

Genetic instability promotes the acquisition of chromosomal imbalances in T1b and T1c breast adenocarcinomas

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In order to evaluate biological and genetic properties of early breast carcinomas we analyzed microdissected tissue from 33 primary breast carcinomas stage T1b and T1c with respect to the nuclear DNA content, the expression pattern of Ki-67, cyclin A, p27^{KIP1}, p53 and p21^{WAF1}, and chromosomal gains and losses. The results show that T1b carcinomas (6–10 mm, $n = 17$) were frequently near-diploid (53%) with low proliferative activity and staining patterns of p53 and p21^{WAF1} that suggest the presence of wild type protein. The majority (12/16) of the T1c tumors (11–20 mm), however, was aneuploid, and proliferative activity and p53 expression were increased. Larger tumor size correlated with an increasing number of chromosomal copy number changes and in particular with regional amplifications. High level copy number increases (amplifications), however, were found exclusively in the aneuploid tumors. Amplification events correlated with elevated cyclin A and reduced p27 expression, respectively. Our results suggest that the sequential acquisition of genomic imbalances during tumor progression is accelerated in aneuploid tumors, and may contribute to the increased malignancy potential.

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1. Introduction

Prognostication of breast cancer is based on morphological and anatomical parameters. For instance, the degree of tissue differentiation as the determining factor for tumor grading serves as an indicator of favorable prognosis in a subgroup of invasive breast carcinomas [12,13]. Furthermore, the earlier breast cancer is detected, the better the prognosis [21,22,24,28]. Stage 1 carcinomas, in particular those with tumor size less than 10 mm are associated with low recurrence risk and low mortality [18,27]. Early clinical stage and curative surgical treatment, however, might not be sufficient to explain the generally good prognosis of these small tumors. It is tempting to speculate that yet poorly defined biological factors contribute to the low malignancy potential and thereby also to the excellent survival rates in this group of breast cancers. Consequently, one can argue that small and generally non-palpable tumors represent slowly proliferating carcinomas, which may not significantly reduce life expectancy even if they remain untreated. However, it is well known that even early cancers that are smaller than 10 mm can metastasize and kill the patient within a short period of time [1,12,19]. This strikingly different clinical course is a reflection of the biological heterogeneity of breast cancer and suggests that subgroups of tumors exist that have a markedly altered propensity to acquire genetic changes responsible for increased tumor aggressiveness.

We hypothesized that small tumors already display distinct biological features that may influence tumor progression. In order to address this question, we performed a multiparameter analysis of T1b (6–10 mm) and T1c (11–20 mm) breast adenocarcinomas. Proliferative activity and the expression patterns of relevant tumor suppressor genes involved in cell cycle regulation were analyzed immunohistochemically. Genomic instability was assessed by DNA-ploidy measurements, and by mapping chromosomal

gains and losses using comparative genomic hybridization (CGH).

2. Materials and methods

2.1. Tumor samples and preparation

33 primary adenocarcinomas of the breast were selected according to macroscopic and microscopic tumor size and only T1b (6–10 mm, $n = 17$) and T1c (11–20 mm, $n = 16$) tumors were included. All patients underwent radical or partial mastectomy and none of the patients received preoperative chemo- or radiotherapy. All tumors were classified according to the AJCC [4] and the Elston and Ellis grading system [3]. A summary of clinico-pathological characteristics of all tumors is presented in Table 1.

The tumor samples were fixed in 4% phosphate buffered formaldehyde and paraffin embedded. From each specimen ten contiguous sections were prepared and used for immunohistochemistry (thickness: 4 μm), and DNA ploidy measurements (8 μm). Genomic DNA for CGH analysis was prepared from two microdissected tissue sections (50 μm) to obtain samples containing more than 80% tumor cells. The tissue was incubated overnight in 1 M NaSCN at 37°C and washed twice in PBS. DNA was isolated by standard proteinase-K digestion, followed by phenol-chloroform extraction and ethanol precipitation. The DNA amount was quantified using a microphotometer.

2.2. Immunohistochemistry

All slides were deparaffinized with xylene, rehydrated and microwaved at 500 W for 2×5 min in 10 mM citrate buffer, pH 6.0. Intrinsic peroxidase activity was blocked with 3% hydrogen peroxide in methanol, followed by incubation with horse serum (1 : 20 dilution) in 0.1 M PBS, pH 6.0. The levels of protein expression were revealed by overnight incubation with the respective antibodies (see below) diluted in 1% (weight/volume) bovine serum albumin and visualized by standard avidin biotin-peroxidase complex technique (Vector Laboratories, Burlingame, CA). The following antibodies were used. The respective dilutions and suppliers are indicated in parentheses: NCL-p27 (1 : 100, Novocastra Laboratories LTD, Newcastle upon Tyne, UK), MIB-1 (1 : 150, Immunotech S.A., Marseille, France), DO1 (1 : 100, Santa Cruz Biotechnology, Inc., USA), WAF1 (1 : 15, Calbiochem), NCL-

cyclin A (1 : 100, Novocastra) and NCL-cyclin E (1 : 40, Novocastra).

All slides were coded and scored according to Porter et al. [14]. Staining patterns that revealed increased levels of p53 in the presence of no detectable p21^{WAF1} staining were interpreted as consistent with wild type protein.

2.3. DNA ploidy

Nuclear DNA was measured by image cytometry on Feulgen stained imprints or histological slides as previously described [23]. DNA histograms were interpreted according to a modified subjective method. The normal control cells were given the value 2c denoting the normal diploid DNA content and all DNA values of tumor cells were expressed in relation to that. The histograms were divided into two groups: cases with a major peak near the 2c region (1.5–2.5c) and <10% cells exceeding 2.5c were classified as near-diploid. DNA profiles with a stemline outside the diploid region and distinctly scattered DNA values exceeding the tetraploid region (>4.5c) were classified as aneuploid.

2.4. Comparative genomic hybridization (CGH)

Normal control DNA was prepared from peripheral blood lymphocytes of karyotypically normal individuals (46,XX) and labeled in a standard nick-translation reaction substituting dTTP with digoxigenin-12-dUTP (Boehringer Mannheim). Tumor DNA was labeled with biotin-16-dUTP (Boehringer Mannheim). For CGH, 500 ng tumor DNA and 500 ng control DNA were ethanol precipitated in the presence of 10 μg salmon sperm DNA and 40 μg of the Cot-1 fraction of human DNA (Gibco BRL, Gaithersburg, MD). The probe was dried and resuspended in 10 μl hybridization solution (50% formamide, $2 \times$ SSC, 10% dextran sulphate), denatured for 5 min and preannealed for 60 min at 37°C. For reliable karyotyping, human centromeric probes for chromosome 4, 8, 14, 20, 22 and X were directly labeled by PCR substituting dTTP with Cy-5 labeled dUTP (Boehringer Mannheim) and cohybridized with the genomic probes.

The probe cocktail was hybridized to human female metaphase chromosomes (46,XX). The metaphase chromosomes were denatured separately (70% formamide, $2 \times$ SSC for 2 min at 75°C), and dehydrated through an ethanol series (70%, 90%, 99%). Hybridization took place under a coverslip for 3 days at 37°C. Posthybridization and detection was

Table 1
Clinicopathological characteristics of T1b (A) and of T1c (B) breast carcinomas

Patient number	Age	Tumor size (mm)	Histol. type	Histol. grade	Lymph node met.	Ploidy
(A)						
1	80	6	Ductal	II	0 of 7	Diploid
2	51	9	Ductal	I	0 of 0	Diploid
3	74	7	Ductal	III	0 of 8	Diploid
4	58	10	Ductal	I	0 of 6	Diploid
5	62	5	Ductal	I	0 of 9	Diploid
6	65	10	Ductal	I	0 of 5	Diploid
7	77	8	Ductal	I	0 of 11	Diploid
8	58	10	Ductal	I	0 of 0	Aneuploid
9	49	10	Ductal	II	2 of 5	Diploid
10	50	10	Ductal	I	0 of 12	Aneuploid
11	36	9	Ductal	III	1 of 9	Aneuploid
12	60	9	Ductal	I	0 of 9	Aneuploid
13	57	7	Ductal	II	0 of 0	Aneuploid
14	36	7	Ductal	III	1 of 8	Aneuploid
15	43	10	Ductal	II	0 of 0	Diploid
16	64	9	Adenoc	II	0 of 11	Aneuploid
17	54	10	Comedo	III	0 of 7	Aneuploid
(B)						
18	53	15	Lobular	III	0 of 1	Diploid
19	41	12	Ductal	III	0 of 6	Aneuploid
20	79	14	Ductal	III	0 of 9	Aneuploid
21	49	13	Ductal	II	0 of 6	Diploid
22	43	11	Ductal	II	0 of 0	Aneuploid
23	51	20	Ductal	III	0 of 6	Diploid
24	64	18	Ductal	II	0 of 5	Aneuploid
25	55	20	Colloid	III	0 of 0	Aneuploid
26	50	11	Ductal	II	0 of 0	Diploid
27	68	15	Ductal	II	0 of 0	Aneuploid
29	48	20	Ductal	III	7 of 7	Aneuploid
29	62	20	Ductal	III	0 of 0	Aneuploid
30	47	20	Ductal	III	0 of 8	Aneuploid
31	69	15	Ductal	III	0 of 1	Aneuploid
32	77	15	Ductal	II	0 of 7	Aneuploid
33	62	12	Colloid	I	0 of 0	Aneuploid

performed as described elsewhere [15]. The chromosomes were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) and mounted in an antifade solution.

2.5. Digital image analysis

Four gray scale images were acquired with filter sets specific for DAPI, FITC, TRITC and Cy-5 (Chroma Technologies, Brattleboro, VT) using a Leica DM-

RBE epifluorescence microscope connected to a CCD camera (Sensys, Photometrics, Tucson, AZ) and Q-FISH software (Leica Microsystems, Cambridge, UK). CGH analysis was performed using Cytovision software (Applied Imaging International, Tyne and Wear, UK) using 10 metaphases per case. Details of the analysis software were described elsewhere [2].

The average number of copy alterations (ANCA) was calculated by dividing the sum of all chromosomal gains and losses by the number of cases in each

group. The average number of regional amplifications (ANRA) was calculated accordingly.

3. Results

3.1. DNA ploidy

53% of the T1b tumors ($n = 17$) were near-diploid, and 47% highly aneuploid (below referred to as diploid and aneuploid, respectively). This ratio changed in the group of larger tumors (T1c, $n = 16$), where 25% were diploid and 75% aneuploid (Table 1).

3.2. Immunohistochemistry

Both diploid and aneuploid T1b tumors were characterized by a low proliferative activity with low expression of Ki-67 (<20% positive cells) and cyclin A (<5% positive cells) and a high number of tumors (71%) expressing p27 (>50% positive cells). Positive p53 staining was infrequent and p53 positivity together with undetectable p21 in the same tumors was found in only one of the aneuploid cases. All tumors revealed a low cyclin E staining pattern (<1% positive cells/case). Among the diploid T1c tumors the staining patterns for all immunohistochemical markers were essentially the same as for the T1b tumors. However, among the aneuploid T1c tumors increased expression of both MIB1 (50%) and cyclin A (50%) was found in addition to decreased p27 expression in 67% of the cases. In 42% of the aneuploid T1c-tumors increased expression of p53 was detected and in 25% of the cases this high expression occurred simultaneously with undetectable expression levels of p21. Only one aneuploid T1c tumor revealed increased expression of cyclin E (>10% positive cells/case).

3.3. Comparative genomic hybridization (CGH)

CGH allows one to screen tumor genomes for genomic imbalances. CGH revealed a recurrent, yet different pattern of chromosomal gains and losses in the two groups of primary breast carcinomas. The most frequent chromosomal imbalances in T1b tumors were gains of chromosome arms 6q (71%), 4q (65%), Xq (59%), 8q (53%), 13q (53%), 5p (47%) and 1q (47%). High-level copy number increases of sub-chromosomal regions (amplifications) were mapped to 4q21–27, 6q12–15, 9p23, 13q21 and 13q21–31. Gain of 20q or 11q13–14, which was reported on in earlier

studies [9,17] was not found in these tumors. The most frequent losses were mapped to chromosome arms 16q (35%), 17p (24%), 19p (24%) and 22q (18%).

In T1c tumors recurrent gains were mapped to chromosome arms 1q (69%), 4q (56%), 8q (56%), 13q (56%), 3q (50%) and 5p (50%). Gain of 20q was found in only one case. The most frequent losses were found to be chromosome arms 17p (50%), 16q (31%), 11q (25%) and 22q (25%). Furthermore, loss of the whole chromosome X was found in 25% of these tumors. Recurrent regional amplifications were mapped to 3q24–25, 8q22–23, 12q21, 17q11.2–12 and Xq21.

In both T1b and T1c tumors a relatively high number of chromosomal aberrations was observed (Figs. 1 and 2).

Among the T1b tumors the average number of copy alterations (ANCA) was 8.8 and the average number of regional amplifications (ANRA) was 0.4. In the fraction of diploid tumors from this group ($n = 9$) an ANCA value of 5.8 and an ANRA value of zero was observed (i.e., no amplified regions were found in this group). The ANCA value of the aneuploid tumors from the same group was 12.3 and the ANRA value 0.8. Differences in tumor grade and the expression levels of MIB-1, cyclin A, p53, p21 and p27 did not reveal any significant correlation to the ANCA and ANRA values.

Among the T1c tumors the ANCA value was 10.8 and the ANRA value 1.5. In the fraction of near-diploid tumors from this group ($n = 4$) the ANCA value was 4.3 and, as for the near-diploid T1b tumors, an ANRA value of zero was found. In contrast, the aneuploid tumors ($n = 9$) revealed an ANCA value of 12.9 and an ANRA value of 2.0, which translates to a greatly increased number of gene amplifications. The differences in ANCA and ANRA values for the different groups are illustrated in Fig. 3.

4. Discussion

Breast tumors are being detected at a significantly earlier stage than even few years ago. This is to a great extent attributable to successful screening programs for breast cancer. At present, as many as 70% of all mammary carcinomas detected in Stockholm and suburbs by mammographic screening are smaller than 10 mm. This trend can be expected to reduce disease specific mortality [5,18]. However, the early detection also imposes a diagnostic challenge because phenotypic characteristics of small lesions are usually less pronounced. Consequently, the importance of bi-

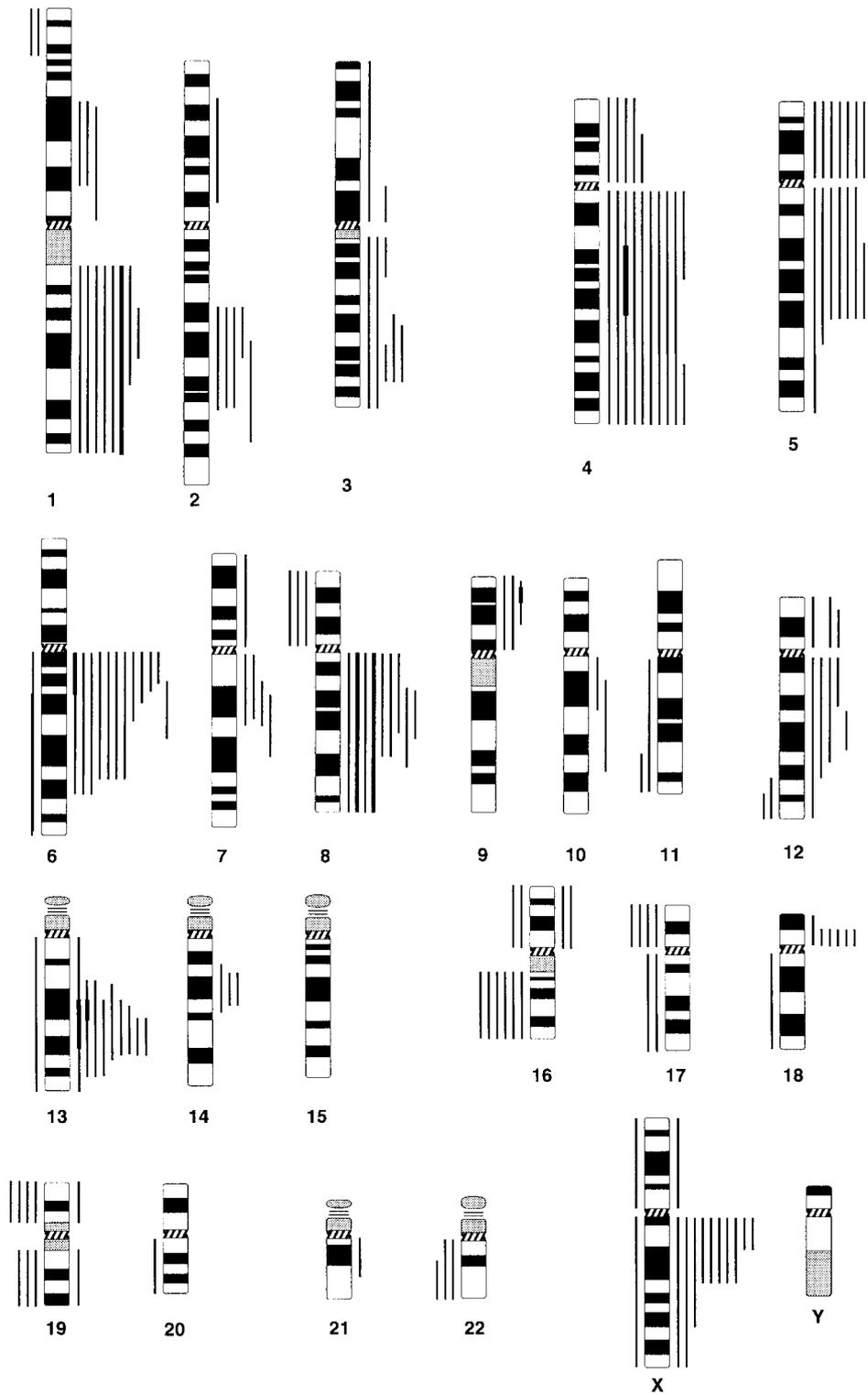


Fig. 1. Karyogram of chromosomal gains and losses in primary breast carcinomas ($n = 17$). All tumors were ≤ 10 mm in size (T1b) and bars on the right side of the chromosome ideogram indicate gains and bars on the left side indicate loss of genetic material. Solid bars denote high-level copy number increases (amplifications).

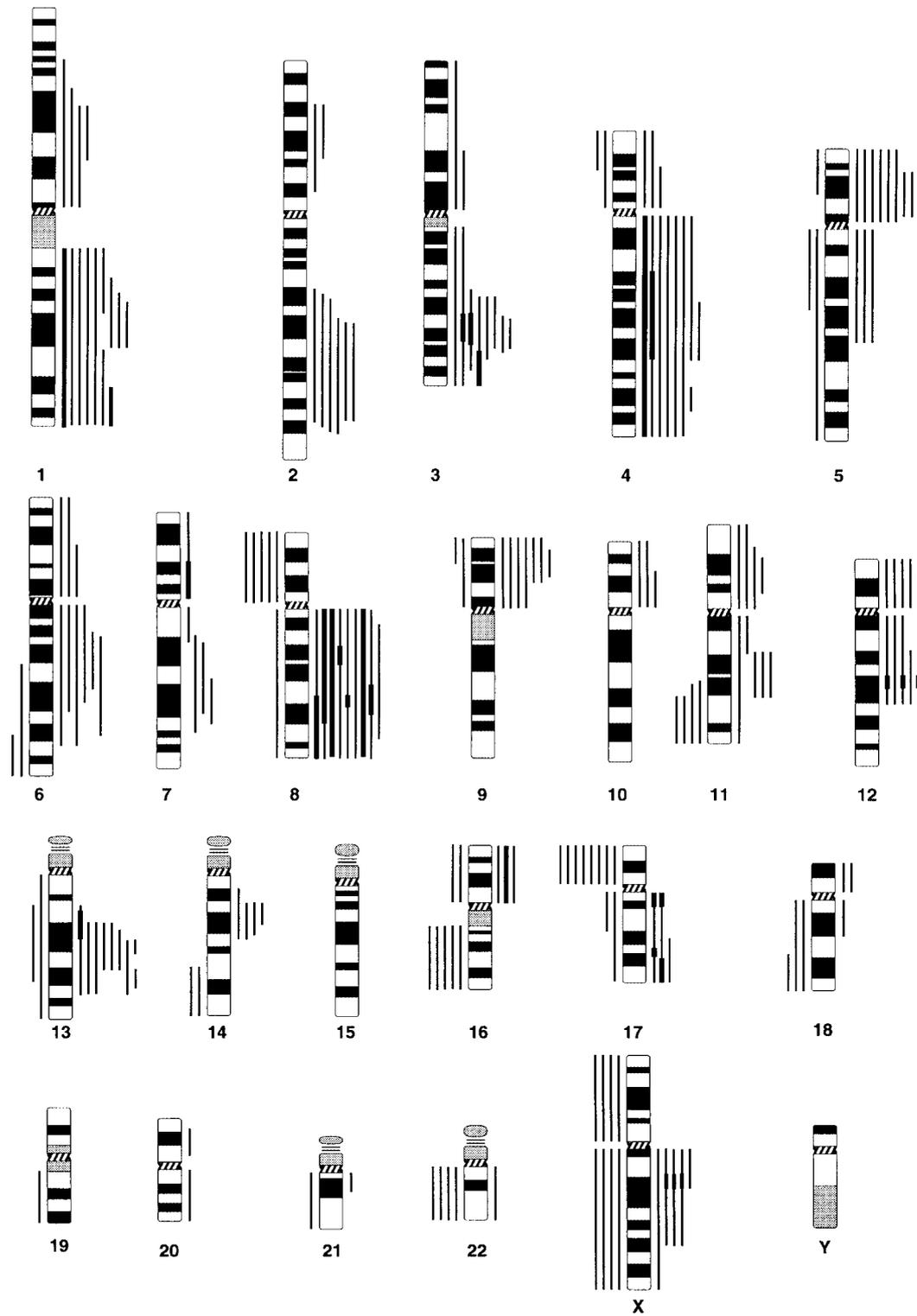


Fig. 2. Karyogram of chromosomal gains and losses in primary breast carcinomas ($n = 16$). All tumors were between 11–20 mm in size (T1c) and bars on the right side of the chromosome ideogram indicate gains and bars on the left side indicate loss of genetic material. Solid bars denote high-level copy number increases (amplifications).

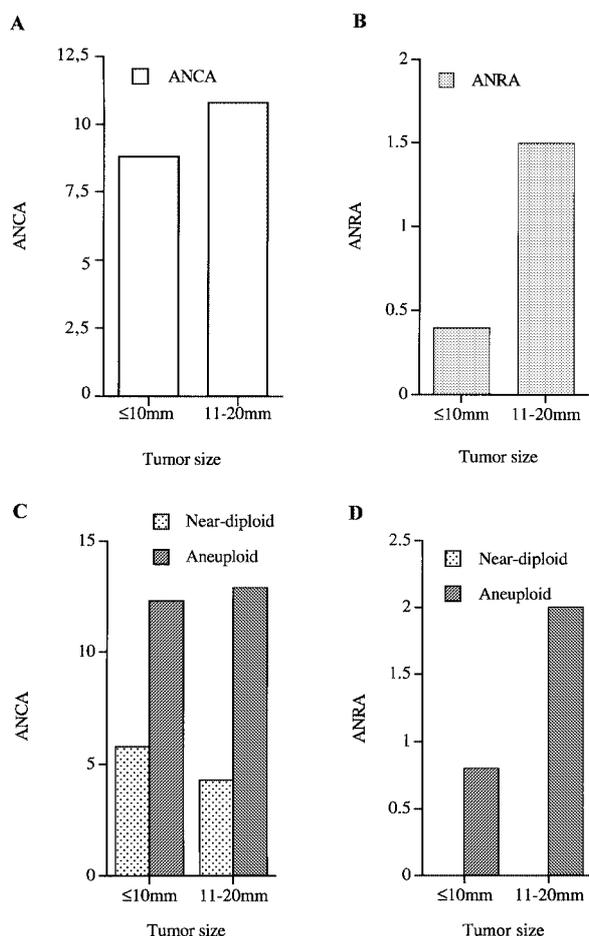


Fig. 3. (A) Average number of copy alterations (ANCA) and (B) average number of regional amplifications (ANRA) in T1b (≤ 10 mm) and T1c (11–20 mm) breast adenocarcinomas, and in relation to tumor size and DNA-ploidy (C, D).

ological and genetic markers for diagnosis and for the prediction of disease progression increases. We have therefore conducted a comprehensive molecular cytogenetic and immunohistochemical study of 33 selected cases of small primary breast carcinomas. The cytogenetic data from T1b (6–10 mm) and T1c (11–20 mm) tumors were compared with the DNA ploidy as determined by image cytometry and with the expression levels of the cell cycle associated proteins Ki-67, cyclin A, cyclin E, p27^{KIP1}, p53 and p21^{WAF1}. This is, to our knowledge, the first such evaluation of small breast carcinomas.

The results reveal that T1b tumors in general have low proliferative activity (indicated by low Ki-67 and low levels of cyclin A and cyclin E), display low or undetectable p53 and p21 levels and express high levels of the cyclin dependent kinase inhibitor p27^{KIP1}.

Diploid and aneuploid tumors were found in this group of selected primary T1b tumors to approximately the same extent (53% diploid and 47% aneuploid). No major difference was found between the diploid and aneuploid T1b tumors either with respect to proliferative activity (cyclin A and Ki-67) or expression levels of p27 and p53. Additionally, in both diploid and aneuploid T1b tumors high-level chromosomal copy number alterations (amplifications) were infrequent. Such amplifications usually occur at later stages of tumorigenesis [16] when cell cycle control is significantly impaired. This result could therefore indicate that the destabilization of the genome has not yet progressed extensively in these small tumor variants.

However, with increasing tumor size (T1c) many of the aneuploid tumors revealed alterations in cell cycle control (as exemplified by decreased levels of p27) and genetic instability (i.e., increased frequency of high-level copy number alterations). This is in clear contrast to the diploid tumors that do not seem to undergo any major genomic destabilization when increasing in size from T1b to T1c tumors.

The immunohistochemical profile of cell cycle proteins that we observed in T1c breast carcinomas has been reported in previous studies to be related to poor outcome [8,11,14,25]. In the present study the clinical follow up times were insufficient to verify this observation.

The T1b tumors generally revealed a relatively high number of chromosomal aberrations (ANCA value of 8.8) and only a slightly increased ANCA value (10.8) was identified in the group of T1c tumors. This shows that small and often non-palpable invasive breast carcinomas detected by screening mammography may have acquired a high grade of genomic instability at time of diagnosis. In a study by Kuukasjarvi et al. and James and colleagues [7,10] numerous numerical chromosomal aberrations were observed already in carcinoma *in situ* lesions of the breast. Along with our data, this indicates that genomic imbalances are present already at preinvasive stages, but that further genomic instability may be a requirement for the expeditious acquisition of additional genetic abnormalities, which correlates to unfavorable prognosis.

The ANCA values found in this study are similar to a previous study of primary breast carcinomas [26] but higher than reported from our laboratory [17]. However, no enrichment for carcinoma cells by virtue of microdissection was performed in the previously published data. The inadvertent contamination with non-tumor tissue could certainly account for the observed discrepancy.

Of note, among the groups of near-diploid T1b and T1c tumors no subchromosomal amplifications were mapped by CGH. This clearly discerns the near-diploid from the aneuploid carcinomas. In the group of aneuploid T1b tumors the ANRA value was found to be 0.8 and this number increased to 2.0 for the aneuploid T1c tumors (Fig. 3D). The ANCA values remained at comparable levels (12.3 and 12.9, respectively, Fig. 3C). This may indicate that a subset of small tumors has an intrinsically low progressive potential. The fact that amplification events were almost exclusively found in the aneuploid, proliferating tumors could potentially be used to define a subgroup that requires more aggressive treatment.

Amplification of specific genes (e.g., c-myc and c-erbB2) has been linked to aggressive tumor behavior. Some of the amplicons identified in this study mapped to the chromosomal location of c-myc (8q24) and c-erbB2 (17q11–12). However, we have also shown recurrent amplifications on chromosomal bands 3q24–25, 8q22–23, 12q21 and Xq21 (Fig. 2) to which no known oncogenes have been mapped to as of yet. As summarized in Figs 1 and 2 there seems to be an increase in gains of 3q, 8q and 9p and loss of 17p and 11q between T1b and T1c tumors. Both groups contain frequent gains of 3q, 5p and 8q. These three chromosomal arms harbor genes (TERC at 3q21–28, TERT at 5p15.33 and TERF at 8q13) involved in the telomerase machinery and are therefore involved in preventing cellular senescence [6,20].

In summary, we could show that the number of chromosomal copy number changes increases with tumor size, and that in particular high level copy number changes (amplifications) are far more prevalent in larger tumors. Our results also indicate that the acquisition of genomic imbalances is accelerated in aneuploid tumors and that this genomic instability correlates with increased cyclin A expression and low expression levels of p27. However, even T1b tumors already display a non-random pattern of chromosomal gains and losses. We anticipate that this knowledge could be translated to the improvement of the diagnosis of early breast lesions using interphase cytogenetics with DNA probes that target the specific chromosomes involved.

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