Genomic imbalances detected by comparative genomic hybridization are prognostic markers in invasive ductal breast carcinomas

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Aims: The aim of this work is the study of the prognostic significance of the chromosomal aberrations described in a series of invasive ductal breast carcinomas.

Methods and results: We analysed by comparative genomic hybridization a group of 70 formalin-fixed paraffin-embedded invasive ductal breast carcinomas. Aberrations showed a frequency similar to previous studies using frozen tumours. Interestingly, we identified gains involving 6q16-q24 more frequently than in other series. We analysed the association among the chromosomal imbalances, 11 histopathological factors, relapse rate and overall survival of patients. Associations showed 16q losses as a potential marker of good prognosis, as they were more frequent in node-negative ($P = 0.025$) and in oestrogen-positive tumours ($P < 0.001$). Furthermore, 100% of bcl-2+ tumours presented this aberration compared with 29.3% in bcl-2– ($P = 0.014$). 1q, 11q, 17q and 20q gains were associated with poor prognosis: 95% of cases with 1q gains were bigger than 20 mm ($P = 0.041$). Tumours with 1q and 11q gains showed a higher relapse rate ($P = 0.063$; $P = 0.066$). Within the good prognosis group of lymph node-negative patients, 17q and 20q gains identify a subgroup with increased relapse rate ($P = 0.039$).

Conclusions: Chromosomal imbalances, together with histopathological factors, may help to predict outcome in breast cancer patients.

Keywords: CGH, prognostic factors, invasive ductal breast carcinomas, 6q gains, 16q losses

Introduction

Breast cancer is the most frequent type of cancer among women in western countries.[1] Because of its incidence and complexity, enormous efforts have been made to increase knowledge about breast cancer biology and to develop new therapeutic strategies.[2] Many new prognostic factors have been suggested, but the prognostic value of a wide number of them is still not confirmed. A recent review of prognostic factors used in breast cancer has considered tumour size, nodal status, micrometastasis, histological grade and type, mitotic figure count and hormone receptor status as the only factors with prognostic value and use in clinical patient management.[3]

Genetic studies have contributed to increase the knowledge of this disease. One of the most useful genetic techniques to study breast cancer has been comparative genomic hybridization (CGH). CGH allows detection of chromosomal imbalances without the need for tumour metaphases and with the possibility of using any source of tissue (fresh, frozen or paraffin-embedded specimens). In breast cancer, the main chromosomal imbalances detected have been gains on 8q, 1q, 11q, 17q and 20q and losses on 16q, 8p, 13q, 17p and 11q23. 1q gains and 16q losses have been reported to

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be involved in early steps in cancer progression; 8q, 11q, 17q and 20q gains and 13q losses have been associated with aggressive phenotypes and have allowed the characterization of new genes such as *AIB1* (20q13).[4–6]

Most of the CGH publications have focused on the description of chromosomal imbalances, but several studies have also reported the associations between chromosomal imbalances and clinicopathological prognostic factors in retrospective analysis, using paraffin-embedded and frozen tissues.[5–7] In breast cancer, no more than three pathological parameters have been considered. The pathological factor more frequently studied has been the histological grade.[8,9] Oestrogen receptor expression (ER),[10] the value of the mean nuclear area, the mitotic index and the apoptotic index[6,11] have also been considered. Tirkkonnen *et al.* studied 55 unselected breast carcinomas where associations with tumour size, nodal status, grade, ER and progesterone receptor (PR) expression, DNA index and S-phase fraction were established.[5] They found gains of 8q correlated with DNA index and high S-phase fraction, as well as the association between Xq loss and oestrogen receptor negativity.

We present the analysis of a homogeneous series of 70 invasive ductal breast carcinomas by CGH. In order to find genetic aberrations that may be used as prognostic markers, we associated the chromosomal imbalances found with 11 histopathological parameters routinely determined in clinical management, the overall survival and the relapse rate.

Materials and methods

PATIENTS

We selected 70 patients diagnosed with invasive ductal breast carcinoma at the Hospital of Navarra between 1991 and 1994. Pathological characterization of the samples was done according to the European Commission Group on Breast Screening Pathology (ECGBP).[12] The mean age of the patients was 62.5 years (range 33–95 years). The mean follow-up time was 64 months (range 6–103 months). Disease-free survival and the presence of local, regional and/or distant metastases were also noted. The 2-year overall survival of the series was 87% (SD = 4.5%) and 75.9% within 5 years (SD = 5.8%). The 2-year relapse rate was 14.6% (SD = 5.1%) and 29.2% (SD = 6.5%) within 5 years.

PATHOLOGICAL FACTORS

From each sample tumour size, histological grade, axillary lymph node status and Nottingham Prognostic Index were evaluated. Immunohistochemical study of ER and PR), p53 and Ki67 were made using the Ventana™ Enhanced DAB Detection Kit. bcl-2 and ErbB-2 expression were studied by the Super Sensitive Immunodetection System (Biogenex). More details about immunohistochemistry methodology are listed in Table 1. DNA ploidy was analysed by cytometry after Feulgen staining. In all cases, positive and negative controls were used. Immunohistochemistry results were scored semiquantitatively in four grades, considering levels 0, 1, 2, and 3 according to the number of positive cells and the intensity of the staining. In order to facilitate the statistical analysis we classified the immunohistochemistry results in positive or negative groups. Table 2 summarizes the pathological features of the samples analysed.

COMPARATIVE GENOMIC HYBRIDIZATION

Genomic DNA was isolated from archival paraffin block samples. Prior to DNA extraction, we selected in each sample the most representative tumour region according to the adjacent haematoxylin–eosin-stained

Table 1. Immunohistochemical stains

Antibody/antigen	Source	Dilution	Antigen retrieval
Oestrogen receptor	ATOM-Ventana	Prediluted	10x antigen retrieval *Citra* solution pressure cooker, 4 min
Progesterone receptor	ATOM-Ventana	Prediluted	10x antigen retrieval *Citra* solution pressure cooker, 4 min
p53	ATOM-Ventana	Prediluted	10x antigen retrieval *Citra* solution pressure cooker, 4 min
erbB-2	BioGenex	Prediluted	10x antigen retrieval *Citra* solution pressure cooker, 4 min
bcl-2	BioGenex	1:100	10x antigen retrieval *Citra* solution pressure cooker, 4 min
Ki67	Dako	1:50	10x antigen retrieval *Citra* solution pressure cooker, 4 min

Table 2. Pathological features of the 57 invasive ductal breast carcinoma samples analysed by CGH in this study

Pathological factors	*n*	%
Tumour size		
<20 mm	10	17.5
≥20 mm	47	82.5
Histological grade		
I	3	5.3
II	34	59.6
III	20	35.1
Node status		
No nodes involved	26	45.6
1–3 nodes involved	16	28.1
4 nodes involved	13	22.8
No data	2	3.5
Oestrogen receptors		
Negative	42	73.6
Positive	15	26.4
Progesterone receptors		
Negative	37	65
Positive	20	35
p53		
Negative	31	54.4
Positive	26	45.6
erbB-2		
Negative	31	54.4
Positive	26	45.6
bcl-2		
Negative	16	28
Positive	41	72
Ki67		
Negative	0	0
Positive	57	100
Ploidy		
Diploid	15	26.3
Aneuploid	41	71.9
No data	1	1.8
Nottingham Prognostic Index		
Good prognosis	10	17.5
Moderate prognosis	24	42.1
Poor prognosis	21	36.8
No data	2	3.5

section. In order to obtain a good quality of DNA, extraction was performed with a protocol slightly modified from Isola *et al.*[13] Briefly, multiple thin sections from tissue were digested with proteinase K at 55°C during 3–5 days. A commercial DNA purification

kit (QIAamp Tissue Kit; Quiagen, Hilden, Germany) was used to purify DNA. Direct DNA labelling was performed using Vysis nick translation kit (Vysis GmbH, Stuttgart, Germany). Digestion time and enzyme concentration were adjusted for each sample. In order to avoid an excessive DNA fragmentation we reduced DNase and nick translation reaction time to 20 min. The labelling of this DNA was improved by increasing the DNA polymerase I concentration. In six cases, preamplification of the DNA using degenerate oligonucleotide-primed PCR methodology (DOP-PCR) was necessary.[14]

Because of the high DNA fragmentation, spectrophotometry usually overestimates DNA concentration from paraffin samples. In order to obtain confirmation we added equal concentrations of both DNAs; sexmismatched experiments were used. For each case, between 8 and 10 metaphases were analysed. Numerical analysis of DNA imbalances was performed using fixed thresholds: 0.8 for losses and 1.2 for gains.

STATISTICAL ANALYSIS

Statistical analysis was performed with SPSS software (Windows v. 9.0). Differences in the average of copy number aberrations among groups characterized by histopathological factors were analysed with Student's *t*-test, one way analysis of variance or the Mann–Whitney *U*-test, and Kruskall–Wallis test depending on the results of Kolmogorov–Smirnov normality test.

The differences in the frequency of the most common gains and losses (those present in >15% of cases) among histopathological groups were analysed by the χ^2 contingency test.

The relationship between histopathological factors and chromosomal aberrations with the overall survival and the relapse rate was analysed by the Kaplan–Meier method. The association of the copy number aberrations to prognosis was analysed by the regression Cox test.

Values of $P \leq 0.05$ were considered to be statistically significant and $0.05 < P \leq 0.1$ were considered to be nearly significant. The size of the different subgroups and the low number of events prevented multivariate analysis.

Results

Technically successful results were obtained in 57 out of 70 tumours (81.4%). CGH detected genetic aberrations in 55/57 tumours (96%). The mean number of copy number aberrations was 5.8 per tumour (SD = 0.45, range 0–15). The number of gains was 3.6 (SD = 0.28, range 0–9) and that of losses 2.2

(SD = 0.27, range 0–8). A summary of all chromosomal aberrations detected is shown in Figure 1. Chromosomal changes affected all chromosomes except chromosome 21. The most frequent gains were on 8q (63.1%), 17q (45.6%), 1q (38.6%), 20q (26.3%), 11q (21%) and 6q (17.5%). The most frequent losses affected at 16q (21%), Xp and Xq (19.3%), 13q (17.5%), 11q (15.7%) and 8p (15.7%).

It was common that the abnormalities affected entire chromosomes or chromosomal arms. However, subregional imbalances were detected in 11q (losses at 11q21-qter), 6q (gains at 6q16-q24, 6q16-q22, 6q22-q24) and 14q (gains at 14q12-q22, 14q11-q24.2, 14q32, 14q14-q24, 14q11-q21). In 11 samples, we found aberrations limited at one band as gains on 1p21, 7q11, 11q13 and 12q15.

We detected slight differences in the average of copy number aberrations between histopathological groups: the average number of gains was higher in tumours >20 mm relative to those of small size (3.9 versus 2.5; $P = 0.058$), and in tumours with p53 accumulation (4.2 versus 3.2; $P = 0.070$). Samples positive for PR expression showed twice as many losses as PR-negative samples ($P = 0.083$). No associations were found between either copy number aberration and survival or copy number aberration and relapse rate.

We compared the aberrations present in >15% of cases with 11 pathological factors. We first confirmed the prognostic value of the histopathological factors: only the size, the node status and the Nottingham Prognostic Index showed significant association with prognosis (Table 3). Chromosomal aberrations found to be significantly associated with pathological features are summarized in Table 4. Of tumours with 1q gains, 95% were >20 mm ($P = 0.041$). Of samples with 6q
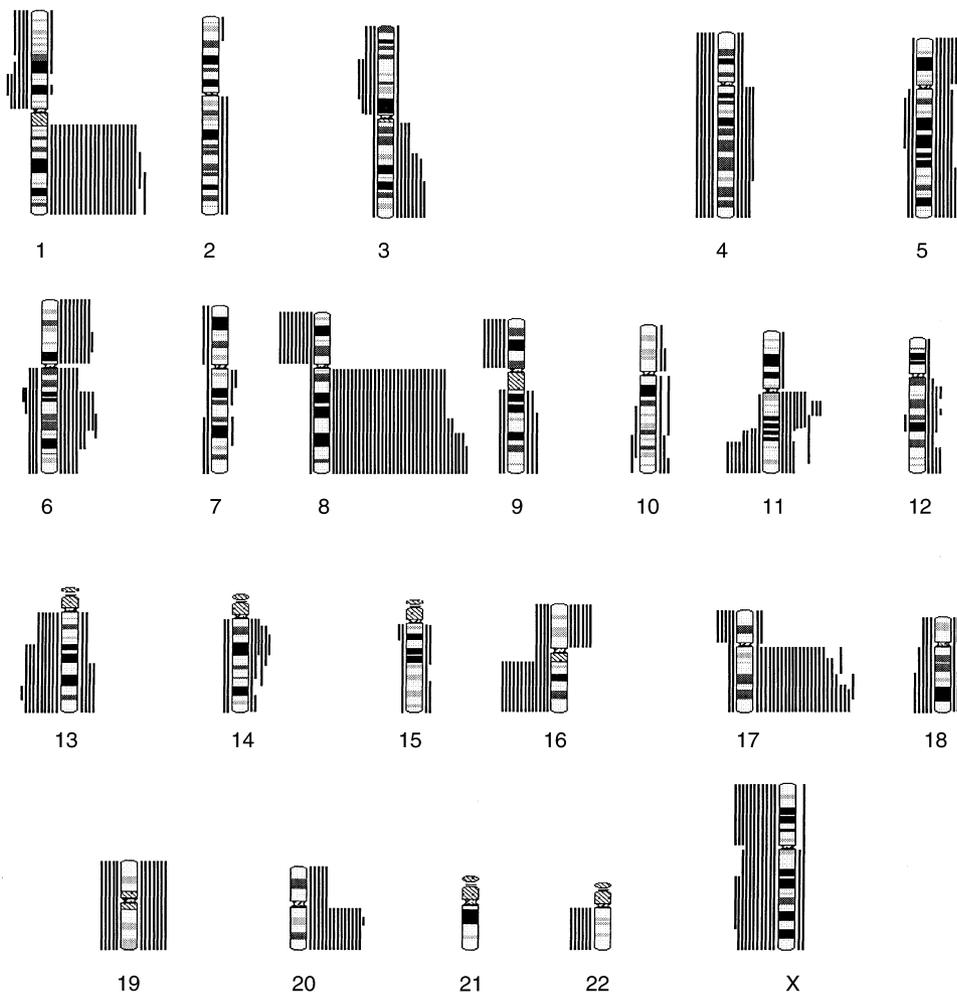

Figure 1. Summary of the genetic imbalances detected in 57 invasive ductal carcinomas of the breast. The vertical lines on the right of the chromosome ideograms represent gains and the vertical lines on the left represent losses.

Table 3. Associations between the pathological factors studied, overall survival and relapse rate

	Overall survival P	Relapse rate P
Size	0.052	0.051
Grade	0.553	0.561
Node status	0.003	0.001
Oestrogen receptor	0.251	0.302
Progesterone receptor	0.185	0.761
p53	0.792	0.463
erbB-2	0.725	0.145
bcl-2	0.137	0.807
Ki67	0.407	0.431
Ploidy	0.286	0.272
Nottingham Prognostic Index	0.032	0.069

gains, 80% showed p53 accumulation ($P = 0.016$). 16q losses were more frequent in node-negative tumours ($P = 0.025$), 46.7% of samples positive for ER also showed 16q losses ($P < 0.001$) and 100% of tumours with this aberration showed a positive bcl-2 over-expression ($P = 0.014$). 8q gains were more frequent in tumours classified within the poor prognostic

group relative to those of the good prognostic group, according to the Nottingham Prognostic Index (81% versus 30%, $P = 0.022$).

Kaplan–Meier analysis revealed a 5-year relapse rate of 41.3% for cases with gains on 1q and a rate of 18.5% for cases without 1q gain ($P = 0.063$). Patients with 11q gains showed a relapse of 50% compared with 23.7% in tumours without this imbalance ($P = 0.066$). Considering node-negative patients only, gains on 17q and 20q were significantly associated with early relapse ($P = 0.039$) (Figure 2).

Discussion

Cytogenetic retrospective analyses are limited to the use of formalin-fixed and paraffin-embedded samples. The quality of the CGH analysis depends largely on the quality of tumour DNA. Especially when working with paraffin-embedded samples, high DNA fragmentation and poor labelling efficiency reduce the sensitivity of detection of chromosomal imbalances.[13] In our study, we achieved a good quality labelling by reducing the concentration of DNase and the digestion time, and increasing concentration of the DNA polymerase I. According to the literature, CGH success rates are between 70% and 90%.[15] When paraffin-embedded tissues are used, lower rates are expected. In our series, with the technical modifications described, we obtained a high success rate of 81.4%.

Genetic changes identified in this series are similar to those described in other breast cancer CGH studies

Table 4. Significant associations between recurrent chromosomal imbalances and pathological factors

|	Chromosomal imbalances	P
Features of good prognosis		
Node-negative tumours	Losses on 16q and 11q	0.025 and 0.040
Oestrogen receptor-positive	Losses on 16q	<0.001
bcl-2-positive	Losses on 16q	0.014
p53-negative	Losses on 11q	0.021
Progesterone receptor-positive	Losses on Xq	0.021
Features of poor prognosis		
Tumours >20 mm	Gains on 1q	0.041
p53-positive	Gains on 6q	0.016
Aneuploid tumours	Losses on 13q	0.027
High Nottingham Prognostic Index	Gains on 8q	0.022
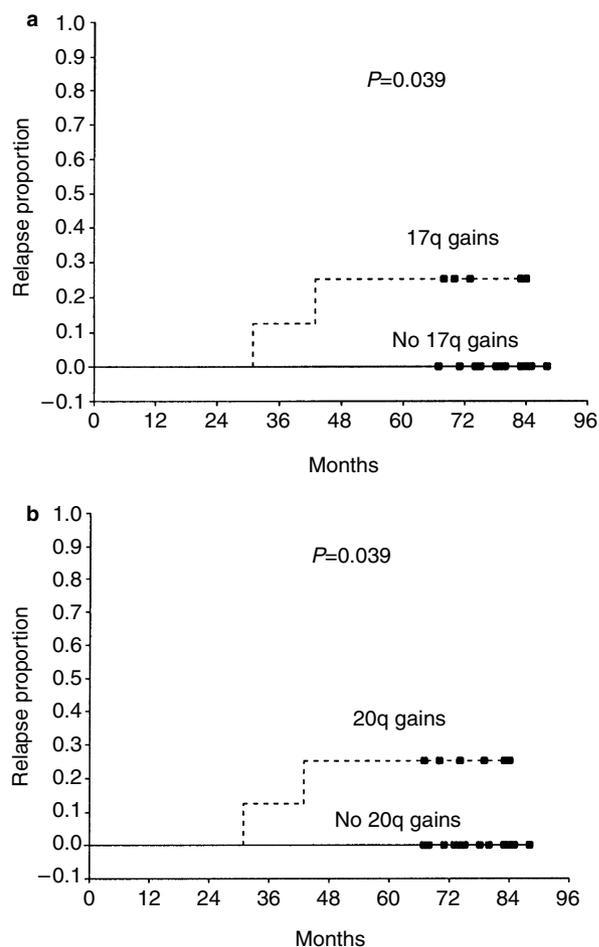
Figure 2. Relapse rate according to (**a**) 17q gains, and (**b**) 20q gains.

using mostly frozen tumours[5,8,10,11,15,16] (Table 5). The different frequencies found between these previous publications and our series for 1q gains and 8p losses could be due to a slightly lower sensitivity of CGH from paraffin samples and/or the presence of normal DNA in tumour samples. Gains on 8q, 1q, 17q, 20q and 11q and losses on 16q, Xq, 13q, 11q and 8p were the most frequent imbalances.

Interestingly, we found gains on 6q more frequently than in other series (Table 5). In these publications losses on 6q were always more frequent than gains.[5,7,8,10,11,15–17] Only Loveday *et al.*[17] showed results similar to ours, with 6q gains more frequent than 6q losses. In our series, gains were limited to bands 6q16-q24, the minimal region involved being limited to 6q22-q23. Hermsen *et al.*[11] and Kuukasjarvi *et al.*[18] showed gains affecting the same bands, more frequent in aneuploid and bad prognosis tumours. Confirming our results, a recent allelotyping analysis of Rodriguez *et al.*[19] showed that the 6q21-q22 region is

most commonly involved in gains, whereas 6q13-q14 and 6q25-q27 are frequently lost. Bands 6q22-q23 harbour the *c-MYB* gene, and c-Myb expression has been associated with oestrogen stimulation and the presence of ER in breast cancer. This gene is amplified in this neoplasia more frequently in tumours with *BRCA1* mutations than in sporadic tumours.[20] In this regard, 20% of our cases with 6q gains showed a previous familiar history of breast cancer, although *BRCA1* mutations have not been analysed. FISH studies analysing *MYB* amplification and other target genes in 6q22-q24 are in progress.

A rarely described gain on 14q11-q24 was detected in our study (minimal region involved 14q13-q21). The *BRF1* gene has been described to be amplified in this region (14q22-q24) in breast cancer lines.[21] Further studies may provide more information about the genes involved.

This study reports associations between chromosomal imbalances, histopathological parameters, overall survival rate and relapse rate in a homogeneous series of breast tumours. Previous CGH studies have considered only one to three pathological parameters, whereas 11 features were analysed in the present work.

The main associations described in our study concern 16q losses with features of good prognosis and 1q, 8q, 11q, 17q and 20q gains with poor prognosis (Table 4). 16q losses were significantly associated with no lymph node involvement, ER-positive expression and overexpression of bcl-2 (Table 4). Axillary lymph node status is the most important predictor of disease-free survival and overall survival in breast cancer,[3] as we confirmed in our series ($P < 0.001$, Table 3). Our series showed that 52.9% of samples without node involvement have 16q losses versus only 11.1% in node-positive cases ($P = 0.025$).

Oestrogen receptor and PR determinations are the main biological markers routinely used to address breast cancer therapy.[22] In our series, ER and PR-positive patients showed a better overall survival and lower relapse rate than those with negative receptors. However, differences were not significant (i.e. ER-positive relapse rate was 19.6% compared with 43.3% in ER-negative tumours, $P = 0.302$). A likely explanation could be the high overall survival of our series due to the fact that most of our patients belong to the Early Detection Programme developed in Navarra. The few number of events in each pathological subgroup could also explain this absence of significance. As previously reported,[10] we detected 16q losses more frequently in ER-positive tumours than in ER-negative (46.6% versus 11.9%, $P < 0.001$), confirming its association with good prognosis.

Table 5. Comparison of the genetic changes described in different breast tumour series

	n	Sample type	Tumour description*	Gains, % 1q	6q	8q	11q	17q	20q	Losses, % 6q	8p	11q	13q	16q	Xq
Kalliomiemi, 1994	33	Cell lines and fresh tumours	–	36	9	27	12	18	18	–	–	–	–	–	–
Ried, 1995	20	Paraffin	–	70	0	50	15	30	10	5	5	0	0	5	–
Isola, 1995	48	Frozen	Node-negative tumours	50	0	48	13	–	15	13	31	13	15	27	–
Tirkkonen, 1997	21	Frozen[21] and paraffin[15]	With BRCA1 mutations	55	6	73	–	–	–	25	–	–	55	–	40
	15		With BRCA2 mutations	–	–	–	–	87	60	60	53	53	73	–	47
Kuukasjarvi, 1997	29	Paraffin	–	66	21	59	–	41	–	14	52	45	21	24	14
Tirkkonen, 1998	55	Frozen	–	67	–	49	18	18	18	18	29	18	25	38	–
Schwendel, 1998	39	Frozen	–	49	5	49	38	28	33	31	20	49	82	25	7
Hermsen, 1998	53	Frozen	Node-negative tumours	28	15	41	19	13	–	17	19	19	–	13	–
Roylance, 1999	90	Frozen	–	63	8	51	12	30	26	25	30	29	30	38	27
Buerguer, 1999	39	Frozen	–	79	–	72	20	10	33	–	49	23	33	26	–
Loveday, 2000	44	Frozen	–	50	45	59	–	–	–	6	36	–	–	16	–
In this study	57	Paraffin	–	39	18	63	21	46	26	9	16	16	18	21	19

*In this column the preselection criteria used by the authors are specified . –, The prevalence of the imbalance is <15% or has not been shown by the authors.

bcl-2 expression has been primarily considered as a predictive marker of response to adjuvant therapy.[23] Most reports have also associated the lack of bcl-2 expression with poor survival.[3,24] Previously to our study, bcl-2 expression has been included only once in CGH publications of breast cancer[25] and it has never been associated with chromosomal imbalances. In our series, all tumours with bcl-2 over-expression showed 16q losses ($P = 0.014$).

The association of 16q losses with other prognostic factors has previously shown the good prognostic value of this imbalance. Buerguer *et al.* found a lower frequency of 16q losses in invasive ductal breast carcinoma of grade III than in invasive ductal breast carcinoma of grade II and ductal in-situ carcinomas.[9,26,27] They also associated this loss with the absence of necrosis and a significantly lower mitotic index and apoptotic index. We also found a higher frequency of 16q losses in grade II than grade III tumours (66.7% versus 25%), but the differences were not significant because of the small size of the subgroups (eight tumours of grade II and three of grade III). Roylance *et al.* and Vos *et al.* found this aberration more frequently in well-differentiated tumours.[8,28] Molecular genetic studies found a statistically significant association of LOH at 16q23.2-24.2 with absence of distant metastases, disease-free survival, and overall survival.[29] Our study confirms these results and shows the association of 16q losses with two of the most important prognostic factors in breast cancer: node involvement and ER expression.

In our study, 1q, 8q, 11q, 17q and 20q gains were significantly associated with poor prognosis (Table 4). 8q gains were more frequent in tumours with poor clinical outcome (those with a high Nottingham Prognostic Index) than in those of the good prognosis group (81% versus 30%, $P = 0.022$). These results are in agreement with several CGH studies where 8q gains were associated with a higher mitotic index, apoptotic index and mean nuclear area,[6,11] poor prognosis in node-negative patients,[7] high DNA index and S-phase fraction,[5] grade III tumours,[8] and aneuploidy.[30] In our series, this aberration was also more frequent in aneuploid tumours (66%) and node-positive tumours (64%), although without statistical significance ($P = 0.290$ and $P = 0.072$, respectively).

Our results also suggest that gains of 1q and 11q are associated with poor prognosis. The 5-year relapse rate was 41.3% for tumours with 1q gains versus 18% for tumours without this aberration ($P = 0.063$). Relapse rate was 50% for tumours with 11q gains compared with 23.7% for tumours without this imbalance ($P = 0.063$). We also found that 95% of tumours with 1q gains were >20 mm ($P = 0.041$). Other CGH publications have correlated 11q13 gains with more aggressive phenotypes such as hypodiploid tumours,[31] intermediate and poorly differentiate tumours[9] and poor prognosis.[11] Consistent with these data, over-expression of genes located in this region (*FGF3*, *FGF4* and cyclin D1) have been associated with a higher relapse rate in node-negative and ER-positive patients.[32,33]

The other associations described in Table 4 may be interpreted by caution because of the small size of the subgroups characterized.

Although no node involvement is the main feature of good prognosis in breast cancer, 30% of node-negative patients relapse within 10 years, and 30% die because of the disease.[34] The association that we have found between 17q gains and 20q gains with poor prognosis in node-negative patients could have clinical impact because it identifies a subgroup with poor prognosis ($P = 0.039$). 17q and 20q gains have been always associated with more aggressive phenotypes.[4,28] Amplifications/over-expression of some genes located in these regions such as *ERBB-2*, Topoisomerase IIα and *AIB1* have been described as responsible for this poor outcome.[35–37]

In conclusion, we present a series of 57 formalin-fixed paraffin-embedded breast tumours analysed by CGH. Successful results were obtained in 81.4% of the samples. Our results show that, with technical modifications, CGH in paraffin samples should be a useful tool for retrospective studies using archival material. These chromosomal imbalances could characterize new genetic markers of breast tumour progression. Together with conventional prognostic factors, losses on 16q and gains on 1q, 8q, 11q, 17q and 20q could help to identify new patient subgroups with a different clinical outcome. Further studies will be required to confirm the prognostic value of these aberrations in individual tumours and to identify the genes involved.

Acknowledgements

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