

# Comparative Genomic Hybridization of Breast Tumors Stratified by Histological Grade Reveals New Insights into the Biological Progression of Breast Cancer

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## Abstract

How does breast cancer progress? There is evidence both to support (S. W. Duffy *et al.*, *Br. J. Cancer*, 64: 1133-1138, 1991; R. Rajakariar *et al.*, *Br. J. Cancer*, 71: 150-154, 1995) and refute (M. Hakama *et al.*, *Lancet*, 345: 221-224, 1995; R. R. Millis *et al.*, *Eur. J. Cancer*, 34: 548-553, 1998) the hypothesis of dedifferentiation; the theory that as breast cancers grow they evolve from well differentiated (grade I) to poorly differentiated (grade III) tumors. We provide evidence to support the view that the majority of grade I tumors do not progress to grade III tumors. Comparative genomic hybridization was used to screen entire genomes of a large sample (40 grade I and 50 grade III) of invasive ductal breast carcinomas, stratified by grade. We found distinct genetic differences between grade I and grade III tumors. Significantly, we found that 65% of grade I tumors lost the long arm of chromosome 16 compared with only 16% of grade III tumors. This pattern of loss leads us to conclude that the majority of grade I tumors do not progress to grade III tumors. These findings have important implications because they suggest that different breast tumor grades may have distinct molecular origins, pathogenesis, and behavior and, therefore, potentially present distinct molecular targets for research and treatment.

## Introduction

Although considerable progress has been made in elucidating the genetic events that underlie the progression of many malignancies (1, 2) those involved in breast cancer progression are poorly understood. Furthermore, the relationship between histological progression and genetic events in breast cancer is not well defined. Studies have begun to examine the genotype-phenotype relationship in carcinoma *in situ* and disease progression (3, 4), and in metastatic progression (5). However, this has only been possible because there are distinct phenotypes with known biology to study. In our understanding of invasive breast cancer, there is a paucity of data relating genotype to phenotype that is compounded by the uncertainty regarding phenotypic progression.

Up to 70% of breast carcinomas are classified as ductal carcinoma of no special type. In a system originally devised by Scarff, Bloom, and Richardson, histological assessment of nuclear pleomorphism, mitotic activity, and tubule formation allows further subdivision by grade (6). Using such a classification, grade I tumors have well differentiated attributes and grade III tumors have poorly differentiated attributes, whereas grade II tumors fall into an intermediate category. Although these phenotypic appearances provide a guide to tumor behavior (6), they do not necessarily relate directly to biological

progression. Indeed, there is considerable controversy regarding biological progression. One hypothesis suggests that as tumors grow they become dedifferentiated in terms of grade (*i.e.*, grade I tumors become grade III tumors over time; Ref. 7). We set out to test the pathological hypothesis of dedifferentiation by taking a novel genetic approach. CGH<sup>2</sup> was used to screen the entire genomes of 90 invasive ductal breast carcinomas, which were stratified by grade (40 grade I and 50 grade III tumors). If dedifferentiation occurs, we expected to find genetic changes in grade I tumors that were a subset of those found in grade III tumors.

## Materials and Methods

**Patients.** Tumors and blood samples were collected from breast cancer patients treated by the Breast Group at Guy's Hospital (London, United Kingdom) between 1988 and 1996. All information regarding the tumors was recorded by the group's pathology laboratory. Tumors were graded using the Nottingham modified criteria of Scarff, Bloom, and Richardson (6). Samples were reviewed by the pathologists (A. H. and R. L.) to confirm tumor grade and to determine the proportion of admixed benign cells. All grade III tumors showed at least 75% tumor cells, although some grade I tumors had more contaminating benign tissue (up to 50%). DNA was extracted from frozen tumor specimens and blood samples using a modification of a standard phenol chloroform extraction method.

**CGH.** CGH was performed with minor modifications of a standard protocol (8). Briefly, tumor and reference DNA were labeled by nick translation. Approximately 1  $\mu$ g each of fluorescein-labeled tumor and Texas red-labeled reference DNA were coprecipitated in the presence of 50  $\mu$ g of human Cot I DNA and resuspended in hybridization mix [50% formamide, 10% dextran sulfate, and 2  $\times$  SSC (pH 7.0)] before hybridization to denatured human metaphase chromosome spreads, prepared from phytohaemagglutinin-stimulated lymphocytes from normal individuals. After hybridization for 72 h, the slides were washed and counterstained with 4,6-diamidino-2-phenylindole, which gives a banding pattern resembling G-bands. Images were captured with a cooled charge-coupled device camera attached to a Zeiss axioskop microscope and then analyzed using Quips software (Vysis, Inc., Downers Grove, IL). Between 5-10 metaphases were analyzed for each tumor. A chromosome region was considered to be lost if the mean hybridization ratio between tumor and normal was  $<0.85:1$  or gained if the ratio was  $>1.15:1$ . These cutoff values were determined based on negative control hybridizations (with normal male *versus* normal female DNA), which showed the mean hybridization ratio, and its SD remained within these limits for all of the autosomes. For the X chromosome, the mean hybridization ratio was below 0.6. Negative control hybridizations were included in each batch of experiments.

## Results and Discussion

We characterized the genetic changes in a sample of 90 invasive ductal breast carcinomas stratified by grade (40 grade I and 50 grade III carcinomas). Fig. 1 shows the gains and losses seen in all of the tumors. The different grades of breast tumor exhibited distinct genetic differences. Three grade I tumors showed no genetic changes at all,

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<sup>2</sup> The abbreviation used is: CGH, comparative genomic hybridization.

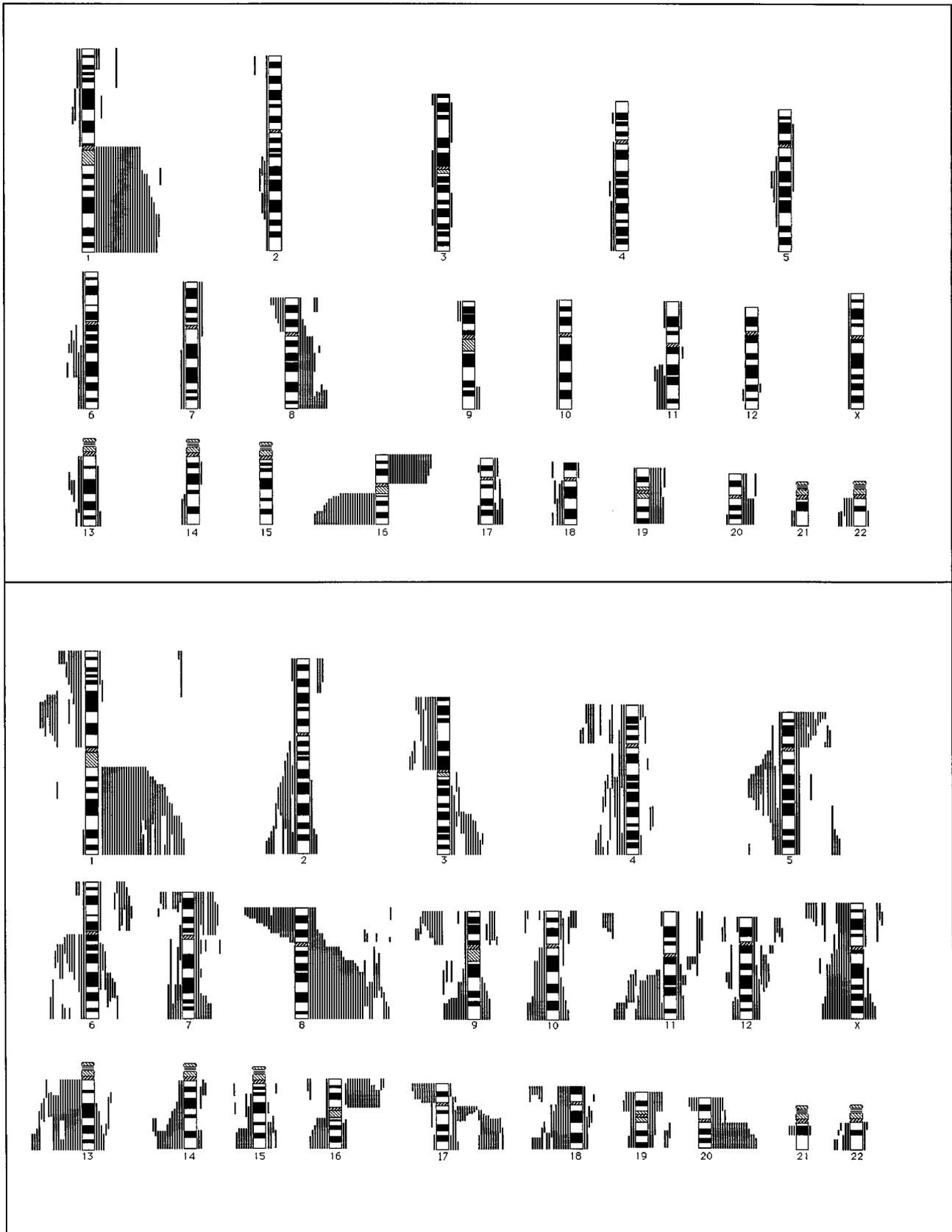


Fig. 1. Chromosomal aberrations detected by CGH in breast carcinomas. *Top*, gains and losses of genetic material in 40 grade I breast carcinomas. Gains are represented by *bars* to the right of the chromosomal ideograms, and losses are represented by *bars* to the left; standard thresholds for gains and losses are used (see "Materials and Methods"). Each *bar* represents data from a single tumor; three tumors had no changes and are not represented. *Bottom*, data from 50 grade III breast carcinomas, represented in the same way. All tumors showed at least one change.

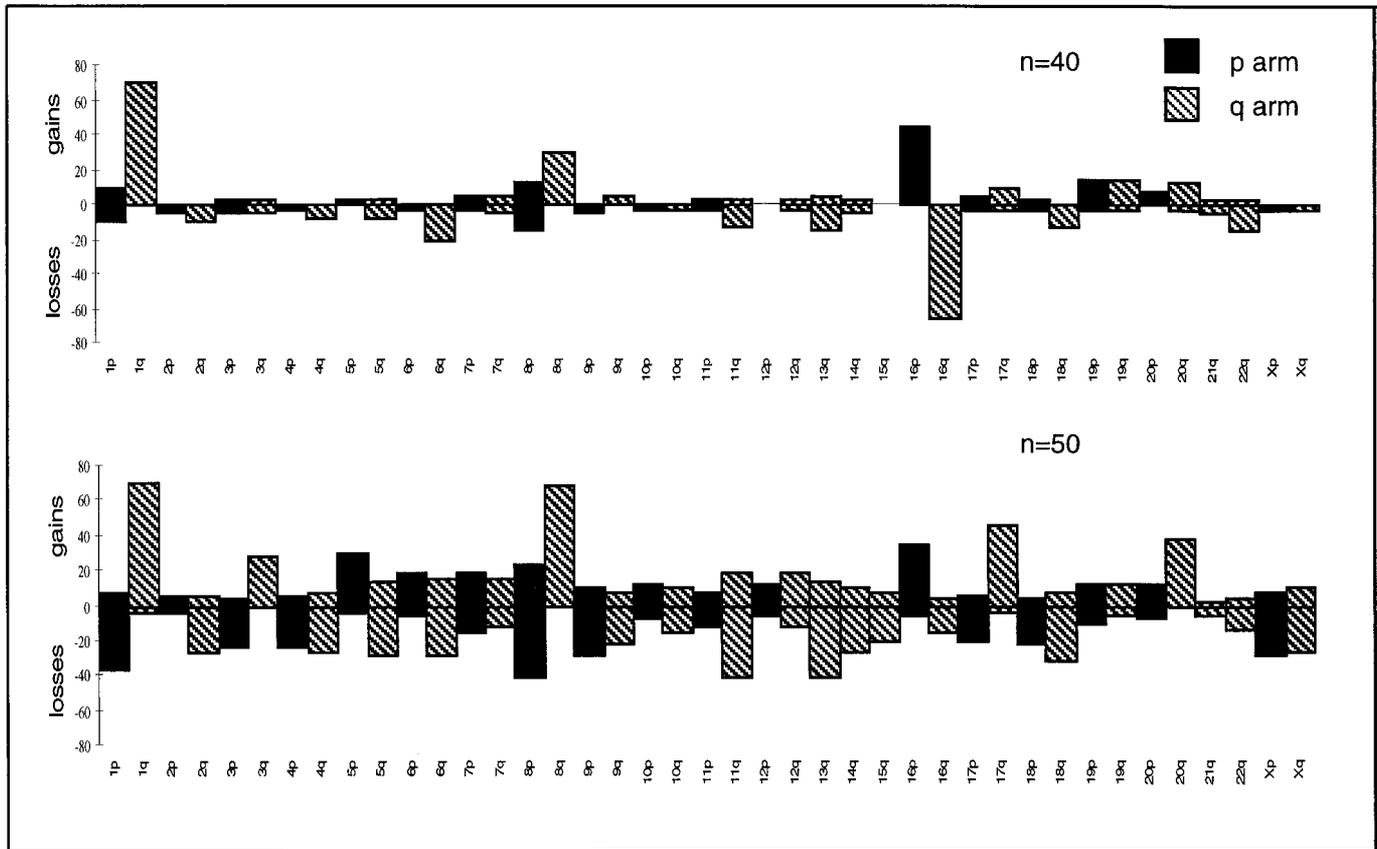


Fig. 2. Frequency of chromosomal aberrations in breast carcinomas. *Top*, chromosomal aberrations in grade I breast carcinomas. Each bar on the X axis represents either the short arm (■) or long arm (▨) of each chromosome. The size of each bar represents the percentage of tumors showing that particular aberration. Gains are represented as positive changes, and losses are represented as negative changes. *Bottom*, chromosomal aberrations in grade III breast carcinomas depicted in the same way. The difference between the *top* and *bottom* graphs with regard to loss of chromosome 16q is clearly apparent.

whereas all grade III tumors showed at least one change. Several genetic regions were unaffected by either gain or loss in the grade I tumors; however, no region of the genome was unaffected in the grade III tumors. The mean number of changes in grade I tumors was 5.4 (range, 0–14), compared with 13.8 (range, 1–33) in grade III tumors. The frequency of gains and losses seen in grade I and grade III breast tumors is shown in Fig. 2. In the grade I tumors, only four regions of change were commonly seen [gains of 1q (70%), 16p (45%), and 8q (30%) and loss of 16q (65%)], whereas many regions were commonly affected in the grade III tumors. The most common regions of gain were 1q (70%), 8q (68%), 17q (46%), 20q (38%), and 16p (34%); common regions of loss were 8p (42%), 11q (42%), 13q (42%), 1p (36%), and 18q (32%). The greatest difference seen between grades was loss of 16q. Sixty-five percent of grade I tumors showed loss of the long arm of chromosome 16, whereas only 16% of grade III tumors showed this change (Fig. 2).

The finding of distinct genetic differences in specific tumor grades is in agreement with data from other groups showing fewer chromosomal aberrations in well differentiated compared with poorly differentiated breast tumors (9). This is consistent with the idea that tumor phenotype and the number of genetic changes are related. However, the striking finding of 16q loss occurring very commonly in grade I tumors, but infrequently in grade III tumors, is very interesting. Surprisingly, genetic material lost in well differentiated tumors was, nevertheless, present in poorly differentiated tumors. These results strongly imply that the majority of grade I tumors do not progress to grade III tumors because regain of genetic material previously lost during tumor progression is extremely unlikely. These data are, there-

fore, inconsistent with the dedifferentiation hypothesis for the origins of most grade III tumors. Although it is possible to envisage a mechanism whereby genetic material could be regained during tumor development (for example, nondisjunction of chromosome 16), we believe this to be much less likely. Because 16q loss occurs as a consistent chromosomal change in grade I tumors, it is likely that cells with loss of this region carry a selective growth advantage. It is difficult to envisage how regaining this region has a further selective advantage. Endoreduplication as a means of regaining chromosome 16 is even less likely because it would result in extra copies of all chromosomes, even allowing for chromosomal losses after endoreduplication this does not account for the pattern of gains and losses seen in the grade III tumors. If most grade I tumors do not progress to grade III tumors this suggests that groups of breast tumors defined by different grades arise by distinct genetic processes and should be regarded as having distinct biological behaviors. This is a view supported by recent morphological studies in which primary tumors and subsequent recurrences and metastases remain, in most cases, within grade (10). The implication of different grades having broadly different biologies is that they may present distinct molecular targets not only for future research but also for treatment.

Loss of 16q clearly plays an important role in tumorigenesis, although the specific function played by deletion of this region is uncertain. Loss of heterozygosity studies of primary breast tumors have identified three minimal regions of deletion (16q22.1, 16q23.2–24.1, and 16q24.3), suggesting the presence of possible tumor suppressor genes (11). A candidate gene for the region 16q24.3 is the cadherin gene, *CDH15* (12), although its expression in breast tissue is

not yet confirmed. Within the 16q21–22.1 region there are an additional five cadherin genes (12). Because disturbance of cell-cell interactions, in which the cadherins are involved, is necessary for invasion and metastasis of tumor cells, all of the cadherins are potential tumor suppressor genes. Of particular interest is the cadherin gene *CDH1*, which maps to 16q22.1. Although it lies outside the minimal area of deletion (13), it is known to play an important role in lobular breast carcinomas (14), whereas its role in ductal carcinomas remains to be established. Another candidate tumor suppressor gene for the region 16q22.1 is *CTCF* (13), which has recently been identified and shown to encode a transcription factor with binding sites in a number of genes including *MYC* and the POLO-like kinase genes. It is envisaged that abnormalities of *CTCF* could lead to dysregulated expression of these target genes, resulting in a positive effect on cell proliferation.

Previous studies have led to the belief that loss of 16q is an early change in breast tumorigenesis. Some evidence for this is from CGH analyses, which have shown 16q loss occurs either as a sole change or in association with only a few chromosomal changes (9). Our findings in grade I tumors confirm these earlier results by showing that loss of 16q occurs predominantly in association with gain of 1q, and in association with only a few other changes (mean number of changes, 3.9), although it was never a sole abnormality in our study. The finding of 16q loss in association with 1q gain is interesting because this is consistent with cytogenetic data that have found der(1;16)(q10;p10) unbalanced translocations in breast cancer (15). However, the grade III tumors that showed 16q loss had a completely different pattern with many other chromosomal abnormalities present (mean number of changes, 15.5), and none with just gain of 1q, thus, suggesting that 16q loss may be an early change only in grade I tumors and that other genetic changes are important for most grade III tumors.

Gains of 8q, 17q, and 20q are changes that have been reported previously as frequent findings in breast cancer (16). However, we found these to be frequent changes only in grade III tumors (see Fig. 2). In grade I tumors, gains of 17q and 20q were seen in only four tumors each. Interestingly, gains of 8q and 20q occurred predominantly in grade III tumors, which are known to have a poorer prognosis (6), supporting previous findings correlating these changes with a shorter overall survival and more aggressive phenotype (17). The findings in grade I tumors of predominant loss of 16q without high frequency gain of 8q and 20q are very similar to changes found in lobular breast carcinomas (18). This leads us to speculate that at the molecular level the origins of these apparently morphologically different breast tumors may be very similar.

Our findings have important implications for the biological study of breast tumors. Most genetic studies to date have analyzed breast tumors as a homogeneous group, without specifically considering grade. As the present study shows, a prerequisite for a meaningful description of grade-specific changes is the selection of a large number of tumors for study. Grade I tumors comprise only 12% of all invasive ductal breast carcinomas (6). Changes that are very prevalent within a subgroup may, therefore, be missed, or their significance diluted, when the whole group is considered. This may explain why previous CGH studies have found loss of 16q ranging from 24–38% in breast tumors (5, 9, 17) and why the importance of gains of 17q and 20q have been overestimated.

In conclusion, this study shows the use of taking a genetic approach to make inferences about the biological progression of breast cancer. By studying a large sample stratified by histological grade, our findings show that the majority of grade I tumors do not progress to grade

III tumors. Breast cancer seems to show a more complex biological behavior than simple progression through histological grades. Therefore, different grades may present discrete therapeutic targets. We suggest that a complete understanding of the genetic events that underlie breast tumorigenesis requires a full appreciation of the relationship between genetic events and histological grade.

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