. In patients with disease recurrence, a high number of genomic aberrations is associated with poor prognosis.

DNA copy number losses in chromosomes 18q and 11p were associated with poor prognosis. To date, three candidate tumor suppressor genes, including *DCC* (20), *DPC4* (21), and *MADR2* (22), have been identified on the long arm of chromosome 18. Two of these genes, *DPC4* and *MADR2*, are particularly interesting because they are important mediators in the TGF- β pathway. Recently, TGF- β has been shown to be a tumor suppressor involved in breast cancer tumorigenesis (23, 24). The tumor-suppressive effects of TGF- β are caused by potent inhibition of cell proliferation via cell cycle arrest in the G₁ phase. Furthermore, it has been demonstrated that TGF- β inhibits telomerase activity (25). Zimonjic *et al.* (26) described an increased tumorigenicity in breast carcinoma cell lines with DNA copy number losses on chromosome 18q. LOH on 18q has been previously reported in 65% of 228 analyzed primary breast cancers (27). This high frequency of 18q losses compared with our study might be explained by a higher sensitivity of LOH analyses and by the higher number of advanced tumors in their study. The authors concluded that there might be three separate

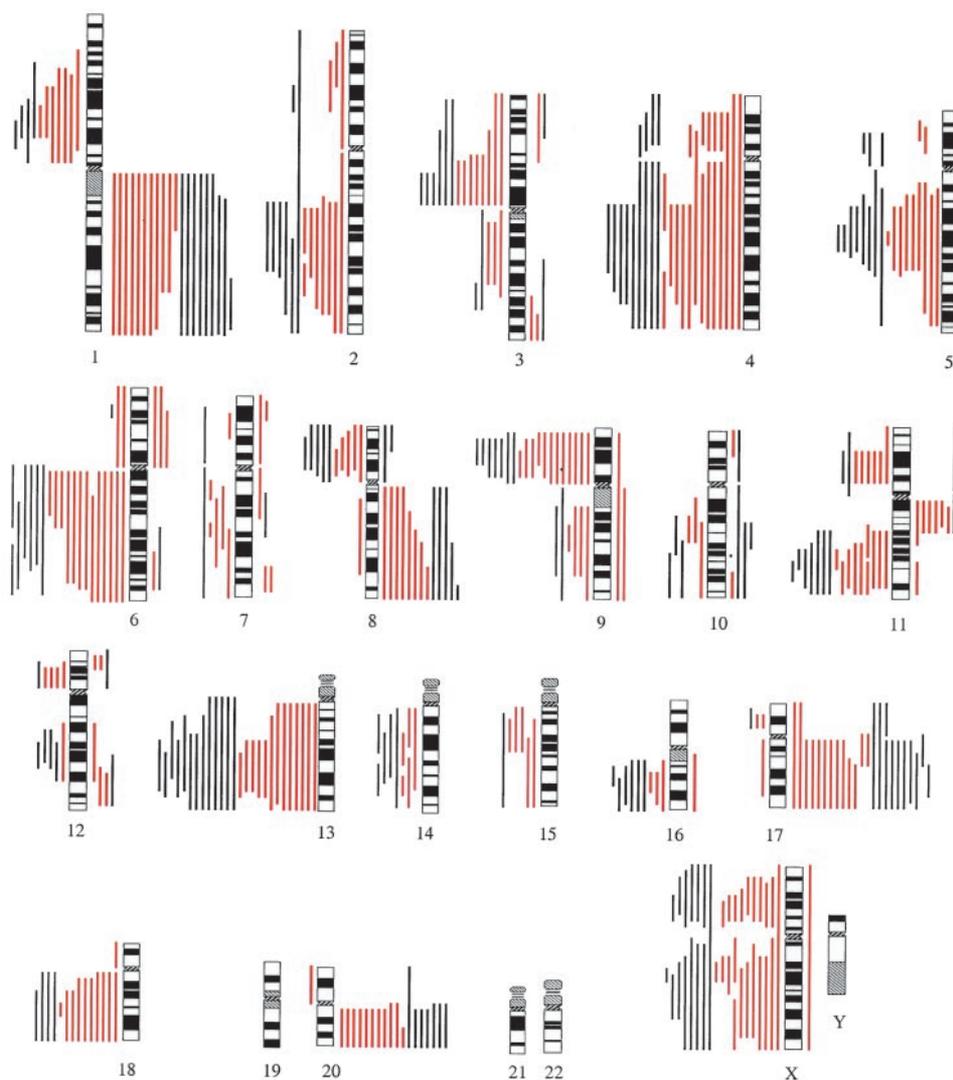


Fig. 1 Summary of all gains (right) and losses (left) of DNA sequences observed by CGH in 40 node-negative invasive ductal carcinomas with (red) and without (black) distant disease recurrence.

LOH target regions on 18q that are associated with unfavorable clinicopathological features. These data also show that gene alterations on 18q might contribute to a more aggressive tumor cell phenotype in invasive ductal breast cancer.

Chromosome 11 is the second most frequent chromosome with structural or numerical aberrations in breast cancer (28). The significant difference in disease-specific survival between tumors with and without 11p losses suggests that a tumor suppressor gene on 11p is involved in ductal carcinoma progression. In two recent reports, a correlation between LOH on two loci at 11p15.5-p15.4 and the presence of metastasis was described (29, 30). Future studies using DNA chips may soon identify other potential target genes on the chromosomes 18q and 11p.

In this study, progression-linked genomic aberrations were identified from formalin-fixed, paraffin-embedded material by CGH. Outcome-related genomic aberrations have been also identified by CGH for other tumors, including renal cell carcinoma (31), cervical carcinoma (19), neuroblastoma (32), and

Table 2 Most frequent genomic aberrations in 40 node-negative invasive ductal carcinomas

Aberration	Total prevalence (%)	Aberrations in recurrent cases/total aberrant
13q-	65	13/26
17q+	57	13/23
4q-	55	14/22
Xq-	50	12/20
1q+	50	11/20
6q-	47	12/19
9p-	45	11/18
20q+	45	11/18
Xp-	42	10/17
3p-	35	8/14
4p-	32	9/13
8q+	32	8/13
2q-	32	8/13
5q-	30	9/12
18q-	27	7/11
1p-	27	7/11
11p-	20	5/8

Table 3 Genomic aberrations, status of relapse, and histological grade in 40 node-negative invasive ductal carcinomas
Mann-Whitney *U* test. Numbers are mean values ± SD.

	Total no. of aberrations/ tumor	<i>P</i>	Total no. of losses/ tumor	<i>P</i>	Total no. of gains/ tumor	<i>P</i>
Status of relapse						
Without (<i>n</i> = 20)	9.3 ± 5.2		6.7 ± 3.9		2.6 ± 1.9	
With (<i>n</i> = 20)	13.3 ± 5.3	0.02	9.5 ± 4.4	0.04	3.8 ± 1.8	0.05
Histological grade						
Grade I/II (<i>n</i> = 30)	10.0 ± 5.3		7.1 ± 4.1		2.9 ± 2.0	
Grade III (<i>n</i> = 10)	15.1 ± 4.6	0.01	11.0 ± 3.8	0.01	4.1 ± 1.4	0.08

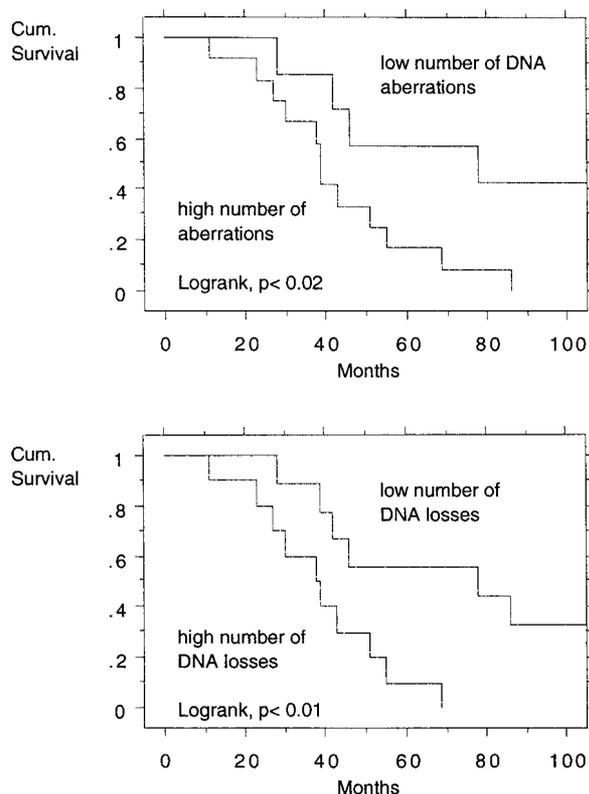


Fig. 2 Overall survival in node-negative patients with disease recurrence (*n* = 20). A, patients with low (≤ 11 ; *n* = 8) and high (> 11 ; *n* = 12) DNA aberrations/tumor. B, patients with low (≤ 9 ; *n* = 10) and high (> 9 ; *n* = 10) DNA sequence losses/tumor.

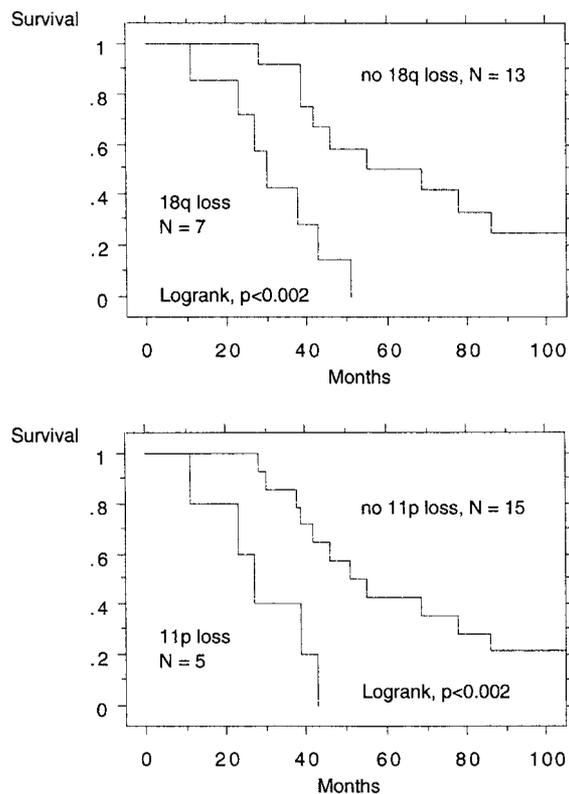


Fig. 3 Overall survival in node-negative patients with recurrence (*n* = 20). A, patients with DNA copy number losses on chromosome 18q. B, patients with DNA copy number losses on chromosome 11p.

others. CGH is primarily a screening method that will not find all copy number alterations. However, the detection of specific chromosomal regions with prognostic importance facilitates the interpretation of DNA microarray experiments. Such experiments normally result in hundreds of candidate genes. The knowledge of chromosomal regions with relation to outcome can narrow the number of candidate genes for further analysis (33, 34). Such target genes would allow a better risk assessment for the individual woman.

The finding of significant genomic differences in specific tumor grades is in agreement with recent CGH data showing fewer aberrations in grade I and II tumors compared with grade

III tumors (9, 12). This is consistent with the model that tumor progression is related to an accumulation of DNA aberrations. The different numbers of 9q aberrations in grade I/II and grade III tumors give a new insight into progression-related events because 9q losses have been rarely described in breast cancer.

In conclusion, we describe a strong association between the number of genomic aberrations detected by CGH and the recurrence risk in node-negative invasive ductal breast carcinomas. In addition, we identified individual chromosome loci associated with unfavorable outcome in breast cancer. Multivariate analysis revealed that histological grade and DNA sequence losses of 18q were independent prognostic parameters in this set of early-stage node-negative ductal breast cancers. The results of the

Table 4 Cox proportional hazard analysis with the parameters histological grade, tumor diameter, loss of 11p, and loss of 18q

Variable	Risk ratio	P
Histological grade	1.9	<0.01
Tumor diameter	1.0	0.06
18q loss	3.0	<0.05
11q loss	0.4	0.1

present study indicate that chromosomes 11p and 18q are chromosomal loci that may harbor important tumor suppressor genes for ductal breast cancer progression.

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