

## Relationship of Extreme Chromosomal Instability with Long-term Survival in a Retrospective Analysis of Primary Breast Cancer

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### Abstract

**Background:** Chromosomal instability (CIN) is thought to be associated with poor prognosis in solid tumors; however, evidence from preclinical and mouse tumor models suggest that CIN may paradoxically enhance or impair cancer cell fitness. Breast cancer prognostic expression signature sets, which reflect tumor CIN status, efficiently delineate outcome in estrogen receptor ER-positive breast cancer in contrast to ER-negative breast cancer, suggesting that the relationship of CIN with prognosis differs in these two breast cancer subtypes.

**Methods:** Direct assessment of CIN requires single-cell analysis methods, such as centromeric FISH, aimed at determining the variation around the modal number of two or more chromosomes within individual tumor nuclei. Here, we document the frequency of tumor CIN by dual centromeric FISH analysis in a retrospective primary breast cancer cohort of 246 patients with survival outcome.

**Results:** There was increased CIN and clonal heterogeneity in ER-negative compared with ER-positive breast cancer. Consistent with a negative impact of CIN on cellular fitness, extreme CIN in ER-negative breast cancer was an independent variable associated with improved long-term survival in multivariate analysis. In contrast, a linear relationship of increasing CIN with poorer prognosis in ER-positive breast cancer was observed, using three independent measures of CIN.

**Conclusions:** The paradoxical relationship between extreme CIN and cancer outcome in the ER-negative cohorts may explain why prognostic expression signatures, reflecting tumor CIN status, fail to predict outcome in this subgroup.

**Impact:** Assessment of tumor CIN status may support risk stratification in ER-negative breast cancer and requires prospective validation. *Cancer Epidemiol Biomarkers Prev*; 20(10); 2183–94. ©2011 AACR.

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### Introduction

Faithful segregation of chromosomes to daughter cells during mitosis maintains chromosome stability and a diploid genome. Altered mitotic spindle assembly checkpoint function, centrosome duplication, kinetochore function, and microtubule stability have been implicated in chromosome missegregation and the ensuing pattern of chromosomal instability (CIN) in cancer model systems (1).

The poor prognosis associated with CIN in solid tumors, including breast cancer (2–4), may relate to the ability of CIN tumors to adapt more readily to environmental or stromal pressures, owing to intratumor heterogeneity and diversity among cancer cells (5–7). Consistent with the proposal that CIN confers adaptive advantage, in murine lung tumors induced by activated KRAS expression, aneuploidy caused by transient MAD2 overexpression promotes rapid tumor relapse following withdrawal of the activated KRAS stimulus (8) and

aneuploidy induction in yeast generates phenotypic variation driving survival under adverse conditions such as drug exposure (9).

However, aneuploidy may also slow the growth of yeast and mammalian cells, and CIN is poorly tolerated by cancer cells when induced through spindle assembly checkpoint inactivation or multipolar mitoses (10–14). Accordingly, elevation of the frequency of chromosome missegregation has been proposed as a strategy to kill tumor cells (15). In summary, preclinical evidence indicates that CIN can have a negative impact upon cancer cell viability, suggesting that extreme CIN *in vivo* may prove both detrimental to cancer cell biological fitness and unsustainable for tumor progression (6).

Identification of methods to predict breast cancer clinical outcome, particularly in estrogen receptor ER-negative disease, are of paramount importance to identify patients at higher risk of relapse who may therefore require more aggressive adjuvant therapy. In this regard, prognostic gene expression approaches, such as Oncotype DX and MammaPrint<sup>®</sup>, which have utility in ER-positive breast cancer, have limited prognostic value in ER-negative disease (16).

Intriguingly, evidence is beginning to emerge that these prognostic expression signatures also reflect breast cancer CIN status as measured by DNA image cytometry-based techniques (17). Conceivably, the combined observations that prognostic expression signatures reflect tumor CIN status but fail to predict outcome in ER-negative breast cancer may be related to a paradoxical relationship between CIN and clinical outcome in ER-negative disease, whereby intermediate and extreme CIN are associated with poor and good prognosis, respectively. Thus, as prognostic signature expression increases, reflecting increased CIN, instead of prognosis worsening, paradoxically, outcome might be improved because of a negative impact of genome instability upon cancer cell biological fitness. Conversely, the robust prognostic relationship of gene signature expression with outcome in ER-positive disease might imply that the association of CIN with risk of death might approximate to a linearly increasing relationship in this subtype.

To test this hypothesis and assess whether extreme CIN might be associated with improved clinical outcome in ER-negative breast cancer in contrast to ER-positive breast cancer, we identified a tissue microarray (TMA) cohort of 246 primary breast carcinomas with associated clinicopathologic details and survival outcome data. Using dual centromeric FISH analysis and standard approaches based on deviation from the modal chromosomal signal (18, 19) and a measure of tumor cell and ecological clonal heterogeneity, the Shannon Diversity Index (SDI; ref. 20), we have estimated the degree of numerical chromosomal heterogeneity within each of these tumors and explored the relationship between tumor CIN and clinical outcome in ER-negative and ER-positive breast cancer. We then test the concordance of our findings using 2 further methods of CIN determi-

nation by comparative genomic hybridization (CGH) and CIN70 expression in a meta-analysis of 832 ER-positive and 356 ER-negative breast cancer patients.

## Methods

### TMA cohort

Archival paraffin-embedded breast cancer tissue was obtained from 246 patients diagnosed at Leeds Teaching Hospitals NHS Trust between 1983 and 1997. TMAs were constructed containing cores, 0.6 mm in diameter and 4  $\mu$ m in thickness, selected from representative tumor areas as determined by a consultant breast histopathologist (A. M. Hanby) from hematoxylin and eosin-stained sections. Full clinicopathologic data were available (See Table 1). Ethical approval was obtained (Leeds East 05/Q1206/136).

### FISH analysis

Centromeric FISH allows an assessment of intercellular heterogeneity of chromosome number in contrast to other techniques such as array CGH (aCGH). Dual color FISH was carried out using 2 centromeric probes (CEP2 and CEP15 labeled with spectrum orange and spectrum green, respectively; Abbott Laboratories) using standard procedures. These chromosomes were selected on the basis of infrequent copy number alterations in a series of breast tumors analyzed by 1 Mb aCGH analysis in our laboratory in order that aberrations would more closely reflect underlying chromosomal numerical instability rather than structural CIN resulting from intrachromosomal rearrangements. Briefly, following dewaxing, the TMA slides were placed in SPoTLight Pretreatment buffer (Invitrogen) at 98°C for 15 minutes and then washed. Three hundred microliters of digestion enzyme was added to each slide, left for 4 minutes at room temperature, and then washed. A solution of 1.5  $\mu$ L of centromeric probes was mixed and diluted to give a final volume of 10  $\mu$ L per slide. Slides were incubated overnight at 37°C. After washing, slides were stained and mounted using DAPI antifade (Invitrogen). Slides were scanned and FISH images captured using a 40 $\times$  objective on the Applied Imaging Ariol System (Applied Imaging), with seven 0.5- $\mu$ m z-stacks. Fifty nuclei per core showing clear and discrete hybridization signals for both chromosomes were scored manually.

### Gene expression data analysis

We obtained raw microarray expression data for 13 publicly available breast cancer cohorts (21–31) and GSE2109 and GSE16446, representing 2,125 individual patients. In addition, we obtained gene expression data from 3 ovarian cancer cohorts (32–34), 2 squamous non-small-cell lung carcinoma (NSCLC) cohorts (33, 35), and 1 gastric cancer cohort (36).

ER status was inferred by *k*-medoids clustering of the Affymetrix probeset 205225\_at representing the *ESR1* gene (37). CIN70 scores were calculated as the mean

**Table 1.** Clinicopathologic characteristics of the Leeds TMA cohort and distribution of histopathologic parameters for all patients across MCD cohorts

	Whole cohort	MCD1	MCD2	MCD3	MCD4	<i>P</i>	adjusted <i>P</i>
Number of patients	246	77	77	59	33		
Age, y							
Median	58	61	57	58	57		
Range	29–92	31–91	35–89	29–92	33–80		
Age (%)						0.1	0.40
>50 y	170 (69)	60 (78)	50 (65)	39 (66)	21 (64)		
≤50 y	76 (31)	17 (22)	27 (35)	20 (34)	12 (36)		
Grade (%)							
1	46 (18)	17 (22)	19 (25)	8 (13)	2 (6)	<b>0.03<sup>a</sup></b>	0.15
2	93 (38)	37 (48)	26 (34)	17 (29)	13 (39)	0.12	0.40
3	106 (43)	23 (30)	31 (40)	34 (58)	18 (55)	<b>0.001<sup>a</sup></b>	<b>0.007<sup>a</sup></b>
Unknown	1 (1)	0 (0)	1 (1)	0 (0)	0 (0)		
ER status (%)						<b>0.02<sup>a</sup></b>	0.12
ER <sup>-</sup>	53 (21)	13 (17)	12 (16)	17 (29)	11 (33)		
ER <sup>+</sup>	192 (78)	63 (82)	65 (84)	42 (71)	22 (67)		
Unknown	1 (1)	1 (1)	0 (0)	0 (0)	0 (0)		
ER/HER2 status (%)							
ER <sup>-</sup> /HER2 <sup>-</sup>	41 (16)	8 (10)	11 (15)	15 (26)	7 (21)		
ER <sup>-</sup> /HER2 <sup>+</sup>	12 (5)	5 (7)	1 (1)	2 (3)	4 (12)		
ER <sup>+</sup> /HER2 <sup>+</sup>	11 (4)	2 (3)	4 (5)	5 (9)	0 (0)		
ER <sup>+</sup> /HER2 <sup>-</sup>	180 (73)	61 (79)	61 (79)	36 (61)	22 (67)		
Unknown	2 (1)	1 (1)	0 (0)	1 (1)	0 (0)		
Node (%)						0.69	1.0
No	118 (48)	38 (49)	34 (44)	32 (54)	14 (42)		
Yes	116 (47)	35 (46)	37 (48)	25 (43)	19 (58)		
Unknown	12 (5)	4 (5)	6 (8)	2 (3)	0 (0)		
Size, cm (%)						0.64	1.0
0–2	117 (47)	36 (47)	38 (50)	30 (51)	13 (39)		
2–5	100 (41)	30 (39)	27 (35)	25 (42)	18 (55)		
≥5	20 (8)	8 (10)	8 (10)	3 (5)	1 (3)		
Unknown	9 (4)	3 (4)	4 (5)	1 (3)	1 (3)		

NOTE: *P* values: Cochran–Armitage trend tests with multiple testing corrections.

<sup>a</sup>Significant results suggest a trend in proportions of histopathologic parameters across MCD cohorts (e.g., increasing proportions of ER-negative patients across MCD cohorts). Each particular tumor grade is tested against the remaining 2 grade types. Tumors with size greater than 2 cm are tested against tumors between 0 and 2 cm.

expression of the 70 genes of the CIN70 signature (2). To combine the breast cancer cohorts, CIN70 scores were centered within each cohort and then divided by the SD. All samples were stratified into CIN70 quartiles on the basis of CIN70 scores of all tumors of the given cancer type. For the analysis of MammaPrint<sup>®</sup> and Genomic Grade Index (GGI), the mean of the expression values of all genes present in the particular expression signature multiplied by 1 or -1 to represent expression direction was used as a score.

#### Single-nucleotide polymorphism-based measurements of structural complexity

Publicly available single-nucleotide polymorphism (SNP) data based on the Affymetrix 100k platform

(38), representing 281 breast tumor specimens with paired expression data (22), were acquired from Gene Expression Omnibus. We determined the Genome Integrity Index (GII) as described (39). To determine the total number of DNA break points, we counted the number of DNA segments with an inferred log<sub>2</sub> ratio of greater/less than ± 0.3. The total number of LOH regions were inferred as described (40). To determine a combined structural complexity score composed of GII/number of break points/LOH score, we linearly transformed each set of scores, with no aberrations being assigned to 0 and the highest number of aberrations assigned a value of 1. We defined the combined structural complexity as the mean of the 3 transformed scores.

## Statistical analysis

For the breast cancer expression and SNP data cohorts and the gastric cancer cohort, survival analysis was conducted with time to relapse or, if not available, time to distant metastasis as outcome variable. For the ovarian and squamous lung cohorts, the outcome variable was overall survival. To derive a CIN score based on centromeric signals, the number of centromeres in 50 nuclei were counted for chromosomes 2 and 15. The mean (chromosomes 15 and 2) percentage deviating from the modal centromere number was used to define 4 CIN score groups (Modal Centromere Deviation groups 1–4: MCD1, 0%–15%; MCD2, 15%–30%; MCD3 30%–45%; and MCD4, >45%) with similar range. To avoid false classification of CIN due to sectioning artifacts and to control for bimodality in diploid tumors, all centromere counts equal to 1 were removed for the derivation of the CIN score. To confirm the validity of this approach to identify tumors with the most extreme CIN, we conducted 2 independent validations. The MCD score was compared with the measure of clonal heterogeneity, the SDI, where centromere counts equal to 1 were included. The SDI ( $H$ ; ref. 20) was estimated for chromosomes 15 and 2 using the formula:

$$H = - \sum_i p_i \ln(p_i)$$

where  $p_i$  is the frequency of centromere signal,  $i$ . We also analyzed a small cohort of normal breast tissue which confirmed including chromosome counts of 1 would result in an overestimation of the percentage deviating from the modal centromere number (unpublished data). The survival analysis for the centromeric FISH data was conducted with time to breast cancer-specific death as outcome variable. A robust Cox proportional hazards (R package *coxrobust*) regression model with a quadratic weighting function was used to conduct a multivariate Cox regression for all ER-negative patients. Cox proportional hazards regression models were conducted for the univariate survival analysis of MCD4 and follow-up times were censored at 100 months.  $P$  values for univariate analysis were estimated by a log-rank statistic. All statistics were conducted in R version 2.11.1. All  $P$  values are 2 sided. All statistical calculations and R scripts can be found in the Supplementary Sweave document.

## Results

### Distribution of MCD in primary breast cancer

To address the frequency of directly quantified tumor CIN in ER-negative compared with ER-positive breast cancer and identify the relationship of CIN with clinical outcome in ER-negative breast cancer, we assessed tumor CIN by centromeric FISH analysis in a TMA cohort of 246 primary breast cancers diagnosed at Leeds Teaching Hospitals NHS trust between 1983 and 1997 (Table 1 and Fig. 1A). Primary breast cancers from this cohort

reflected the diversity of histologic tumor grade, tumor size, nodal involvement, and ER and HER2 receptor status encountered in common clinical practice. Patients received conventional (neo)-adjuvant chemotherapy at the time, consisting of predominantly CMF (cyclophosphamide, methotrexate, and 5-fluorouracil) or FEC (5-fluorouracil, epirubicin, and cyclophosphamide). No patients received either taxane-based chemotherapy or, if HER2-positive, trastuzumab, as this cohort preceded the dates of introduction of these agents. Radiotherapy was given following breast conserving surgery. All ER-positive patients received 5 years of tamoxifen.

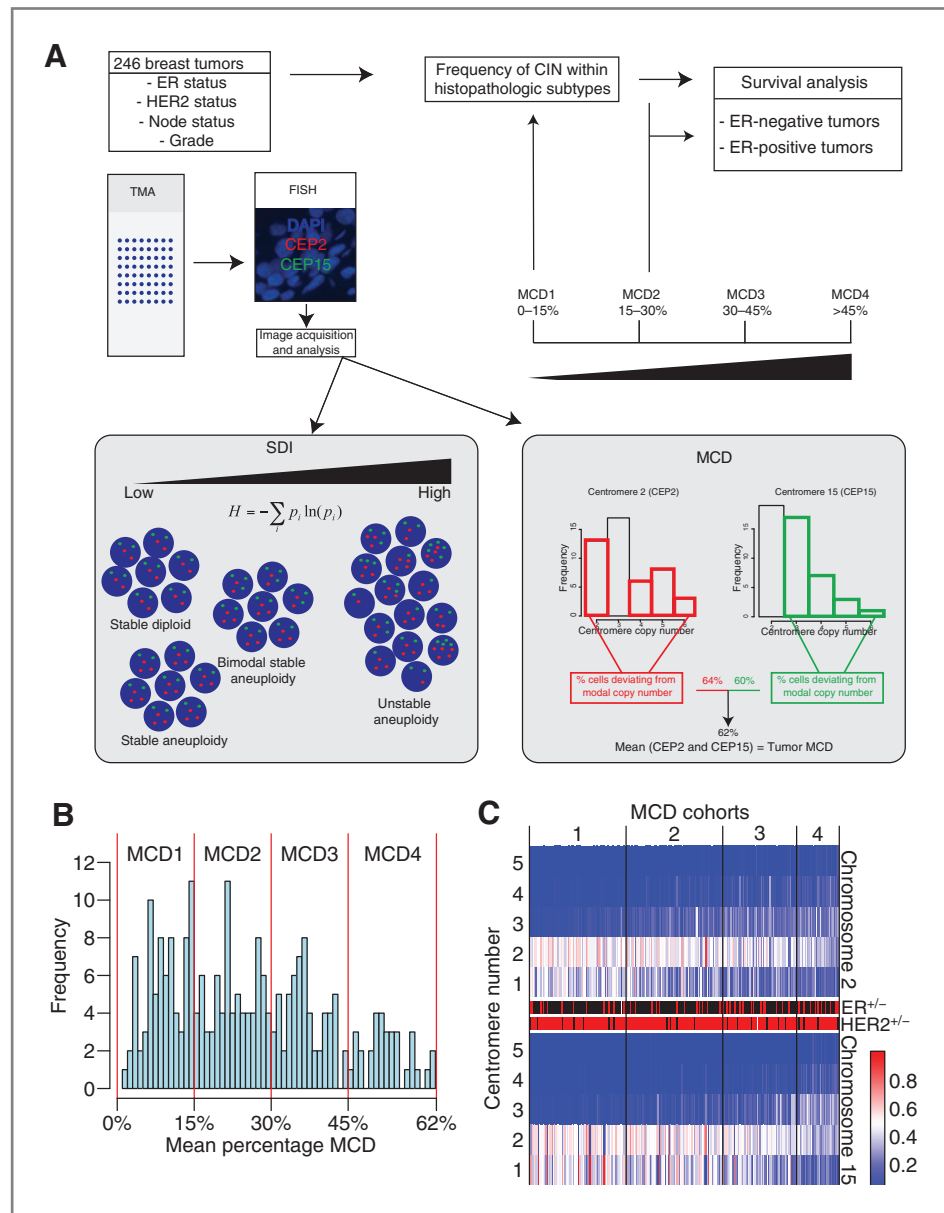
Studies have previously shown that the use of FISH probes for 2 chromosomes is sufficient to segregate diploid from aneuploid tumors (41, 42). This technique also enables an estimation of clonal heterogeneity within the tumor and allows the differentiation between CIN (aneuploid tumors with high clonal heterogeneity) and stable aneuploidy (low clonal heterogeneity; refs. 18, 43). Therefore, we used centromeric FISH analysis of chromosomes 2 and 15 to assess the mean percentage of nuclei within each tumor deviating from the modal centromere number, using established methods (18, 41).

The percentage of nuclei with centromeric signals deviating from the centromere mode was assessed for each of the 246 tumors to establish the range of tumor numerical CIN across the cohort. We calculated the percentage of nuclei deviating from the modal centromeric signal for chromosomes 2 and 15 separately. A summary measure, the average MCD, was assessed by calculating the mean percentage of nuclei that deviated from the mode for both centromeric probes within each tumor (Fig. 1A). We separated tumors into 4 groups of increasing numerical CIN based on increasing MCD (MCD groups 1–4: MCD1, 0.0%–15%; MCD2, 15%–30%; MCD3, 30%–45%; and MCD4, >45%; Fig. 1A and B) and displayed tumors according to the distribution of centromeric signals, HER2, and ER status (Fig. 1C and Supplementary Fig. S1). The MCD4 cohort was classified as a MCD score greater than 45%, concordant with the "unstable aneuploidy" definition previously defined by Ling and colleagues in an analysis of 20 breast tumors (18).

### Relationship between tumor MCD and clonal heterogeneity

Deviation from the modal centromere number provides an estimate of tumor CIN (18). However, this measure may similarly classify unstable aneuploid tumors with high clone heterogeneity (e.g., 60% of nuclei with 2 centromeres and 25% with 3, 5% with 4, and 10% with 5 centromeric signals) together with stable aneuploid tumors with relatively few clones making up a large proportion of the tumor (e.g., 60% nuclei with 2 centromeres and 40% nuclei with 3 centromeres). To more directly assess clone heterogeneity within each tumor and assess whether the MCD4 cohort represents the tumors with the most extreme CIN, we calculated the SDI that integrates both the number and abundance of

**Figure 1.** Distribution of average MCD across the Leeds primary breast cancer cohort. **A**, flowchart of experimental procedures and breast cancer cohort CIN analysis. **B**, histogram distribution of the percentage of nuclei with deviation from the modal centromere signals and allocation of MCD1 to MCD4 cohorts. The y-axis shows the frequency of the MCD score. The MCD scores were grouped into 4 cohorts with similar range. **C**, heatmap of centromeric distribution for chromosomes 2 and 15. Each column represents 1 tumor ranked according to MCD scores, with corresponding ER status: ER negative (red) and ER positive (black); HER2 status: HER2 negative (red) and HER2 positive (black). The rows of the heatmap indicate the centromere number of each chromosome per nucleus and colors the percentage of nuclei having the particular centromere number (blue, 0%; red, 100%). For diagrammatic purposes, the figure only portrays centromere counts between 1 and 5.



tumor clones within each tumor across the cohort according to published methods (20). Consistent with a close relationship of MCD with clonal heterogeneity in breast cancer, there was a highly significant correlation between MCD and the SDI for chromosome 2 (Fig. 2A; Pearson's correlation coefficient = 0.941,  $P < 0.0001$ ) and chromosome 15 (Fig. 2B; Pearson's correlation coefficient = 0.902,  $P < 0.0001$ ). These data suggest that tumors in the MCD4 cohort have the highest SDI and thus the greatest clonal heterogeneity (Fig. 2C; Student's  $t$  test,  $P < 0.0001$ ), indicating that these tumors have the most extreme chromosomal numerical heterogeneity (Supplementary Fig. S2A and B) and, within the experimental limitations of our analysis of paraffin-embedded tumor microarrays, can be designated

extreme CIN (MCD4 representative images; Fig. 2D). To assess the concordance of CIN status defined by MCD cohort with fluorescence-activated cell sorting FACS-based measures of DNA index, through a parallel analysis of 29 tumors for which FISH data and DNA index data derived from FACS analysis were present, we confirmed a significant enrichment of MCD3 and MCD4 cohorts within the aneuploid tumors classified by FACS (Fisher's exact test,  $P = 0.014$ ; data not shown).

**ER-negative breast cancers display significantly greater numerical CIN**

Previous evidence from a DNA image cytometry analysis of 48 breast cancers showed that higher risk ER-negative breast cancer subtypes were more frequently

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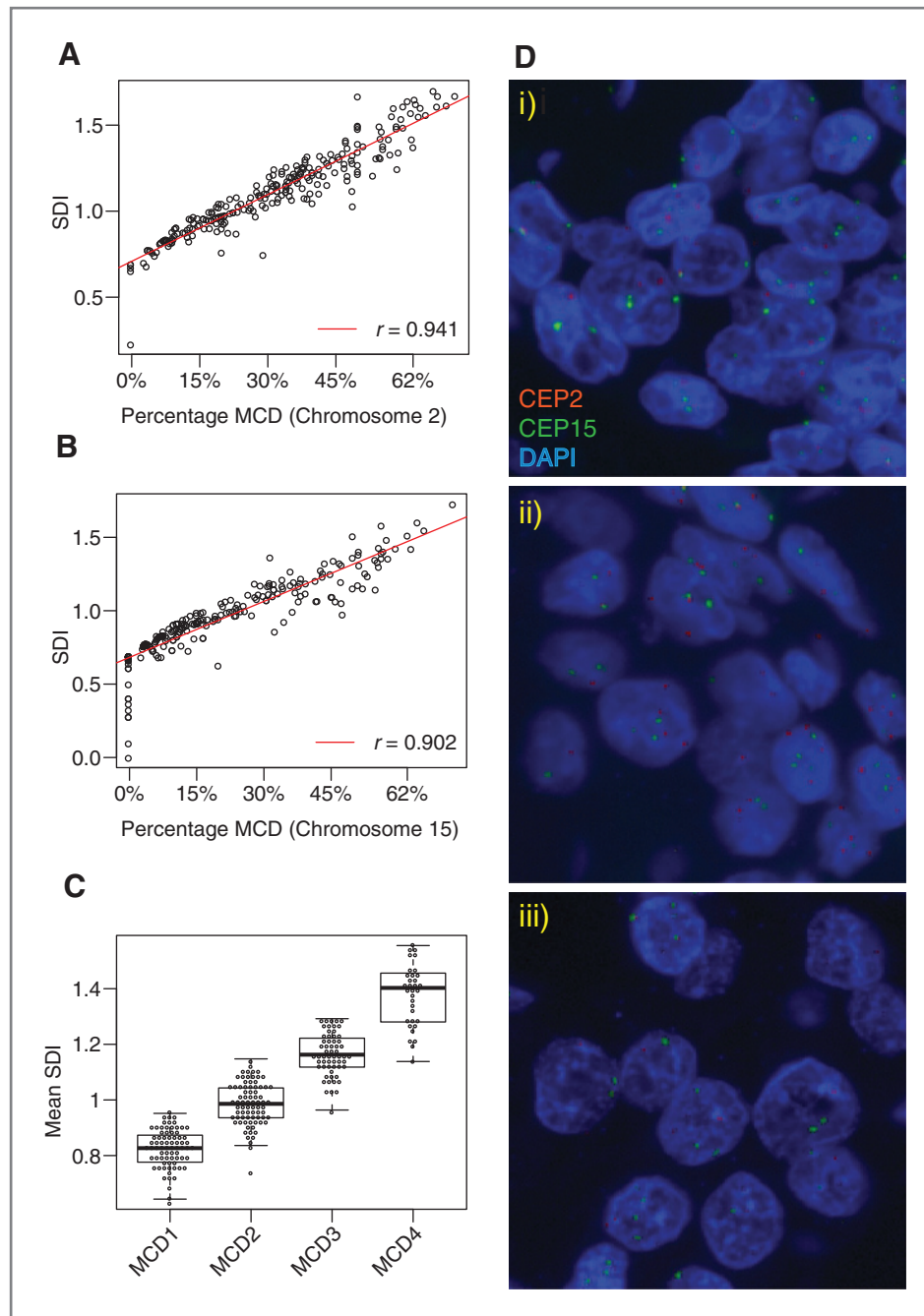


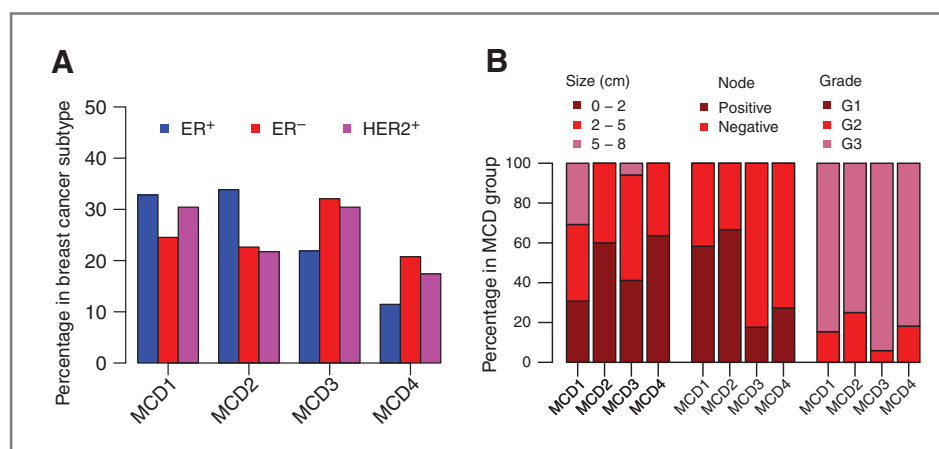
Figure 2. Relationship of CIN scores to SDI: correlation of MCD with SDI for chromosome 2 (A) and chromosome 15 (B). C, the MCD4 extreme CIN cohort has the highest median (chromosomes 15 and 2) SDI. D, representative images of MCD4 cohort tumors. Centromeric probes of chromosomes 2 and 15 are labeled in red and green, respectively. DAPI, 4',6-diamidino-2-phenylindole.

chromosomally unstable than lower risk ER-positive breast cancers (17). Consistent with these data, we noted significant differences in the proportion of ER-negative compared with ER-positive subtypes between the 4 MCD groups ( $P = 0.02$ ; Cochran–Armitage trend test), with overrepresentation of ER-negative compared with ER-positive tumors within the MCD3 and the extreme CIN MCD4 cohorts (Fig. 3A and Table 1). After correcting for multiple testing across all histopathologic subtypes, we noted a significant difference in the proportion of grade 3

tumors between the 4 MCD groups ( $P = 0.007$ ; Cochran–Armitage trend test), with overrepresentation of grade 3 tumors in the higher MCD cohorts 3 and 4 (Table 1). Neither node-positive ( $P = 0.69$ ) nor larger ( $P = 0.64$ ) tumors appeared to show significant differences between the MCD cohorts.

In contrast, in ER-negative tumors, MCD3 and MCD4 cohorts were significantly more likely to be node negative than tumors in MCD1 and MCD2 cohorts (Fig. 3B; Fisher’s exact test,  $P = 0.004$ ). Tumors were not significantly

**Figure 3.** Relationship of histopathologic parameters to MCD cohort. A, distribution of ER and HER2 status across each MCD cohort. The frequency of ER<sup>-</sup> (including HER2<sup>+/-</sup> tumors), ER<sup>+</sup> (including HER2<sup>+/-</sup> tumors), and HER2<sup>+</sup> (including ER<sup>+/-</sup> tumors) breast cancers within each MCD cohort. B, relationship of histopathologic parameters with MCD cohort in ER-negative breast cancer.



larger or of higher grade in MCD3 and MCD4 than in MCD1 and MCD2 cohorts (Fig. 3B).

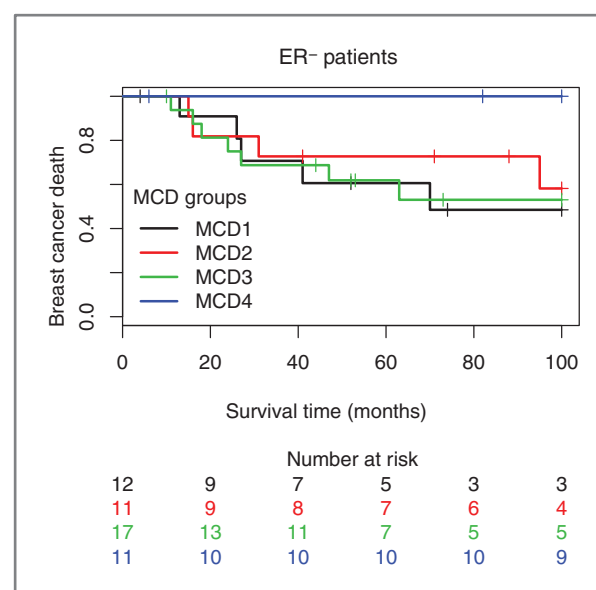
**Relationship of extreme CIN with breast cancer clinical outcome**

Preclinical observations showing that CIN can negatively impact upon tumor biological fitness and the limited utility of prognostic expression signatures in ER-negative breast cancer, such as MammaPrint<sup>®</sup> and Oncotype DX which may also reflect tumor CIN status (17), led us to investigate whether ER-negative tumors with extreme CIN might be associated with a better outcome relative to the rest of the cohort or tumors with intermediate levels of CIN.

Consistent with a positive prognostic relationship between CIN and breast cancer outcome, patients with ER-negative breast cancers in the extreme CIN MCD4 cohort had a significantly better breast cancer survival than patients in the intermediate and lower MCD cohorts (Fig. 4; log-rank test,  $P = 0.014$ ) and specifically in a comparison with the MCD3 intermediate cohort alone ( $P = 0.015$ ). To determine that these results do not simply reflect the diversity of treatment given to this cohort, we restricted our analysis to patients treated with adjuvant chemotherapy only. Significantly improved outcome was also seen in the extreme CIN MCD4 cohort when the analysis was repeated in patients treated with adjuvant chemotherapy (Supplementary Fig. S3;  $P = 0.04$ ). In a multivariate model including MCD4, tumor grade, HER2 status, chemotherapy exposure, size, and nodal status, MCD4 was a significant independent positive prognostic variable in ER-negative breast cancer (Table 2;  $P = 0.02$ ). When repeating the multivariate analysis based on MCD by using the mean SDI to separate the ER-negative breast cancers into 4 increasingly clonally heterogeneous tumor groups with equal range, consistent with the results of the MCD approach, the most extreme CIN group (SDI4) was also a significant positive predictor of outcome in multivariate analysis (Supplementary Table S1;  $P = 0.005$ ).

**Concordance of CIN with prognostic expression signatures in ER-negative and ER-positive breast cancer**

Breast cancer gene expression signatures, such as MammaPrint<sup>®</sup>, prognosticate in ER-positive but not in ER-negative breast cancer. We therefore hypothesized that the association between CIN and outcome in ER-positive breast cancer may approximate to a linear rather than a nonmonotonic relationship as witnessed in ER-negative breast cancer. For this reason, we assessed the relationship of CIN with clinical outcome in an independent cohort of 356 ER-negative patients compared with 832 ER-positive breast cancer using 2 further measurements of CIN, the



**Figure 4.** Association of MCD cohort with breast cancer death. Kaplan-Meier survival curves for the 4 MCD cohorts across all ER-negative breast cancer patients. The y-axis shows breast cancer-specific survival probabilities and the x-axis shows survival time in months.

**Table 2.** Multivariate analysis of prognostic variables in ER-negative breast cancer with CIN grouped by MCD

	HR	95% CI		P
		Lower	Upper	
Size	1.1051	1.0238	1.1193	<b>0.0103<sup>a</sup></b>
Grade	14.7902	1.7581	124.4247	<b>0.0132<sup>a</sup></b>
HER2 status	7.1390	1.3560	37.5875	<b>0.0204<sup>a</sup></b>
CIN, extreme (MCD4)	0.0827	0.0097	0.7066	<b>0.0228<sup>a</sup></b>
Chemotherapy	1.8154	0.5743	5.7386	0.3099
Node status	1.6023	0.4673	5.4942	0.4533

NOTE: Analysis results of the multivariate robust Cox proportional hazard regression for all ER-negative patients with covariates size, grade, HER2 status, chemotherapy, node status, and MCD4 versus all other MCD cohorts taken together. The table shows the HR, upper and lower 95% CIs, and *P* values for each covariate. The covariates are ranked by *P* values.

<sup>a</sup>Significant covariates.

CIN70 expression signature and an aCGH-based structural complexity score (reported by our group in ref. 44). In each case, we compared the results to our centromeric FISH analysis using centromeres 2 and 15 in the Leeds cohort of 53 patients with ER-negative and 192 patients with ER-positive breast cancer (Fig. 5).

Patients with ER-negative breast cancer in the extreme CIN (fourth quartiles) have improved outcome across the 3 measures of CIN (Fig. 5A). The same relationship was observed with ovarian, gastric, and NSCLC where the extreme CIN (fourth quartile determined by CIN70 expression) is associated with improved prognosis (Supplementary Fig. S4). Consistent with the concordance of breast cancer prognostic signatures with breast cancer CIN status as reported by Habermann and colleagues (17), a similar relationship was observed in ER-negative breast cancer using MammaPrint<sup>®</sup> and the GGI, where fourth quartile expression was associated with relatively improved outcome compared with intermediate expression quartiles (Fig. 5B).

Given the ability of prognostic signatures to both mirror tumor CIN status (17) and predict outcome in ER-positive breast cancer, we hypothesized that in contrast to ER-negative breast cancer, there would be a near linear relationship between CIN and breast cancer outcome. Accordingly, in ER-positive breast cancer, the extreme CIN fourth quartile was associated with the worst prognosis across all 3 measurements of CIN, including direct CIN assessment using CEP2 and CEP15 in the independent Leeds cohort of 192 patients (Fig. 5C), concordant with the HR for the quartiles of MammaPrint<sup>®</sup> and GGI expression (Fig. 5D).

In summary, the MCD4 cohort is associated with the most extreme clonal heterogeneity and CIN and relatively enriched with ER-negative breast cancers. Consistent with evidence suggesting there is a negative impact of aneuploidy on cell biological fitness, patients

with ER-negative breast cancer in the extreme CIN MCD4 cohort have a significantly improved survival outcome in multivariate analysis. In contrast, consistent with the ability of prognostic expression signature sets to reflect CIN status and define outcome in ER-positive in contrast to ER-negative breast cancer, increasing CIN status, defined by MCD, CIN70 expression, or aCGH-based measurements of structural chromosome complexity, appears to be associated with increased risk of cancer death in ER-positive but not in ER-negative breast cancer.

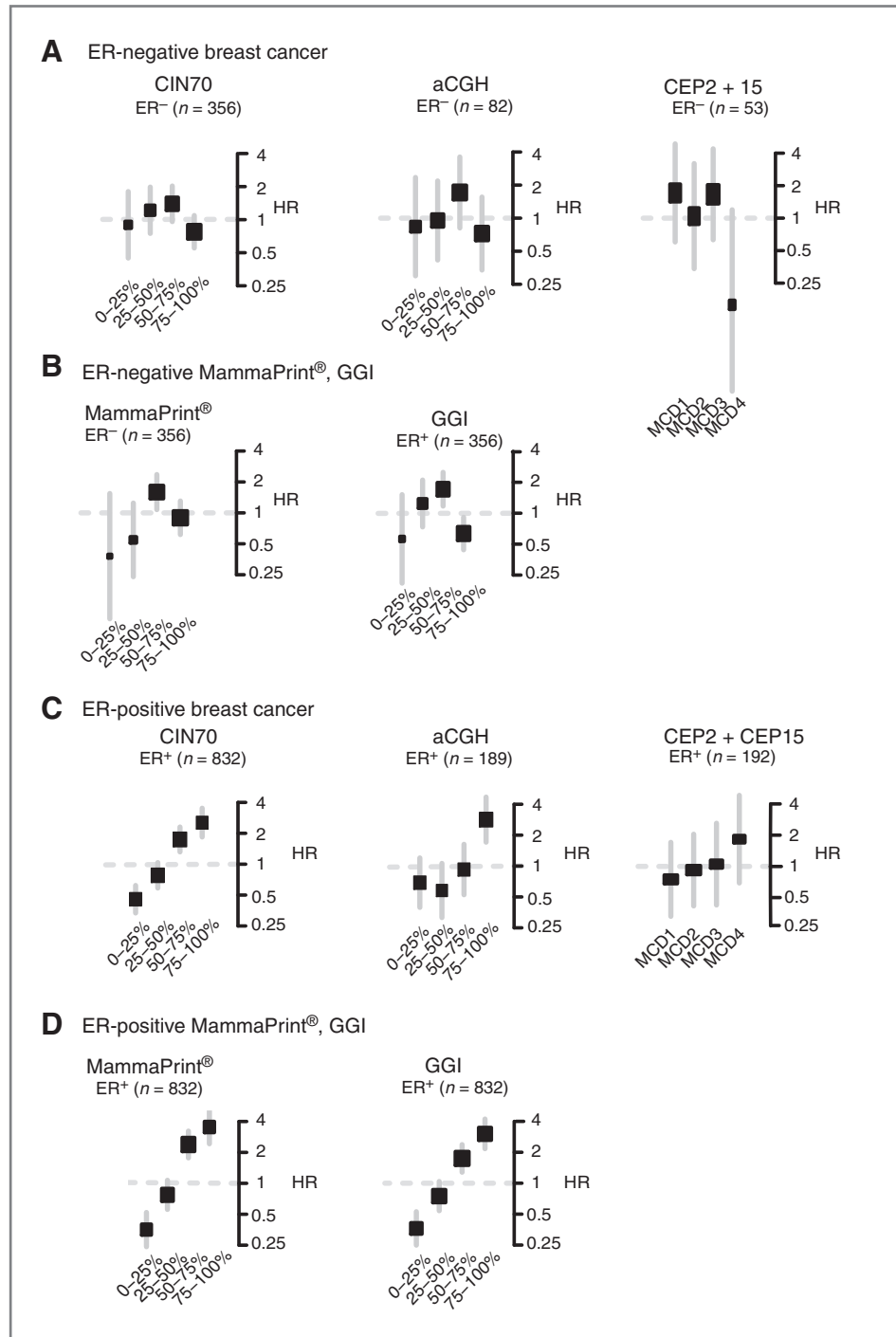
## Discussion

Developments in breast cancer prognostication over the last 10 years have provided gene expression-based approaches to assess risk of tumor relapse in the adjuvant setting, such as the Oncotype DX recurrence score and MammaPrint<sup>®</sup>, that are applicable to patients with ER-positive breast cancer (45). In part, these signatures provide information about tumor proliferative capacity and identify high-risk ER-positive, highly proliferative luminal B tumors (16). Recently, Habermann and colleagues showed that both Oncotype DX and MammaPrint signatures closely reflect tumor CIN status as quantified by DNA image cytometry in breast cancer (17).

Heterogeneity in terms of clinical outcome is well recognized for ER-negative breast cancer (46). Furthermore, predicting relapse in patients with ER-negative disease, using prognostic gene expression approaches that effectively predict outcome in ER-positive breast cancer, has proven challenging, with the overwhelming majority of ER-negative and node negative tumors classified as high risk using MammaPrint<sup>®</sup> (47, 48). Therefore, identification of independent and distinct biological processes influencing breast cancer clinical outcome, particularly in ER-negative disease, remains an important



**Figure 5.** Association of CIN with clinical outcome. CIN status is determined by quartile distribution using CIN70, aCGH structural chromosome complexity score, and MCD (CEP2 and 15). A, HRs for ER-negative breast cancer across CIN quartiles determined by CIN70, combined structural chromosomal complexity (aCGH) score, and MCD. B, HRs for ER-negative breast cancer associated with quartiles of MammaPrint® and GGI expression signatures. C, HRs for ER-positive breast cancer with CIN scored by CIN70, combined structural instability (aCGH), and MCD. D, HRs for ER-positive breast cancer associated with quartiles of MammaPrint® and GGI expression signatures. HRs represent risk of death for centromeric analysis and risk of recurrence or development of metastasis for gene expression/CGH analyses.



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research area to help refine prognostic signatures and provide new methods to stratify good from poor prognosis disease (49).

There is a seemingly paradoxical relationship between tumor karyotypic instability and tumor biological behavior. Aneuploidy and CIN can both enhance and negatively impact upon cell biological fitness (8, 9, 12-14).

Furthermore, proliferation-associated prognostic signature sets, which may also mirror CIN status, fail to predict outcome in ER-negative breast cancer. These observations prompted us to formally address the relationship of directly quantified tumor CIN with clinical outcome in patients with primary ER-negative compared with ER-positive breast cancer.

Using 2 centromeric FISH probes and estimating the deviation from the modal chromosome signals in nuclei from 246 breast cancers, we find that tumors with the greatest deviation from the modal chromosomal signal are enriched for grade 3 and ER-negative breast cancers. These data are consistent with previous observations of tumor CIN measured using DNA image cytometry in a cohort of 48 breast cancers (17). The association of higher MCD cohort with grade 3 tumors is not surprising given the Bloom–Richardson scoring for tumor grade in breast cancer, where nuclear pleomorphism forms 1 of the 3 components of the scoring system.

The relationship of increasing tumor CIN status, across 4 MCD cohorts, with clinical outcome in ER-negative compared with ER-positive breast cancer was addressed. The selection of the MCD cohorts was based on an unbiased stratification of increasing chromosomal numerical heterogeneity into 4 groups of similar range with the most extreme CIN MCD4 cohort (>45%) corresponding to the unstable aneuploid category defined by Lingle and colleagues (18). Consistent with preclinical evidence suggesting that CIN may have a negative impact on organism fitness, we have observed that ER-negative tumors in the extreme CIN MCD4 cohort are associated with the best prognosis and that MCD4 may serve as an independent favorable prognostic variable in ER-negative breast cancer in multivariate analysis. Notably, although node-negative status was associated with higher MCD3 and MCD4 cohorts, in a multivariate model, MCD4 remained a significant predictor of favorable outcome in ER-negative breast cancer.

These data are concordant with a parallel analysis by our group using the CIN70 expression signature as a surrogate of genome instability (Fig. 5; ref. 44). We have shown that extreme CIN70 expression, which correlates with structural chromosome complexity and numerical chromosome instability, is associated with improved prognosis in multivariate analysis of retrospective independent cohorts of patients with ER-negative breast cancer (Fig. 5A). Importantly, the same phenomenon is observed in gastric, squamous NSCLC, and ovarian cancer (ref. 44 and Supplementary Fig. S4), indicating that the association of extreme CIN with improved prognosis may be more widely apparent across solid tumors. In contrast, in ER-positive breast cancer, the risk of death increases with increasing CIN status determined by CIN70 expression, aCGH-based structural chromosomal complexity and numerical CIN determined by CEP2 and CEP15 (Fig. 5C). These data are consistent with the hypothesis that breast cancer prognostic expression signature sets, which may reflect tumor CIN status, have utility in ER-positive but not in ER-negative disease due to an approximately linear relationship between CIN status and risk of death in ER-positive but not in ER-negative breast cancer (Fig. 5B and D). The mechanistic basis for this differential relationship of CIN with clinical outcome in ER-positive

breast cancer compared with ER-negative breast cancer, NSCLC, gastric, and ovarian cancer is intriguing and requires further investigation.

Although directly quantified numerical CIN has been well described in breast cancer, to our knowledge this is the largest study to assess numerical CIN directly using 2 centromeric FISH probes, for structurally stable chromosomes, across a panel of breast cancers of mixed size, grade, and ER status. The selection of structurally stable chromosomes may help to minimize errors in CIN frequency assessment due to selection of chromosomes based on copy number alterations that might confer tumor cell survival advantage. Lingle and colleagues studied chromosome number in 20 breast tumors and identified 9 breast cancers (45%), with approximately 45% of tumor cells deviating from the modal chromosomal signal, that were classified as unstable aneuploid (18). The "extreme CIN" MCD4 cohort reported here (>45% of tumor cells deviating from the modal chromosomal signal) correlates closely with the unstable aneuploid cohort of Lingle and colleagues. When we use a direct statistical measure of clonal heterogeneity, the SDI, we confirm that tumors in the MCD4 cohort are the most chromosomally unstable and clonally heterogeneous. On the basis of our retrospective data from a cohort of 246 primary breast cancers, 13% of all primary breast cancers, 21% of ER-negative, 18% of HER2-positive, and 11% of ER-positive tumors would be represented in the extreme CIN MCD4 cohort (Supplementary Table S2).

These data from a small retrospective clinical cohort of ER-negative breast cancer patients, with validation by surrogate measures of CIN in an independent analysis of 356 patients, must be interpreted with caution and require prospective validation in larger patient cohorts with defined tumor stage and treatment history. At this stage, we have limited insight into the prognostic or predictive relevance of extreme CIN with patient outcome. Conceivably, ER-negative tumors in the extreme CIN cohort have an improved outcome because of their sensitivity to adjuvant chemotherapy regimens relative to tumors with intermediate CIN. We are testing such a hypothesis to distinguish the prognostic from predictive relevance of genome instability in a cohort of 3,300 patients treated within the TACT adjuvant chemotherapy trial.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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