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Research Article

Cancer Prevention Research

Copy Number Imbalances between Screen- and Symptom-Detected Breast Cancers and Impact on Disease-Free Survival

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Abstract

Screening mammography results in the increased detection of indolent tumors. We hypothesized that screen- and symptom-detected tumors would show genotypic differences as copy number imbalances (CNI) that, in part, explain differences in the clinical behavior between screen- and symptom-detected breast tumors. We evaluated 850 women aged 40 and above diagnosed with stage I and II breast cancer at the University of Texas MD Anderson Cancer Center between 1985 and 2000 with information available on method of tumor detection (screen vs. symptoms). CNIs in screen- and symptom-detected tumors were identified using high-density molecular inversion probe arrays. Cox proportional modeling was used to estimate the effect of method of tumor detection on disease-free survival after adjusting for age, stage, and the CNIs. The majority of tumors were symptom detected ($n = 603$) compared with screen detected ($n = 247$). Copy number gains in chromosomes 2p, 3q, 8q, 11p, and 20q were associated with method of breast cancer detection ($P < 0.00001$). We estimated that 32% and 63% of the survival advantage of screen detection was accounted for by age, stage, nuclear grade, and Ki67 in women aged 50 to 70 and aged 40 to 87, respectively. In each age category, an additional 20% of the survival advantage was accounted for by CNIs associated with method of detection. Specific CNIs differ between screen- and symptom-detected tumors and explain part of the survival advantage associated with screen-detected tumors. Measurement of tumor genotype has the potential to improve discrimination between indolent and aggressive screen-detected tumors and aids patient and physician decision making about use of surgical and adjuvant treatments. *Cancer Prev Res; 4(10); 1609–16. ©2011 AACR.*

Introduction

A recent meta-analysis of randomized studies showed that mammography screening reduces breast cancer mortality in women age 39 to 59 and age 60 to 69 by approximately 15% and 32%, respectively (1). Even after adjusting for earlier stage at diagnosis, several cohort studies have shown that screen detection remains a significant prognostic factor conveying approximately a 2-fold disease-specific survival advantage over symptom-detected breast cancer (2–6). This stage-adjusted reduction in breast cancer mortality has been partially attributed to higher detection rates of indolent tumors with low metastatic potential by screening. Studies using the prognostic molecular subtypes defined by expression profiling (i.e., luminal, HER2-positive, and basal) have shown that screen-detected tumors are more likely to be luminal A and less likely to be basal like, consistent with improved outcomes (7). However, molecular subtyping explains only an additional 10% of the survival advantage associated with screen-detected breast tumors after adjusting for stage (7). Thus, there are yet unknown biological differences between screen- and symptom-detected breast tumors that contribute to the survival advantage associated with screening.

Copy number imbalances (CNI) occur frequently in breast tumors (>90%) and in patterns that are thought to distinguish distinct genetic paths to tumorigenesis and predict clinical outcome (8–11). To our knowledge, no studies have evaluated differences in the tumor genotypes of screen- versus symptom-detected breast tumors. It is possible that screen-detected breast tumors exhibit more favorable, indolent tumor genotypes than symptom-detected tumors and that differences in tumor genotype

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may explain some of the disease-free survival benefit. We used high-density molecular inversion probe (MIP) arrays to investigate for the differences between CNIs in breast tumors detected by screening versus symptoms in a well-characterized cohort of patients with early-stage breast cancer and long-term follow-up. The identification of fixed genetic imbalances in DNA that discriminate more indolent from aggressive screen-detected tumors could significantly improve identification of clinically meaningful disease among women undergoing mammography screening and aid in treatment decision making.

Materials and Methods

Patient population

The Early Stage Breast Cancer Repository (ESBCR) is a retrospective cohort of 2,409 women diagnosed with American Joint Committee on Cancer pathologic stage I or II breast cancer and surgically treated at University of Texas MD Anderson Cancer Center (MDACC) between 1985 and 2000. Criteria for eligibility and cohort details have been previously described (12). Briefly, detailed clinical information including patient age, stage, tumor size, lymph node status, modified Black's nuclear grade, estrogen receptor (ER) and progesterone receptor (PR) status, and primary treatment including surgery, radiation therapy, chemotherapy, and endocrine therapy were abstracted from medical charts. Patient's formalin-fixed, paraffin-embedded (FFPE) breast tumor specimens were accessed from the MDACC Department of Pathology Tumor Bank. Screen-detected tumors were defined as those detected via a screening mammogram. Symptom-detected breast tumors were those identified through patient reported symptoms including palpable breast lump, breast pain, or nipple discharge. Follow-up information for patients in the ESBCR is obtained by direct review of the medical records and linkage to the MDACC Tumor Registry, which mails annual follow-up letters to each patient registered at MDACC known to be alive to determine their clinical status. The MDACC Tumor Registry checks the social security death index and the Texas Bureau of Vital Statistics for the status of patients who fail to respond to the letters.

The ESBCR patients selected for the MIP assay of DNA copy number were enriched to include all African American ($n = 196$) and Hispanic patients ($n = 208$) and a random sample of Caucasian patients over sampled for recurrences ($n = 808$). There were 241 patients excluded from the analyses because of insufficient tumor for DNA extraction, DNA extraction failure, or MIP assay failure. Women younger than age 40 at the time of breast cancer diagnosis were also excluded ($n = 121$). There were 850 patients included in the final study analyses (Fig. 1).

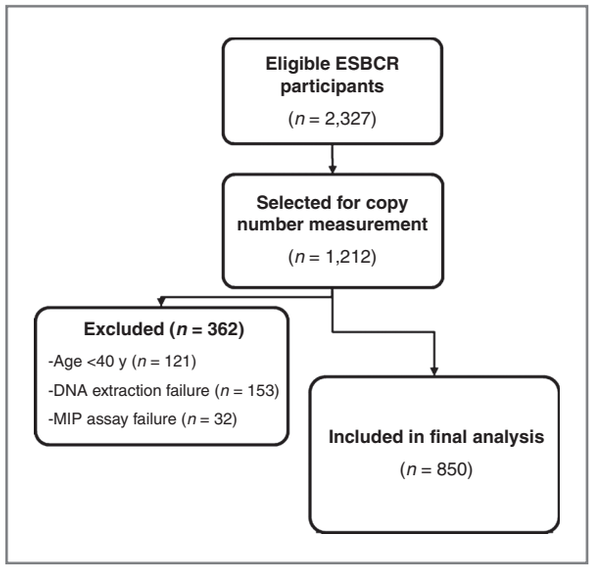

Figure 1. Participant selection for analysis.

Definition of tumor subtypes

We approximated tumor subtypes from clinically validated immunohistochemical (IHC) analyses of ER, PR, HER2, and Ki67 (13). All samples were tissue arrayed and standard IHC staining using monoclonal antibodies were applied to detect Ki67 (1:100; DAKO), ER (1:35; Novocastra), and PR (1:200; DAKO) with tissue from normal breast and uterus used as controls. The stained tissue microarray slides were scanned using the Applied Imaging Ariol SL-50 System. The percentage of positive tumor cells was assessed by manually counting the total number of positively stained tumor nuclei and dividing by the total number of tumor nuclei and converting this fraction to a percentage. ER and PR were interpreted as positive when 5% or more of the tumor nuclei were positive. The ER and PR status was obtained from 2 sources: medical records (primary source) and tissue microarray (secondary source). The agreement in ER and PR status between the 2 sources were 84.8% and 76.4%, respectively. MIP array–based HER2 copy number proved superior to immunohistochemistry (area under receiver operator characteristic curve was 0.94) and equivalent to FISH for *HER2* gene amplification. We used MIP copy number to determine HER2 amplification in cases that were ambiguous by IHC (2+) and FISH data were unavailable. Tumors were classified into approximated subtypes on the basis of IHC and FISH results with luminal A (ER or PR positive, HER2 negative, Ki67 < 15%), luminal B (ER or PR positive, HER2 negative, Ki67 $\geq$ 15%), HER2 positive (HER2 positive and ER or PR positive or ER and PR negative), and triple negative (ER and PR negative and HER2 negative).

Tumor DNA extraction

DNA was extracted and processed for copy number analyses from FFPE tissues as described previously (14). Briefly, 5 to 10 (5 μm) macrodissected tumor sections were pooled and treated 3 times with proteinase K in ATL Tissue Lysis Buffer (Qiagen). Following lysis, the samples were applied to uncoated Argylla Particles

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(Argylla Technologies) and processed according to manufacturer recommendations (http://www.argylla.com).

MIP-based arrays for copy number measurement

DNA from FFPE tumor was prepared. For 10% of the cases, DNA from a nontumor lymph node was used as an internal normal referent. The samples were shipped for processing for copy number measurement at the Affymetrix MIP Laboratory, which was blinded to all sample information including matched normals and duplicates. The MIP assay has been described in detail (14–18) including platform validation using representative but independent samples from the ESBCR (17). Data quality was assessed using the sample 2-point relative standard error (2p-RSE; ref. 19). The majority (95%) of FFPE tumors samples applied to the MIP arrays passed the 2p-RSE threshold (GSE31424).

Determination of copy number change

DNA copy number differences were analyzed using AsCNAR software (http://genome.umin.jp) for single-nucleotide polymorphism mapping data. Data collected from matched normal were used for normalizing the copy number data. For each sample, we generated full genome MIP quantifications (330K MIPs). To reduce the data dimension, we computed the running median within groups of 25 consecutive MIPs, yielding 13,175 data points per sample. Circular Binary Segmentation (20) was used to convert the data to a list of segments for each sample. DNA copy number differences were analyzed with the R package DNAcopy (www.r-project.org) using thresholds of 2.5 for one copy gained and 1.5 for one copy lost. The parameter α (significance level for acceptance of change points) used in the segmentation algorithm was set to 0.01. We recombined consecutive segments if their gain/loss calls agreed for at least 99.5% of the samples. This procedure yielded 1,593 segments, representing the entire genome.

Statistical methods

We computed frequency tables of the patient's clinical characteristics by method of detection using the χ^2 test. We conducted Fisher's exact test (2 tailed) with random permutations of the samples to compare CNIs between tumors detected by screening versus symptoms. We also adjusted for multiple comparisons using a false discovery rate (21). Breast cancer disease-free survival was calculated from the date of diagnosis to the primary endpoint of the study defined as the occurrence of local lymph node or breast recurrence, metastasis to contralateral breast, chest wall or other sites, or breast cancer–related death. Patients not known to have a breast cancer event at the date of last contact were censored. We used Cox proportional hazards regression model to estimate the HR and 95% CI first for the association between method of detection and disease-free survival and then adjusted for each of the clinical variables (age at diagnosis, year at diagnosis, ethnicity, tumor size, lymph node status, treatment, tumor subtype, nuclear grade, histology, and the CNIs found to differ between screen- and symptom-detected tumors). The method of Freedman and colleagues was used to calculate the percentage of the effect of method of detection on disease-free survival accounted for by the variables (22). The Freedman statistic is defined as: $P = 100\ (1 - a/b)$, where a is the adjusted and b is the unadjusted logarithm of the HR. The null hypothesis is that the Freedman statistic = 0% meaning that the adjustment makes no difference. Significance at the 0.05 level implies that adjusting for the variable significantly explains part of the association between method of detection and breast cancer disease-free survival.

Results

Characteristics of the ESBCR patient population

The majority of breast tumors were detected as a result of symptoms (70%) compared with screening (30%; Table 1). The proportion of screen-detected tumors increased from 13.8% between the years 1985 and 1989 to 54% in the years 1995 and 2000. As anticipated, compared with symptom-detected tumors, screen-detected tumors were more likely to be smaller ($P < 0.001$), lymph node negative ($P < 0.001$), low nuclear grade ($P < 0.001$), and Ki67 low ($P < 0.01$). Screen-detected tumors were also more likely to be luminal A (46%), and symptom-detected tumors were more likely to be HER2 positive (19%) and triple negative (21%). At the time of analysis, 241 patients (28%) of the study population had experienced disease progression or breast cancer–related death.

CNIs in screen- and symptom-detected breast cancers

We identified CNIs involving 22 segments in 5 chromosomes (2, 3, 8, 11, 20) that were statistically significantly associated with method of breast cancer detection (Table 2). The 22 segments identified corresponded to a threshold of 0.05 of the false discovery rate. The 22 segments corresponded to 5 distinct chromosomal regions, each one of which contained one or more adjacent segments. In particular, the 5 regions at 2p11.2, 3q27.1-q29, 8q24.13, 11p13, and 20q13.13-q13.32 contained 1, 5, 1, 8, and 7 segments, respectively. The copy number gains/losses for these 5 regions were determined by the median value across segments in each region. Recurrent copy number gains in the 5 distinct chromosomal regions were significantly more common among symptom-detected than screen-detected tumors ($P < 0.0001$).

The effect of clinical variables and CNIs on breast cancer–specific survival

The HR for the association between method of breast cancer detection and breast cancer disease-free survival and then adjusting for the clinical factors individually are shown in Table 3. In the unadjusted analysis, patients with screen-detected breast tumors had a statistically significant 34% improvement in disease-free survival compared with patients with symptom-detected tumors. The HR for the effect of screening on disease-free survival was attenuated

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Table 1. Patient and clinical characteristics of study population

Factors	Screen detected ($N = 247$)	Symptom detected ($N = 603$)	P
Year at diagnosis			
1985–1989	34 (13.8)	169 (28)	<.001
1990–1994	75 (30.4)	192 (31.8)	
≥1995	135 (54.6)	226 (37.5)	
Missing	3 (1.2)	16 (2.7)	
Age at diagnosis, y			
40–49	55 (22.3)	201 (33.3)	<0.01
50–59	82 (33.2)	188 (31.2)	
60–70	60 (24.3)	130 (21.6)	
71–87	50 (20.2)	84 (13.9)	
Ethnicity			
White	193 (78.1)	434 (72)	0.13
Black	23 (9.4)	85 (14)	
Hispanic	30 (12.1)	77 (12.8)	
Other	1 (0.4)	7 (1.2)	
Tumor size, cm			
≤2	193 (78.1)	300 (49.8)	<0.001
2–5	47 (19.1)	260 (43.1)	
>5	1 (0.4)	16 (2.7)	
Missing	6 (2.4)	27 (4.4)	
Nodal status			
Negative	160 (64.8)	342 (56.7)	0.06
Positive	84 (34)	244 (40.5)	
Missing	3 (1.2)	17 (2.8)	
Nuclear grade			
1	35 (14.2)	52 (8.6)	<0.01
2	131 (53)	292 (48.4)	
3	62 (25.1)	217 (36)	
Missing	19 (7.7)	42 (7)	
Histology			
Invasive ductal	225 (91.1)	562 (93.2)	0.22
Invasive lobular	20 (8.1)	35 (5.8)	
Other	2 (0.8)	6 (1)	
Ki67			
Low (<15%)	126 (51)	222 (36.8)	<0.001
High (≥15%)	84 (34)	307 (50.9)	
Missing	37 (15)	74 (12.3)	
Tumor subtype			
Luminal A	114 (46.2)	170 (28.2)	<0.001
Luminal B	48 (19.4)	148 (24.5)	
HER2 positive	29 (11.7)	114 (18.9)	
Triple negative	28 (11.3)	126 (20.9)	
Missing	28 (11.3)	45 (7.5)	
Chemotherapy			
None	170 (68.8)	290 (48.1)	<0.001
Anthracycline	40 (16.2)	205 (34)	
Anthracycline + taxane	27 (10.9)	72 (11.9)	
Other	4 (1.7)	13 (2.2)	
Missing	6 (2.4)	23 (3.8)	
Hormone therapy			
Tamoxifen	136 (55.1)	254 (42.1)	<0.01
Other	3 (1.2)	17 (2.8)	
None	107 (43.3)	321 (53.2)	
Missing	1 (0.4)	11 (1.8)	

NOTE: All values are *n* (%).

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Table 2. CNIs associated with method of breast cancer detection ($P < 0.0001$)

Chromosome	Cytobands	Start	Stop	Screen detected (% gains)	Symptom detected (% gains)	Breast cancer–related genes
2	p11.2	84,813,597	87,104,100	0	4	–
3	q27.1-q29	185,054,739	198,436,531	3	10	*ABCC5*, *ADIPOQ*, *RFC4*
8	q24.13	124,313,073	125,474,900	23	35	–
11	p13	31,571,247	36,662,678	1	7	*CD44*
20	q13.13-q13.32	46,536,148	57,711,655	8	18	*AURKA*, *BMP7*, *GNAS*, *TUBB1*

from 0.66 (95% CI: 0.50–0.88) to 0.86 (95% CI: 0.59–1.24) after adjusting for age at diagnosis, tumor size, nodal status, nuclear grade, and Ki67. The HR was further attenuated with the addition of the 5 CNIs to 0.93 (95% CI: 0.64–1.36). The Freedman statistic of the proportion of the survival advantage associated with screen detection that was attributed to the clinical variables (age at diagnosis, tumor size, nodal status, nuclear grade, and Ki67) was 63% ($P = 0.04$). Further adjustment of the model to incorporate the 5 CNIs changed the proportion of the screen detection effect on survival to 82% and thus explained a further 20% of disease-free survival advantage associated with method of detection. We conducted a separate analysis excluding patients younger than 50 years and older than 70 years, thus making the study population more balanced for method of detection (Table 4). The proportion of the survival advantage associated with screen detection attributed to the clinical variables (age at diagnosis, tumor size, nodal status, nuclear grade, and Ki67) was 32%. The addition of the 5 CNIs increased the Freedman statistic to 50%.

Discussion

Screening mammography increases the detection of indolent tumors that are associated with more favorable prognosis, otherwise known as length time bias. Indeed, screen-detected tumors are more likely to be low nuclear grade, better differentiated, ER positive, and less likely to have abnormal DNA content (aneuploidy; refs. 23–28) and as we show here, discrete copy number gains. Several epidemiologic studies have shown that despite adjustment for an earlier stage at diagnosis and favorable prognostic tumor characteristics, the survival advantage associated with screen-detected tumors persists (2, 4, 5, 29), suggesting an incomplete understanding of the underlying favorable biology of screen-detected tumors. Consequently, it has been recommended that method of breast cancer detection be accounted for as an important confounder in epidemiologic and clinical studies that address breast cancer outcomes (3, 4). This study represents a novel approach using the examination of the tumor genotype to define biological markers that differ between screen- and

Table 3. Attenuation of the association between method of detection and disease-free survival after adjusting for clinical variables and CNIs for study population ($n = 850$)

Factors adjusted for	HER (95% CI) for screen vs. symptom detected	Freedman statistic, %	Freedman statistic, *P*
None	0.66 (0.50–0.88)	–	
Race	0.66 (0.49–0.88)	0.13	0.97
Histology	0.66 (0.5–0.89)	1.8	0.53
Tumor subtype	0.68 (0.50–0.93)	7.0	0.38
Ki67	0.70 (0.50–0.98)	15	0.21
Hormonal therapy	0.70 (0.53–0.94)	15.1	0.06
Nodal status	0.71 (0.53–0.95)	17.9	0.04
Chemotherapy	0.71 (0.53-0.96)	17.4	0.14
5 CNIs	0.72 (0.53–0.96)	20.3	0.06
Nuclear grade	0.73 (0.55–0.99)	25.8	0.08
Age at diagnosis	0.74 (0.54–1.003)	27	0.12
Tumor size	0.75 (0.55–1.01)	29.5	0.04
Tumor size + nodal status + age + grade + Ki67	0.86 (0.59–1.24)	63.5	0.04
Tumor size + nodal status + age + grade + Ki67 + 5 CNIs	0.93 (0.64–1.36)	82.4	0.02

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Table 4. Attenuation of the association between method of detection and disease-free survival after adjusting for clinical variables and CNIs for study cohort aged 50 to 70 ($n = 460$)

Factors adjusted for	HR (95% CI) for screen vs. symptom detected	Freedman statistic, %	Freedman statistic, *P*
None	0.65 (0.44–0.98)	–	–
Race	0.66 (0.43–0.99)	0	0.99
Histology	0.66 (0.44–0.99)	1.4	0.99
Tumor subtype	0.69 (0.45–1.08)	13.3	0.41
Ki67	0.69 (0.44–1.09)	11.9	0.55
Hormonal therapy	0.67 (0.45–1.01)	6.8	0.80
Nodal status	0.72 (0.48–1.09)	23.1	0.14
Chemotherapy	0.71 (0.46–1.09)	18.8	0.26
5 CNIs	0.72 (0.47–1.1)	22.1	0.16
Nuclear grade	0.71 (0.46–1.09)	17.4	0.35
Age at diagnosis	0.66 (0.44–0.98)	0.4	0.72
Tumor size	0.76 (0.49–1.19)	34.9	0.09
Tumor size + nodal status + age + grade + Ki67	0.75 (0.44–1.27)	31.7	0.11
Tumor size + nodal status + age + grade + Ki67 + 5 CNIs	0.81 (0.47–1.39)	50.0	0.06

symptom-detected tumors and that could contribute to the prognosis of patients with early-stage breast cancer.

We identified statistically significant differences in the frequency of gains in 2p, 3q, 8q, 11p, and 20q between screen- and symptom-detected tumors. Gunther and colleagues showed that invasive ductal cancers are more likely to have gains in 8q than invasive lobular cancers (26% vs. 69%, respectively; ref. 30). Nishizaki and colleagues (31) and Courjal and Theillet (32) found similar results as Gunther and colleagues and also showed a higher frequency of gains in 20q in invasive ductal cancers. Although screening mammograms are more likely to miss tumors of invasive lobular histology presumably because of the lack of a stromal response (23), there was no significant difference in histology by method of detection in our study. Gains in 2p have been associated with high-grade tumors and ER negativity (33) whereas gains in 3q, 8q, 11p, and 20q have been consistently associated with breast cancers of higher malignant potential including familial (*BRCA1* and *BRCA2*), basal-like, and luminal-B tumors (34–39). Our study findings are therefore highly compatible with the concept that specific CNIs underlie breast tumor biology as driver events and that their detection with advanced technologies such as MIP arrays may prove useful in discriminating between tumors with differing malignant potential.

Although the exact biological mechanisms for the quantitative differences in the copy number gains identified in this study are unknown, chr3q27.1-q29 contains the adiponentin gene (*ADIPOQ*), which suppresses cell proliferation, induces apoptosis, and inhibits angiogenesis (40–44). There were, however, a higher number of copy number gains in the chr3q27.1-q29 segment in symptom-detected than in screen-detected tumors. The *CD44* gene is contained in chr11p13 and *in vitro* studies have shown that its expression is associated with epithelial-mesenchymal transition and pathways regulating cell migration (45, 46). Overexpression of the *CD44* gene in breast tumors has been positively associated with lymph node involvement and metastasis and the presence of $CD44^+/CD24^-$ cells is enriched in the highly aggressive basal-like breast tumors (47, 48). To our knowledge, there are no breast cancer candidate genes currently implicated in chr2p11.2 and chr11p13 regions.

A potential harm of mammography screening is that it can lead to overdiagnosis with the subsequent treatment of tumors that would never have been clinically aggressive over the lifetime of a patient (49). It is estimated that 4% to 30% of invasive breast cancers are overdiagnosed in a screened population of women (50–52), and the likelihood of overdiagnosis is highest among women at oldest age groups (53). When the study population was balanced for screen- versus symptom-detected tumors (i.e., restricted to age 50–70), age, stage, nuclear grade, and Ki67 accounted for 30% of the survival advantage associated with screen detection similar to results observed in a prior study (29). The addition of the 5 CNIs increased the proportion of the screen detection effect on survival to 50%. These results suggest that other host or molecular factors likely contribute to breast tumor biology and metastatic potential.

The study has several strengths including the use of a well-characterized cohort of early-stage breast cancer patients with long-term follow-up and high-quality copy number data derived from the novel MIP technology. However, one of the study limitations is the lack of information on whether the symptom-detected tumors in the study cohort represented cancers detected in the interval between screening examinations or tumors that were missed during screening mammography or were self-detected in an unscreened population of women. Whether these categories of symptom-detected tumors differ biologically is not clear, although associations with

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increased aggressiveness have been reported for "true" interval cancers (54). An additional limitation is the lack of information on the type of mammography screening that patients in the ESBCR received. The ESBCR patient population spans 15 years of diagnosis (1985–2000) during which time significant improvements in mammography technology led to increased sensitivity and specificity of mammography screening (55). The time period of the ESBCR was also prior to the routine incorporation of combined histologic grade in the pathology reports of patients with breast cancer diagnosed at MDACC. Genomic changes in breast tumors significantly correlate with the underlying components of histologic grade, that is, tubular formation, nuclear pleomorphism, and mitosis (56). Therefore, the use of nuclear grade in this study may have lead to an overestimation of the contribution of CNIs to disease-free survival. Confirmation of these study results will be needed within the context of a randomized screening trial or a population-based cohort of patients undergoing current state-of-the-art screening mammograms with long-term follow-up. The challenge of future studies will be in identifying populations of breast cancer patients who have not received systemic therapy to more precisely measure the influence of CNIs on length time bias and their impact on disease-free survival.

The characterization of a tumor genotype that appears to differ between screen- and symptom-detected tumors contributes to the survival advantage associated with screen-detected tumors and represents an advance in knowledge of the role of CNIs in breast tumors. A greater understanding of the biological relevance of the quantitative differences in copy number gains in the identified chromosomal regions is needed to identify screen-detected tumors that may not need intervention creating new treatment paradigms for women presenting at mammography with indolent disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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