

## **Deletion of chromosome 11q predicts response to anthracycline-based chemotherapy in early breast cancer**

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## **ABSTRACT**

Despite recent consensus on eligibility of adjuvant systemic therapy in lymph-node negative breast cancer (NNBC) patients based on clinico-pathological criteria, specific biological markers are needed to predict sensitivity to the different therapeutic options. We examined the feasibility of developing a genomic predictor of chemotherapy response and recurrence risk in 185 patients with NNBC using assembled arrays containing 2,460 BAC clones for scanning the genome for DNA copy number changes. After surgery, 90 patients received anthracycline-based chemotherapy whereas ninety-five did not. Tamoxifen was administered to patients with hormone-receptor positive tumors. Association of genomic and clinico-pathological data and outcome was computed using Cox proportional hazard models and multiple testing adjustment procedures. Analysis of NNBC genomes revealed a common genomic signature. Specific DNA copy number aberrations were associated with hormonal receptor status, but not with other clinico-pathological parameters. In patients treated with chemotherapy, none of the genomic changes was significantly correlated with recurrence. In patients not receiving chemotherapy, deletion of eight BAC clones clustered to chromosome 11q was independently associated with relapse (DFS at 10 years $\pm$ SE, 40 $\pm$ 14% vs. 86 $\pm$ 6%; $p$ <00001). Patients with 11q deletion did not show more aggressive clinical-pathological features than those without 11q loss. The adverse influence of 11q deletion in clinical outcome was confirmed in an independent validation series of 88 NNBC patients. Our data suggest that NNBC patients with 11q deletion may benefit from anthracycline-based chemotherapy despite other clinical, pathological or genetic features. However, these initial findings should be evaluated in randomized clinical trials.

## **INTRODUCTION**

The use of adjuvant systemic chemotherapy and/or endocrine therapy in the treatment of lymph-node negative breast cancer (NNBC) has increased greatly in the last decade.(1-5) Despite recent consensus, the group of patients with NNBC that will obtain clinical benefit from the use of adjuvant chemotherapy is still a problematic debate. In patient with young age, with large sized or high pathological grade tumors, or with hormone receptor–negative tumors, systemic chemotherapy improves the odds of disease-free and overall survival.(1, 2, 5, 6) Among the different chemotherapy regimens, those containing anthracyclines are on average more effective.(5, 6) Therapy with trastuzumab seems to be effective in the subset of *HER2* positive NNBC.(7) However, despite obvious therapeutic advances, approximately one fourth of NNBC patients will have tumor recurrence that is potentially treatable but ultimately fatal. These data highlight the need for more sensitive and specific therapy-predictive indicators to refine the use of the multiple treatment options.

Using gene expression profiling, several investigators have reported gene-expression signatures of breast tumor cells that were more powerful predictors of disease outcome than standard clinical and histological criteria.(8-12) However, the apparent variability and lack of reproducibility observed among these previous transcriptional analyses and the requirements for high-quality RNA obtained from fresh tissues have limited their application to the clinical setting. A different type of microarray technology, termed comparative genomic hybridization (CGH) to microarrays (array CGH), allows a quantitative detection of DNA copy number changes in tumor genomes with high resolution.(13, 14) This method enables the identification of precise areas in which genetic changes occur, including loss of genomic material (deletion) and genomic gain (amplification). These genomic alterations usually result in damage of specific genes involved in cancer development and progression. In breast cancer, definition of recurrent genomic aberrations has revealed loci encoding genes involved in the pathogenesis of the

disease,(15-18) some of which have been correlated with the different pathological subtypes.(19) As the most prominent example, amplification of chromosome 17q12 targets *HER2* gene, which results deregulated at the RNA and protein levels.(20) Recently, array CGH has also proven its value for predicting clinical outcome in prostate carcinoma, lymphoma, gastric carcinoma and acute myeloid leukemia.(21-23) However, to date, no similar studies have been attempted in breast cancer.

In the present study, we examined the feasibility of developing a predictor of recurrence risk and therapeutic response for 185 NNBC patients using array CGH for scanning the entire genome for DNA copy number changes.

## **METHODS**

### **STUDY DESIGN AND SELECTION OF PATIENTS**

From September 1979 to June 2000, over 3.100 new breast cancer patients were diagnosed at the University of Valencia. Of them, 1.482 tumors were criopreserved at -80°C. For this study, biopsies were randomly selected based on the following criteria for inclusion: (1) diagnosis of primary invasive breast carcinoma of any size; 2) treatment by modified radical mastectomy or breast-conserving surgery, including dissection of axillary lymph nodes, followed by radiotherapy if indicated; 3) the apical axillary lymph nodes were tumor-negative (pathological examination, pN0); and 4) complete clinical data were available. Over 363 samples fulfilled the criteria. Previous to DNA extraction, these frozen tumor sections were stained by hematoxylin/eosin (H&E) and reviewed for tumor infiltration: only those with clear >50% of tumoral cells were selected. A cohort of 185 patients fulfilled these final criteria. Clinico-pathological variables including tumor size, histological grade and subtype and ER and PR status were determined following standard methods as reported.(24) Human investigations were performed after approval by an institutional review board on scientific and ethical affairs.

### **MICROARRAY-BASED COMPARATIVE GENOMIC HYBRIDIZATION (ARRAY CGH)**

**DNA extraction, hybridization and imaging.** Frozen tumors were included in OCT compound. Previous to DNA extraction, H&E stained tumor sections were examined to select samples with more than 50% of tumoral cells. Around 20-30 sections of 25µm were used for DNA extraction. After removing the OCT with PBS washes, DNA was extracted as previously described.(24) Genome-wide analysis of DNA-copy number changes was performed using array CGH on a microchip with ~2.460 BAC and P1 clones printed in triplicate (UCSF Hum Array 2.0) with a resolution of 1.4 Mb across the genome.(25) Methods and analytical procedures have been described elsewhere in detail.(23, 25) Briefly, 0.5 µg of test (tumor) and reference genomic DNAs were labeled by random priming using Cy3

and Cy5, respectively. After 48 hour of hybridization, slides were washed and mounted with DAPI. The images of the arrays were captured using a CCD camera, and the “UCSF SPOT” 2.0 software (available at <http://www.jainlab.org/downloads.html>) was used to analyze the images and measure tumoral vs. control fluorescence intensity ratios that were converted to the log<sub>2</sub> scale. A second program, the “UCSF SPROC” was used to associate clones with each spot and to create a mapping information file that allows the data to be plotted relative to the position of the BACs on the draft human genome sequence (<http://genome.cse.ucsc.edu>; May 2004 freeze). A formal data filtering procedure was then performed, and a SPROC output file consisting of averaged ratios of the triplicate spots for each clone, standard deviations of the replicates and plotting positions for each clone on the array, was obtained (Figure 1). For visualization of genomic data, the TreeView program 1.60 (Stanford, CA) was used. To confirm array CGH data, CGH to chromosomes was performed in 44 biopsies included in the study.

**Interphase FISH analysis.** To confirm specific gains and losses of BAC clones observed in the array CGH analyses, fluorescence in situ hybridization (FISH) studies using individual BAC clones as probes on isolated nuclei from frozen tumor sections was performed using a reported technique.(26) The gene loci examined corresponded to 5 overrepresented and 4 deleted BAC clones, using appropriate centromeric probes as controls. A total of 100 cells were examined on each of the 22 tumors examined. These clones were obtained from RZPD German Resource Center (Berlin, Germany) or purchased from Vysis (Downers Grove, IL, US).

## **STATISTICAL ANALYSIS**

**Array preprocessing.** In order to process the genomic data obtained with array CGH and to compare the genomic alterations with different clinical phenotypes we used a previously described analytical model.(27) Clones with ratios missing in 2 or more replicate spots (out of 3) were excluded from further analysis, as well as when the standard deviation of the replicates log<sub>2</sub> ratios was above 0.2. In addition, clones that were successfully mapped to May 2004 release of human genome sequence and

were declared present in more than 75% of the samples were included in the final analysis. Duplicate clones were averaged. The final dataset contained 2117 unique BACs, and clone values were missing in a median of 5.4% of the samples. Arrays were normalized by subtracting the median of each array from the average log<sub>2</sub> ratio for every clone.

**Copy number changes identification.** The array CGH data were analyzed using Hidden Markov Model (HMM) as implemented in the Bioconductor package aCGH using the default tuning parameters.<sup>(27)</sup> Log<sub>2</sub> ratios as ordered in the genome were segmented into regions of constant copy number. In addition, the HMM model was employed to impute missing values by using the estimated copy number ratio for the segment containing the clone(s) with missing values. Clones with missing values located between segmented regions were assigned the mean value of the segment that is closer in genomic distance. Thus, each clone was assigned a segment value referred to as its "smoothed" value. Median absolute deviation (MAD) of the difference between the observed and smoothed values was used to estimate the tumor-specific experimental variation. All of the tumors had MAD less than 0.22. Clones for each array were assigned into three groups: gained - the smoothed log<sub>2</sub> ratio of a clone in a particular tumor was higher than 3 times the MAD; lost - when the smoothed log<sub>2</sub> ratio was less than 3 times the -MAD; and finally, not changed - when the log<sub>2</sub> ratio can not be assigned to the lost or gained groups.

**Association of copy number with phenotypes.** Smoothed, imputed data was used to study association with the following phenotypes: age, tumor size, histological grade and subtype, stage, estrogen and progesterone receptor status, and recurrence/survival. For example, for the right censored data we used Cox proportional hazards model, where we tested for difference in survival given different baseline log<sub>2</sub> ratio for a given clone. We corrected for multiple hypotheses testing by controlling the False Discovery Rate. Significance was claimed at the FDR < 0.05, which corresponds to the expectation of at most 5% of false discoveries among the loci declared significant. In addition, we

tested for difference in recurrence/survival outcome for patients subgroups defined by the treatment assigned to them.

**Cross-tabulation of clinical variables.** We used Fisher's two-sided exact test 2x2 crosstabs to compare genomic events or clinical variables among both groups of treatment. To evaluate differences in disease-free survival, Kaplan-Maier survival curves for the sets of patients were examined.

**GO validation.** Finally, we performed a statistical analysis to check if there were any gene Ontology (GO) categories that were enriched in the genes located in the region of deletion in chromosome 11q23-q24 with respect to the whole genome

## **CLINICAL SERIES FOR VALIDATION OF ARRAY CGH RESULTS**

To validate the possible association of chromosome 11q deletion with increased relapse rate, this was tested in a validation group of 88 tumor biopsy samples from an independent cohort of NNBC patients. These were 18 Spanish patients treated in different Institutions within the Valencia area whose genomes were analyzed with array CGH as described above. In addition, data from 70 patients were obtained from a recently published series of American breast cancer patients analyzed using similar whole-genome array CGH techniques (Chin et al, submitted). All patients fulfilled the reported inclusion criteria of the study. Kaplan-Maier survival curves for the two sets of patients were evaluated. Clinico-pathological characteristics of the validation series are shown in Supplemental Table S1.



## **RESULTS**

### **CHARACTERISTICS OF THE PATIENTS**

Clinico-pathological characteristics of the 185 patients and tumors are summarized in Table 1. Based on the clinico-pathological features, 90 women received anthracycline-based chemotherapy (CHEMO group) whereas 95 patients did not (non-CHEMO group). In both groups, women with ER/PR positive tumors were treated with tamoxifen: 42 in the CHEMO group (47%) and 56 in the non-CHEMO group (59%) (Supplemental Table S2). With a median duration of follow-up time of 82 months (range, 9 to 218 months), 45 of the 185 patients (24%) have relapsed. Median duration of follow-up time for patients who are free of disease was 96 months in both CHEMO and non-CHEMO groups. Death from the disease was assessed in 16 of 185 patients (9%). We initially determined differences in clinico-pathological features and outcome between patients in the CHEMO group vs. those in the non-CHEMO group. Women in CHEMO group were younger (mean, 51 vs. 67 years;  $p=0,003$ ) and had a more frequent pre-menopausal status (43 vs. 17%;  $p=0,001$ ). We did not observe statistically significant differences in any other histopathological feature (Table 1 and Supplemental Figure S1A). In addition, no statistically significant differences in disease-free survival (DFS) and overall survival (OS) were found between CHEMO and non-CHEMO groups (Supplemental Figure S1B and S1C).

### **GENOMIC PROFILING OF LYMPH-NODE NEGATIVE BREAST CANCER**

Array CGH analysis was performed in the 185 tumor biopsies, and allowed the identification of specific regions of gain and loss throughout the genome with high resolution in all of the biopsies. All tumors showed genomic changes (Figure 2). A total of 112 clones that were mapped to 40 different chromosome loci in 9 different chromosome arms were found. These corresponded to 23 genomic gains and 17 genomic losses involving regions known to be commonly involved in breast cancer as well as uncharacterized genomic aberrations. The most common gains corresponded to chromosomes

1q31 and 20q12 (91 of 185, 49%), 8q24.2 (40%), 17q21 (39%), 1q32, 8q23.1 and 20q13.1 (35%), 1q23 (34%) and 8q24.1 at *MYC* gene locus (32%). In addition, high-level amplification (defined as log<sub>2</sub> ratio >1 observed in at least 10 different samples) was identified in 5 different regions of chromosomes 11q13-q14 at *CCND1* gene (17 of 185 tumors, 9%), *HER2* (13 tumors, 7%), 1q31 and 8p12 at *FGFR1* gene (11 tumors, 6%) and 8q21-q24.1 including *MYC* gene (10 tumors, 5%). The most frequently deleted regions were observed at chromosomes 13q14-q22 (66 of 185 tumors, 36%), 17p12-p13 including *P53* gene locus (34%), 16q21-q22 including the *CDH1* gene (30%), and 11q21-q25 (29%), 16q24 and 16p12-p13.1 (26%), 11q12 (25%), 8p21.3-p22 (25%) and 22q11.2. A total of 18 homozygous deletions (defined as log<sub>2</sub> ratio below -1,4) were identified, being the loss of 13q21.3-q22 at *KLF12* gene observed in two different tumors. The full array CGH data has been deposited in GEO database (Platform GPL3632).

To initially validate the array CGH results, a subset of 44 samples was also analyzed with CGH to chromosomes, and the two techniques showed concordant values (Supplemental Figure S2). To further validate our data, the analysis of 9 individual BAC clones in 22 frozen tumor sections using fluorescence in situ hybridization (FISH) also showed a high concordance with array CGH results (Supplemental Table S3). The array CGH data defined a common genomic signature of NNBC (Figure 2; detailed information in Supplemental Table S4).

## **CORRELATION OF GENOMIC ALTERATIONS AND CLINICOPATHOLOGICAL FEATURES**

We tested the association of clinical and pathological variables with each of the BAC clones in the 185 patients. After adjustment for multiple testing, these analyses showed that the only variables correlated with genomic changes were ER and PR status (Figure 3). Tumors that showed expression of ER (ER+) presented with frequent gain of chromosomes 1q21-q43 (35% vs. 14%;  $p < 0,05$ ), and 16p12 (17% vs. 1%;  $p < 0,01$ ) and losses of chromosome 16q21-q24 (25% vs. 7%;  $p < 0,01$ ). Tumors negative for PR

(PR-) also presented with frequent deletion of chromosomes 4p13-p16 (19 vs. 5%;  $p<0,001$ ) and 5q11.2-q31 (16 vs. 3%;  $p<0,001$ ) (Figure 3B and Supplemental Table S5). We also determined the correlation of the genomic status of 17q12 locus at *HER2* gene (determined by array CGH analysis) with other clinical variables. Amplification or gain of *HER2* gene was observed in 29 tumors (16%) and was correlated with negativity for PR expression ( $p=0,007$ ), but not with other clinico-pathological features.

### **ASSOCIATION OF GENOMIC ABNORMALITIES WITH CLINICAL OUTCOME: 11q LOSS PREDICTS RESPONSE TO CHEMOTHERAPY**

We compared the genomic profiles of tumors in the CHEMO and non-CHEMO groups. None of the abnormal BAC clones showed a significantly different distribution between the two cohorts, indicating that both groups were comparable at the genomic level (Figure 2). To develop a genomic predictor of clinical outcome, we examined the association of the genomic aberrations with disease recurrence in the two differently treated cohorts. In the CHEMO group, after adjustment for multiple testing, none of the abnormal BAC clones was associated with tumor relapse (Figure 4A). In the non-CHEMO group, however, there were statistically significant differences ( $p<0,05$ ) in 8 BAC clones that showed more common deletion in tumor recurrences with respect to non-recurrences (Figure 4B). Notably, these 8 clones clustered to the long arm of chromosome 11 from 11q23.1 to 11q24.1, spanning ~9 Mb. in size (Figure 4C). Therefore, deletion of chromosome 11q was associated with decreased DFS in NNBC patients in the non-CHEMO group (DFS  $\pm$  SE at 10 years,  $40\pm 14\%$  vs.  $86\pm 6\%$ ,  $p<0,0001$ ) but not in the non-CHEMO group (DFS at 10 years  $\pm$  SE,  $92 \pm 21\%$  vs.  $65 \pm 9\%$ ,  $p=0,13$ ). Analysis of the association of the genomic changes with OS in the two treatment cohorts did not reveal any significant correlation, probably due to the low number of patients who have died of the disease so far. Finally, we performed a statistical analysis to check if there were any GO categories that were enriched in the genes located in the region of deletion in chromosome 11q23.1-q24.1. Among them, DNA repair genes

and meiotic-related genes were significantly enriched (hypergeometric test pvalue <0.00092). Four genes belonged to this category (*CHK1*, *H2A*, *ATM* and *ZW10*).

## **CHARACTERISTICS OF PATIENTS WITH 11q DELETIONS**

To determine whether the negative impact of 11q deletion on DFS was dependent on other clinical and biological features, we compared the clinical and biological characteristics of the 54 patients with 11q deletion vs. those 131 patients without deletion of 11q. In the whole group of 185 patients, there were no statistically significant differences for age, clinical stage, hormonal status, tumor size and grade, and expression of ER/PR for 11q-deleted vs. non-deleted tumors (Supplemental Table S6). These data indicate that the influence of 11q deletions in the relapse rate of the patients in the non-CHEMO group is independent of other known clinical and pathological features. We also analyzed possible differences in patients with and without 11q deletion for genetic alterations reported in correlation with aggressive breast cancer (*HER2*, *CCND1*, *MYC* and *FGFR1* amplifications and *P53/P16* deletions). We did not observe changes in the distribution of these genomic alterations, with the exception of *CCND1* amplification that was more common in tumors harboring deletion of chromosome 11q: among 17 cases with *CCND1* amplification, 12 (70%) presented deletion of 11q whereas only 42 cases (25%) showed 11q deletion among the 168 non-amplified *CCND1* cases ( $p < 0,001$ ). This association can probably be explained by the proximity of *CCND1* gene (which maps to 11q13 band) to the 11q23.1-q24.1 deletion (Supplemental Figure S4B). Notably, genomic amplification of *CCND1* was not associated with decreased DFS in both the non-CHEMO and CHEMO groups. In summary, tumors with 11q deletion do not show a more aggressive phenotype or genotype that can distinguish them from those without this chromosome deletion.

## **VALIDATION OF 11q DELETION AS A THERAPY-PREDICTIVE INDICATOR**

To validate the association of chromosome 11q deletion with worse outcome in patients not receiving anthracycline-based chemotherapy, we analyzed a second series (validation group) of 88 NNBC biopsies. After surgery, 27 of the patients received chemotherapy whereas the remaining did not. Sixty-two patients with ER/PR positive expression received hormonal therapy based on tamoxifen. In the group treated with chemotherapy, 6 of 15 patients (40%) without 11q deletion relapsed whereas 3 of 12 patients (25%) with 11q deletion had a recurrence ( $p=0,23$ ). However, in the group not receiving chemotherapy, tumor recurrence was observed in 4 of 33 patients (12%) without 11q deletion and in 10 of 28 patients (35%) with 11q deletion ( $p=0,02$ ) (Figure 5). Kaplan-Meier curves also showed that deletion of chromosome 11q was associated with inferior DFS in patients not treated with chemotherapy (DFS  $\pm$  SE at 10 years,  $65\pm 13\%$  vs.  $88\pm 8\%$ ,  $p<0,1$ ). Notably, in the non-CHEMO group, patients with 11q deletion had a tendency to show a superior DFS compared to those without 11q loss ( $73\pm 18\%$  vs.  $50\pm 18\%$ ,  $p<0,7$ ). These differences, however, did not reach statistically significant values, probably because of the limited number of patients and the relatively short median follow-up time (Figure 5D). In summary, the results observed in this validation set were coincident with the results obtained in the training set of 185 patients, and confirms that deletion of 11q is associated with relapse in patients with NNBC who are not treated with anthracycline-based chemotherapy.

## DISCUSSION

Following current therapeutic guidelines, one fourth of NNBC patients will have tumor recurrence and ultimately die of the disease. In addition, many patients treated with systemic therapy who will never have disease recurrence could have been cured with surgery alone. These over- and under-treatments are owing to limitations of the current prognostic factors, which largely rely on clinical characteristics and classical histopathological features.(2-4). Recently, *HER2* amplification/over-expression has been accepted as a risk factor for prognostication in the St Gallen criteria and moreover, recent reports demonstrate that a recombinant monoclonal antibody against HER2 combined with chemotherapy improves outcomes among women with HER2-positive breast cancer.(4, 7, 28) Still, as this therapy will benefit ~20% of NNBC women with HER2-positive tumors, there is an urgent need of similar therapy-predictive factors to tailor optimal individualized therapies in the remaining women. In the present study, by using CGH to BAC microarrays for scanning NNBC genomes, we have identified the single deletion of chromosome 11q as a novel genomic marker that predicts response to anthracycline-based chemotherapy. Thus, patients receiving anthracycline-based chemotherapy with 11q deletion had lower tumor relapse rates (although it was not statistically significant) than those not having 11q deletion. On the contrary, in the group of patients not receiving chemotherapy, tumors with 11q deletion relapsed more frequently than those without 11q loss. Notably, the presence of 11q deletion in tumors was not correlated with classical prognostic factors such as age, clinical stage, tumor size, histological grade and subtype, and ER and PR expression status, nor with other genetic alterations correlated with poor outcome in breast cancer (*HER2*, *MYC* and *FGFR1* amplification and *P53* and *P16* deletion).(20, 29) There was however a regional association between 11q deletion and amplification of *CCND1* gene at 11q13 band, although genomic amplification of *CCND1* did not influence patient outcome. Thus, the adverse outcome of 11q deletion in the non-CHEMO group was independent of all tested prognostic factors. Therefore, our data suggest that NNBC patients with 11q deleted tumors may benefit from the

use of systemic chemotherapy that could be considered as the first treatment option for these patients despite other clinical, histopathological and genetic characteristics.

In the attempt of delineating the minimal region of common loss of 11q, we observed that most tumors in our study showed large 11q deletions extending from bands 11q21 to 11q25. These data confirm previous studies using LOH, standard CGH and array CGH techniques that have reported the loss of large areas of 11q as a common event in breast cancer.(17, 19, 30-35) The wide region defined in our study overlaps with all these early reports. A number of candidate target tumor suppressor genes involved in breast and/or ovarian cancers are mapped to this chromosome region, including the ataxia-teleangiectasia mutated gene *ATM*,(36) the gene coding for the CHK1 kinase, which coordinates cell cycle progression and preserves genome integrity,(37) the tumor suppressor in lung cancer-1 gene *TSLC1*,(38) and the breast cancer suppressor candidate-1 gene *BCSC1*.(39) As a consequence of the chromosome deletion, we may speculate that any of these genes (or several of them) become altered in the tumor cells. Further support to the hypothesis suggesting that 11q may contain gene/s important for the suppression of tumorigenesis was provided using microcell-mediated chromosome 11 transfer into cancer cells.(40, 41) In addition to the deletion of one allele with loss-of-function mutation of the remaining allele as the causative mechanism of inactivation of the putative 11q tumor suppressor genes, the 11q loss may also act as haploinsufficient mutation, being the deletion of one chromosome enough to perturb gene/s that contribute to cancer progression through a gene-dosage effect.(42) Our studies lead us to hypothesize that the large non-random deletions of chromosome 11q are the consequence of a yet-unidentified common molecular hit occurring in tumor cells at early stages that confers chromosome instability and predisposition to regional 11q loss. Further ongoing genetic and functional studies will address these initial questions.

The bottom line of our findings is that the loss of chromosome 11q makes tumor cells responsive to anthracycline-based chemotherapy. Why 11q-deleted cells become more sensitive to chemotherapy is currently unknown. Theoretically, adjuvant systemic chemotherapy is administered to the patients to kill any remaining malignant cell (wherever they may be) after surgical removal of the tumor. Cellular DNA is the primary target for anthracyclines, by binding and inserting between DNA bases, leading to chromatin unfolding and aggregation. These chromatin structural changes primarily interfere with DNA replication and transcription, thus leading to the apoptosis undergone by the cells treated with anthracyclines.(43) The selectivity of these drugs for targeting cancer cells but not non-tumoral cells may reside in the lower ability of cancer cells to repair the damage induced by the drugs.(43) We may speculate that deletion of chromosome 11q in breast tumor cells leads to functional impairment of gene/s involved in DNA repair, thus contributing to the increased sensitivity to anthracyclines reported in our study. Indeed, we have observed that genes involved in DNA repair were enriched in the deleted 11q region with respect to the rest of the genome. Two of the most prominent candidate targets for 11q deletion in breast cancer are 1) the ataxia-teleangiectasia mutated gene *ATM* that codifies for a protein involved in DNA repair and cell cycle control;(44, 45) and 2) the cell cycle checkpoint kinase *CHK1*, that acts downstream of ATM in response to DNA damage.(37) Other candidate targets include the gene encoding for the H2AFX histone, which is critical for facilitating the assembly of specific DNA-repair complexes on damaged DNA;(46) and the gene encoding for the mitotic checkpoint protein ZW10.(47) In this theoretical scenario, 11q-deleted tumor cells that become deficient for these DNA repair genes cannot detect and/or repair DNA damage induced by anthracyclines whereas tumor cells with intact chromosome 11q and functional DNA repair proteins are able of repairing DNA efficiently, evade apoptosis, and ultimately metastasize.(48, 49) We may also suggest that deletion of 11q targets the non-coding microRNA gene *miR125b-1*, which is specifically down-regulated in breast cancer and may potentially regulate oncogenes such as *ETS1*, that plays a role in cell growth and has been shown over-expressed in breast cancer.(50)



Finally, our study could be valuable in the clinical management of patients with NNBC, by adding the 11q deletion status to the currently accepted prognostic and therapy-predictive markers.(4) According to our data, tumors should be screened for the presence or absence of 11q deletion at diagnosis using rapid quantitative PCR, FISH and/or mini-array CGH devices using a reduced set of BAC clones. These diagnostic tests should allow clinicians to prospectively identify patients who are candidates to receive anthracycline-based chemotherapy, such as standard AC/FAC, which are widely used as front-line therapies in NNBC, irrespective of other clinico-pathological features. In patients presenting factors that imply a good prognosis, such as age >35, clinical stage I, low-grade tumors sized >1 cm, and ER/PR positivity, systemic chemotherapy could be avoided only if 11q deletion is not identified. Although our study requires further validation and refinement, future application of 11q deletion measurement as a novel therapeutic indicator in NNBC is clearly challenged.

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## **TABLES**

**Table 1. Clinico-pathological features of 185 patients and tumors with NNBC.** Differences between patients treated with chemotherapy (CHEMO group) and patients who did not receive chemotherapy after surgery (non-CHEMO group).

## **SUPPLEMENTAL TABLES**

**S1.** Clinico-pathological characteristics of the 88 NNBC patients and tumors in the validation series.

**S2.** Chemotherapeutic schemes of 185 breast cancer patients.

**S3.** FISH analysis of frozen tumor samples. Correlation with array CGH results.

**S4.** Description of common regions of genomic gain and amplification, hemizygous loss and homozygous deletion in lymph node negative breast tumors. Mb position is based on UCSC Genome Browser Human May 2004 version, <http://genome.cse.ucsc.edu/>

**S5.** Correlation of abnormal BAC clones in ER+ vs. ER- tumors and in PR+ vs. PR- tumors.

**S6.** Clinico-pathological and genetic characteristics, and survival rates of patients with 11q deletion vs. those without 11q deletion. In *CCND1* and *HER2* amplification subgroups, no separate statistical analysis for CHEMO and non-CHEMO groups could be performed because of the small number of patients. NS, non significant.

## **FIGURES**

**Figure 1. Representation and description of genome-wide array CGH technique.** Genome-wide analysis of DNA-copy number changes was performed using array CGH on a microchip with ~2.460 BAC and P1 clones printed in triplicate. Briefly, 0.5  $\mu$ g of test (tumor) and reference genomic DNAs were labeled by random priming using Cy3 and Cy5, respectively. After 48 hour of hybridization, slides were washed and mounted with DAPI. The images of the arrays were captured using a CCD camera, and the "UCSF SPOT" 2.0 software (available at <http://www.jainlab.org/downloads.html>) was used to analyze the images and measure tumoral vs. control fluorescence intensity ratios that were converted to the log<sub>2</sub> scale. A second program, the "UCSF SPROC" was used to associate clones with each spot and to create a mapping information file that allows the data to be plotted relative to the position of the BACs on the draft human genome sequence (<http://genome.cse.ucsc.edu>; May 2004 freeze). A formal data filtering procedure was then performed, and a SPROC output file consisting of averaged ratios of the triplicate spots for each clone, standard deviations of the replicates and plotting positions for each clone on the array, was obtained.

**Figure 2. Representation of array CGH results of 185 NNBC.** Genomic gains and losses are depicted in green and red, respectively.

**Figure 3. Correlation of ER/PR expression status with genomic data.** **A.** The genomic gain of BAC clones mapped to 1q21-q43 and to 16p12 chromosome regions and the genomic loss of clones mapped to 16q21-q24 were associated with positive ER (marked with arrowheads). **B.** The progesterone receptor (PR) status is not significantly associated with abnormalities of 1q, 16p or 16q arms, but a borderline significance in chromosome 16 is observed. Instead, the genomic loss of clones mapped to 4p13-16 and 5q11.2-q31 were observed associated statistically with negative PR (marked with arrowheads). **C.** Frequency plot of all BAC clones in chromosome 16 comparing ER positive (C2) versus ER negative (C1) tumors. **D.** Representation of log<sub>2</sub>ratios from clones in chromosome 16 in one ER-positive breast tumor.



**Figure 4. Association of genomic results with clinical outcome in NNBC patients.** Association of genomic results with clinical outcome in NNBC patients. **A.** In the CHEMO group, after adjustment for multiple testing, none of the 2,460 BAC clones was associated with tumor relapse. **B.** In the non-CHEMO group, however, there were statistically significant differences ( $p < 0,05$ ) in 8 BAC clones, clustered to the long arm of chromosome 11, that showed more common deletion in the group of tumor recurrences. Kaplan Meier curves showed differences in disease-free survival (DFS) for 11q deleted tumors vs. those without deletion in the non-CHEMO group (DFS  $\pm$  ES at 10 years,  $40 \pm 14\%$  vs.  $86 \pm 6\%$ ,  $p < 0,0001$ ) but not in the non-CHEMO group. Considering the patients harboring deletion of chromosome 11q, five of 31 patients in the CHEMO group (16%) had recurrence of the disease whereas the relapse rate was much higher in the non-CHEMO group: 14 of 23 with 11q deletion (62%) had a relapse ( $p < 0,0001$ ). Among the 59 patients in the CHEMO group who did not show deletion of 11q, 19 (30%) presented recurrence of the disease whereas only 8 of 72 without 11q deletion (11%) in the non-CHEMO group relapsed. **C.** Representation of the region of deletion in chromosome 11q. The eight clones clustered to chromosome 11 from bands 11q23.1 to 11q24.1 are highlighted in yellow. When the adjusted value for statistical significance was of  $< 0,1$  instead of  $< 0,05$ , the number of BAC clones correlated with relapse increased to 24, all of them mapped to 11q21-q25.

**Figure 5. Correlation of 11q deletion in the test and validation series.** **A.** Bars show the comparative relapse rates of tumors with 11q vs. non-deleted in 185 NNBC patients (training set). **B.** Relapse rates in the validation set of 88 NNBC patients. **C.** The distribution of recurrences between the 11q vs. non-11q subgroups is shown in the training and validation sets. **D.** Kaplan–Meier curves show differences in DFS for the validation group, resembling the data obtained in the training set of 185 patients.

#### **SUPPLEMENTAL FIGURES.**

**S1. A.** Boxplot representing differences between presentation age in both treatment groups ( $p=0,003$ ).

Kaplan Meier curves for **(B)** Disease Free Survival (DFS) and **(C)** Overall Survival (OS) in CHEMO group and non-CHEMO group.

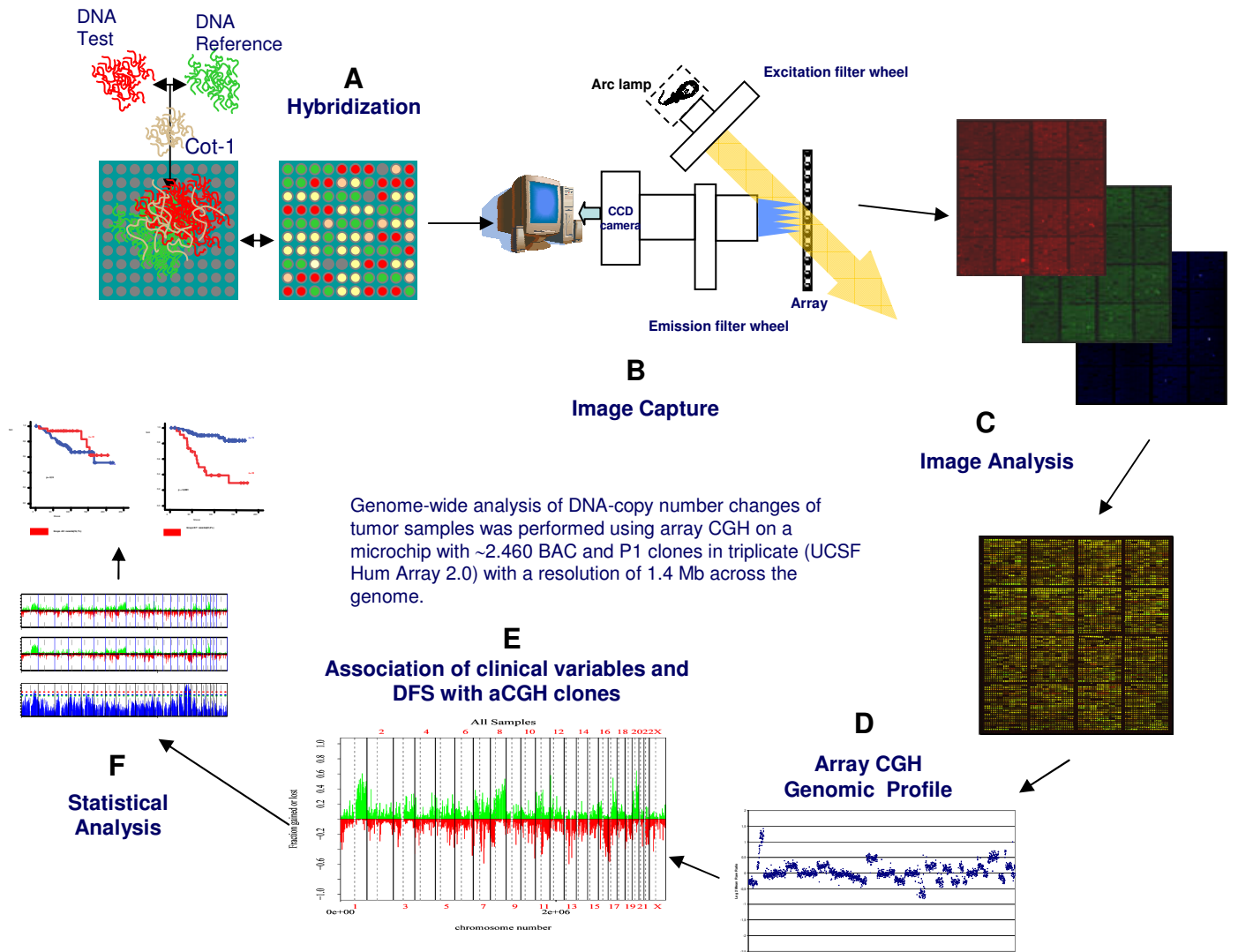
**S2.** Comparison of array CGH and CGH to metaphase chromosome techniques in 44 NNBC samples.

## **ACKNOWLEDGEMENTS**

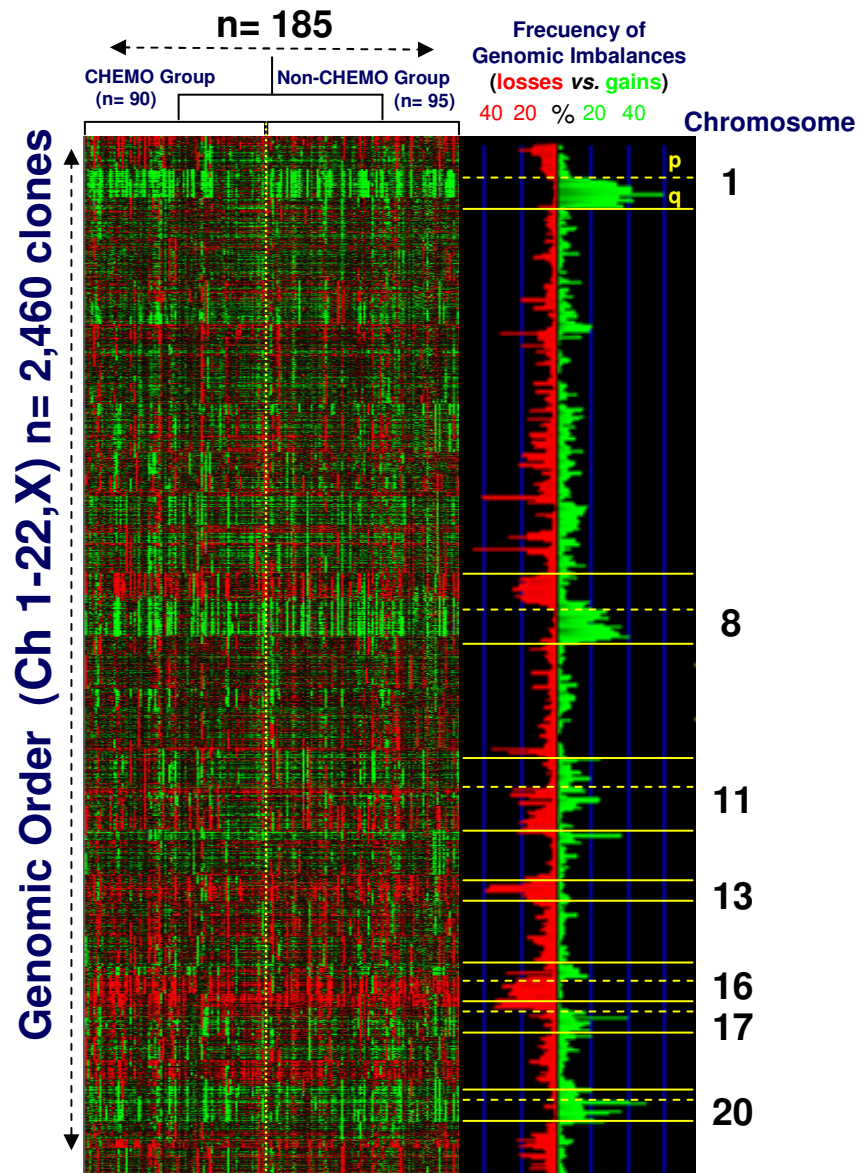
We thank Javier Arsuaga (San Francisco State University, CA, US) for his advices in mathematical analysis; Angel Rubio (Centro de Estudios e Investigaciones Técnicas de Gipuzkoa, Spain) for Gene Ontology analysis; Allan Balmain (UCSF, San Francisco, CA, US) for critical review of the manuscript; and Rick Segraves (UCSF, San Francisco, CA, US) for technical support.

Clinical Characteristics		All Patients		Adjuvant Chemotherapy (CHEMO)		No Adjuvant Chemotherapy (non-CHEMO)		p-value
		Nº	%	Nº	%	Nº	%	
Follow-up (Months)	Range Median	N = 185 9,2 - 218 82		N = 90 9 - 218 85		N = 95 11 - 174 77		NS
Recurrence		45	24%	23	26%	22	23%	NS
Age (years)	range	21 - 86		21 - 73		30 - 86		0,003
	mean	47	25%	48	25%	50	25%	
		58	50%	51	50%	67	50%	
		70	75%	62	75%	75	75%	
	<35	11	6%	9	10%	2	2%	
35-50	54	29%	32	36%	22	23%		
51-55	16	19%	13	14%	3	3%		
>55	104	56%	36	40%	68	72%		
Hormonal Status	Post-Pre- No data	115	63%	48	53%	67	70%	0,001
		55	30%	39	43%	16	17%	
		14	7%	3	4%	11	13%	
Tumoral Size	T1 < 2cm	70	39%	27	30%	43	48%	NS
	T2 2 - 5cm	100	55%	56	63%	44	48%	
	T3 >5cm	10	6%	6	7%	4	4%	
Histologic Type	Ductal Infiltr	146	79%	76	85%	70	74%	NS
	Lobulillar Infiltr	13	7%	7	8%	6	6%	
	Other	26	14%	7	7%	19	20%	
Hormonal Receptors	ER -	60	32%	33	36%	27	28%	NS
	ER+	101	55%	42	47%	59	62%	
	No data	24	13%	14	16%	9	10%	
	PgR -	65	35%	32	36%	33	35%	
	PgR+	96	52%	43	48%	53	56%	
No data	24	13%	15	16%	9	9%		
Histologic Grade	I	41	22%	13	14%	28	30%	NS
	II	90	49%	44	49%	46	48%	
	III	17	9%	12	14%	5	5%	
	No data	37	20%	21	23%	16	17%	

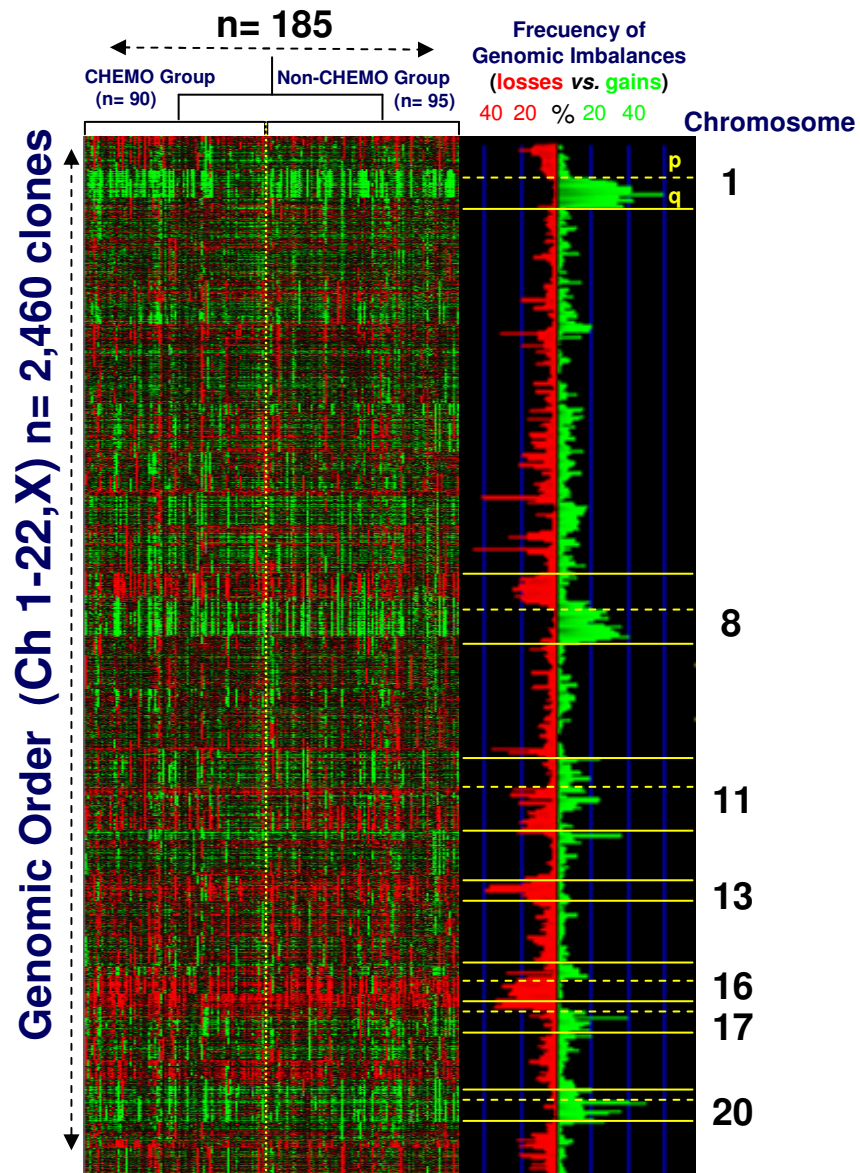
TABLE 1



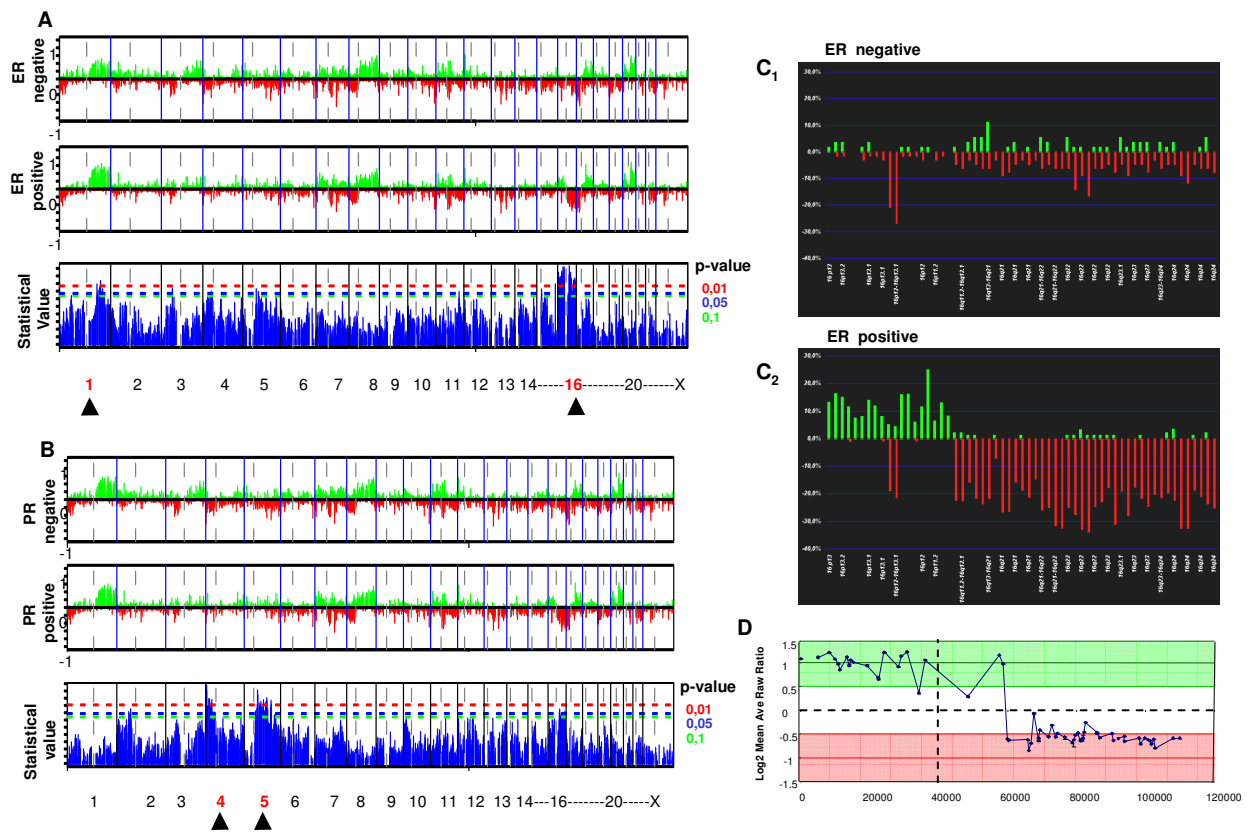
**Figure 1A**



**Figure 1B**

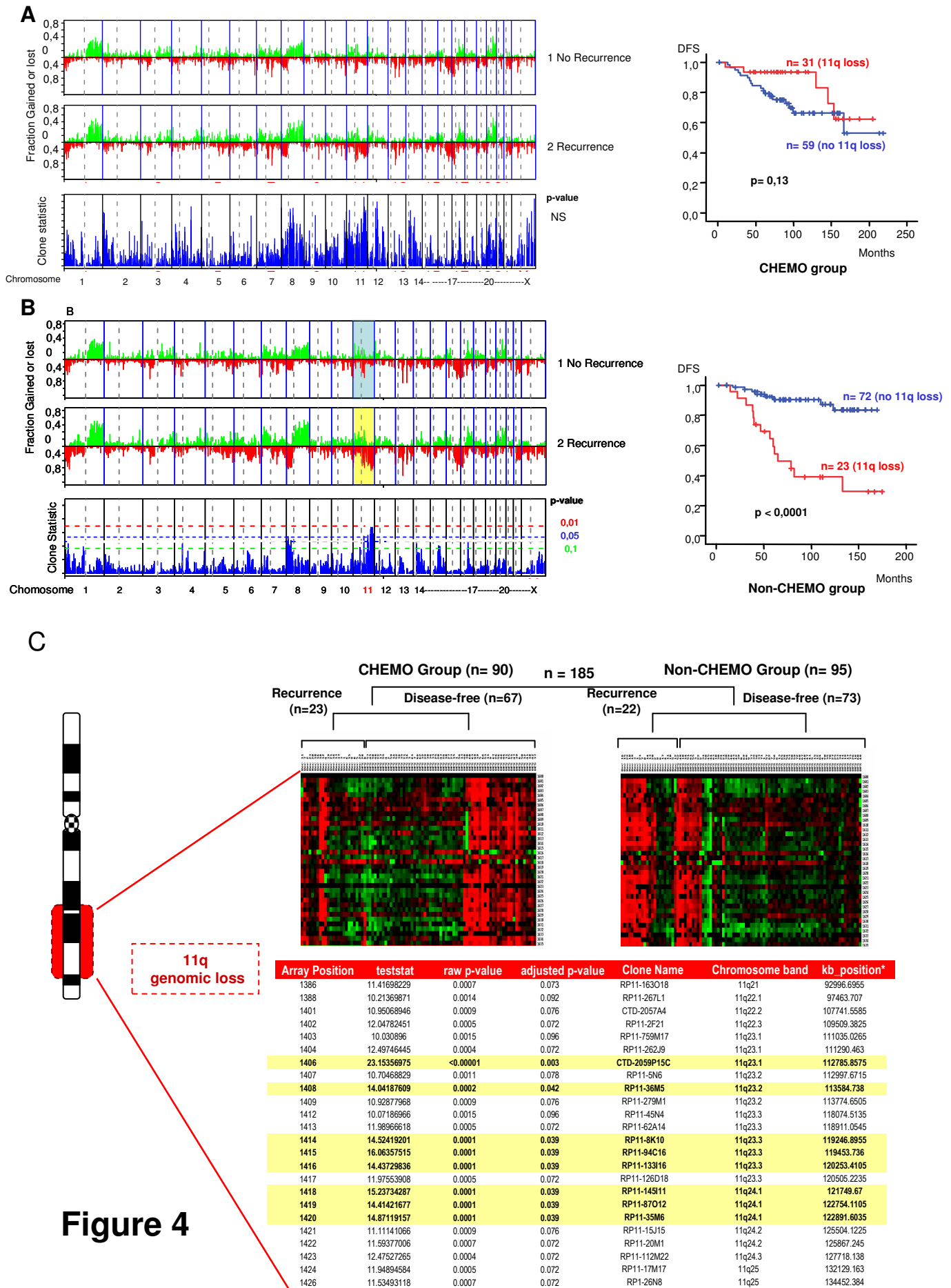


**Figure 2**



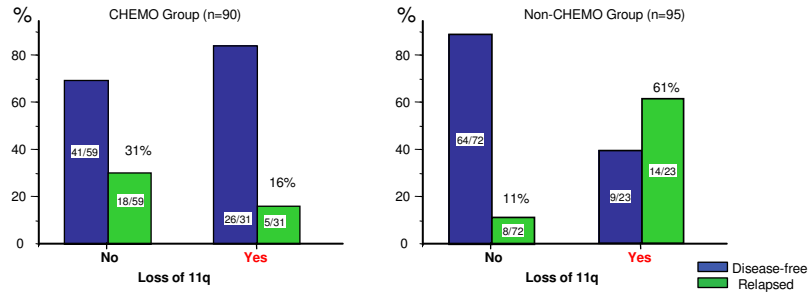
**Figure 3**



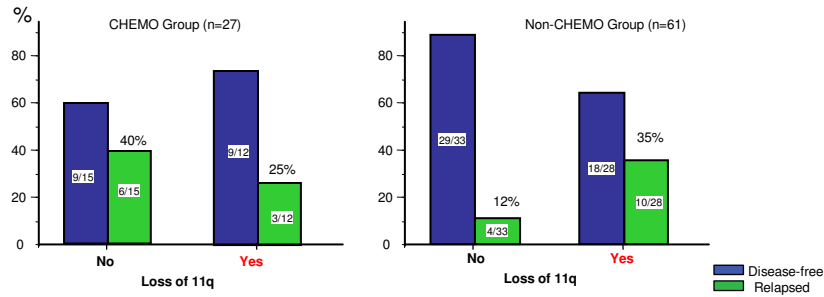


**Figure 4**

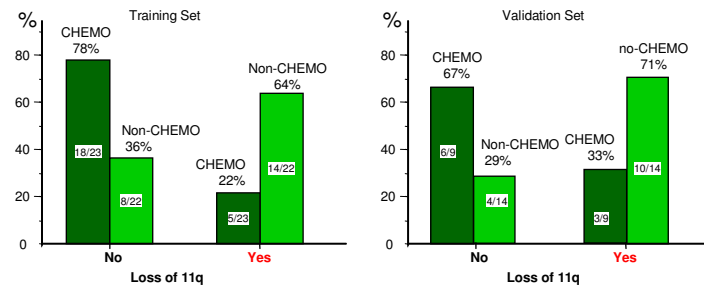
### A Training Set



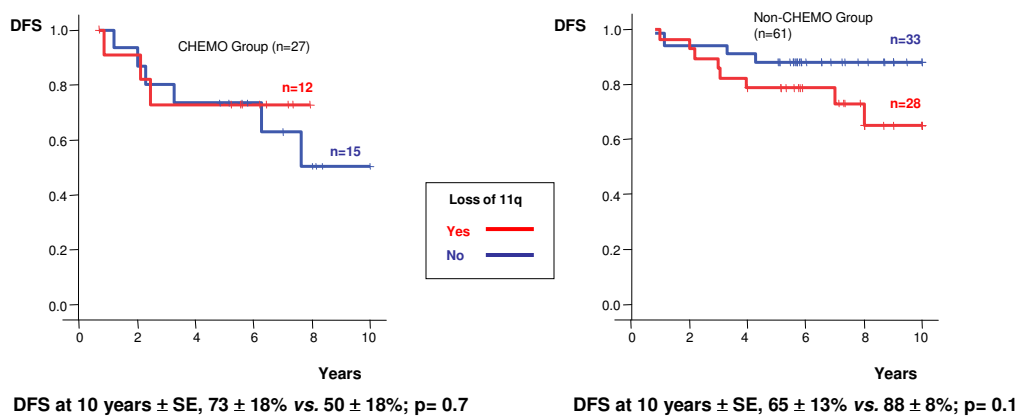
### B Validation Set



### C Recurrence groups



### D Validation Set



**Figure 5**