

The Genomic Landscape of Male Breast Cancers

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Abstract

Purpose: Male breast cancer is rare, and its genomic landscape has yet to be fully characterized. Lacking studies in men, treatment of males with breast cancer is extrapolated from results in females with breast cancer. We sought to define whether male breast cancers harbor somatic genetic alterations in genes frequently altered in female breast cancers.

Experimental Design: All male breast cancers were estrogen receptor-positive, and all but two were HER2-negative. Fifty-nine male breast cancers were subtyped by immunohistochemistry, and tumor-normal pairs were microdissected and subjected to massively parallel sequencing targeting all exons of 241 genes frequently mutated in female breast cancers or DNA-repair related. The repertoires of somatic mutations and copy number alterations of male breast cancers were compared with that of subtype-matched female breast cancers.

Results: Twenty-nine percent and 71% of male breast cancers were immunohistochemically classified as luminal A-like or luminal B-like, respectively. Male breast cancers displayed a

heterogeneous repertoire of somatic genetic alterations that to some extent recapitulated that of estrogen receptor (ER)-positive/HER2-negative female breast cancers, including recurrent mutations affecting PIK3CA (20%) and GATA3 (15%). ER-positive/HER2-negative male breast cancers, however, less frequently harbored 16q losses, and PIK3CA and TP53 mutations than ER-positive/HER2-negative female breast cancers. In addition, male breast cancers were found to be significantly enriched for mutations affecting DNA repair-related genes.

Conclusions: Male breast cancers less frequently harbor somatic genetic alterations typical of ER-positive/HER2-negative female breast cancers, such as PIK3CA and TP53 mutations and losses of 16q, suggesting that at least a subset of male breast cancers are driven by a distinct repertoire of somatic changes. Given the genomic differences, caution may be needed in the application of biologic and therapeutic findings from studies of female breast cancers to male breast cancers.

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Introduction

Male breast cancer is an uncommon disease, accounting for <1% of all breast cancers (1), with approximately 2,350 new cases diagnosed per year in the United States (2). In contrast to female

breast cancers, male breast cancers are usually diagnosed at later stage and older age (3). Risk factors of male breast cancers are largely related to physiologic changes in estrogen levels, hereditary syndromes (e.g., Klinefelter syndrome), and BRCA2 or PALB2 germline mutations (4, 5). The majority of male breast cancers are estrogen receptor (ER)-positive invasive ductal carcinomas of no special type (IDC-NSTs). Unlike female breast cancers, male breast cancers rarely display HER2 gene amplification or a triple-negative phenotype (6). Given its rarity, there have been no prospective clinical trials for patients with male breast cancer (7), and critical treatment decisions for these patients are generally extrapolated from small single-institution retrospective studies and prior knowledge obtained from studies carried out in females with breast cancer (8).

From a clinicopathologic standpoint, male breast cancers are similar to estrogen receptor (ER)-positive/luminal female breast cancers. Patients with male breast cancer and female breast cancer appear to have similar prognosis when matched according to age at diagnosis and clinicopathologic characteristics (9, 10). Even when prognostic differences were documented [i.e., female breast cancers had better 5-year overall survival (OS) than male breast cancers in the early but not in the late stages; ref. 11], these differences could be attributed to the lack of mammographic screening in men, higher likelihood for non-breast cancer-related mortality in men, and deficiencies in data collection and reporting by cancer registries (11).

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Translational Relevance

Male breast cancer is an uncommon disease accounting for <1% of all invasive breast cancers. Given its rarity, there have been no prospective clinical trials for male patients with breast cancer, and critical treatment decisions are generally based on extrapolations from prior knowledge obtained through studies of female patients with breast cancer. Here we found that although overall male breast cancers and female breast cancers appeared to be characterized by similar patterns of somatic genetic alterations, they differ in their mutational repertoire and the mutational frequency of the most commonly mutated genes. Male breast cancers less frequently harbor *PIK3CA* and *TP53* mutations than female breast cancers of the same immunohistochemical profile and display enrichment for potentially pathogenic mutations affecting DNA repair-related genes. Our data suggest that caution should be exercised in the extrapolation of biologic and therapeutic findings from studies performed in female breast cancers to male breast cancers, and that further studies to define targetable alterations in the latter are warranted.

The genetic landscape of male breast cancers has yet to be fully characterized. Massively parallel sequencing (MPS) studies have provided a comprehensive portrait of the genetic landscape of female breast cancers and demonstrated that they are characterized by complex genomes (reviewed in ref. 12). These studies have identified bona fide driver genetic alterations in breast cancer, in particular recurrent mutations affecting *PIK3CA*, *TP53*, *GATA3*, *MAP3K1*, *MAP2K4*, *KMT2C*, and *CDH1* in ER-positive breast cancers (12, 13). It should be noted, however, that only a few male breast cancers were included in these studies. In The Cancer Genome Atlas (TCGA) project, six male breast cancers were included, of which all were of luminal subtype, two harbored *PIK3CA* somatic mutations, and none had *TP53* mutations (13). The repertoire of genetic alterations affecting 48 common cancer genes has been analyzed in familial male breast cancers (BRCA1, BRCA2, and BRCAX patients; ref. 14); these tumors were found to display a mutational landscape similar to that of luminal A female breast cancers, with *PIK3CA* being the most commonly mutated gene (17%) in addition to infrequent *TP53* and *PTEN* mutations (4% and 2%, respectively; ref. 14). Substantial differences were detected, however, between BRCA2 and "BRCAX" patients. While *TP53* mutations were only found in BRCA2 cases (14), *PIK3CA* mutations were significantly more prevalent in BRCAX patients (15), suggesting that BRCA2-associated male breast cancers may have distinct genomic features, possibly due to the DNA repair defect caused by *BRCA2* mutations.

Recent studies have shown that male breast cancers and female breast cancers of luminal molecular subtype harbor similar copy number alterations (CNAs), including recurrent 1q gain and 16q loss (16, 17). Of note, these two CNAs (1q gain/16q loss), which often co-occur with *PIK3CA* mutations, define a genetic signature present not only in low-grade/luminal A-like, but also in a subset of high-grade/luminal B-like breast cancers (13, 18, 19). By integrating CNAs and gene expression, Johansson and colleagues (20) identified 30 candidate driver genes in male breast cancers, of which only a minority included known cancer genes (*MAP2K4*, *LHP*, *ZNF217*), suggesting that male breast cancers may display a

constellation of candidate driver genes distinct from that of female breast cancers (20).

Given the limited genetic characterization of male breast cancers and the apparent clinicopathologic similarities between ER-positive male breast cancers and female breast cancers, here we sought (i) to define the repertoire of somatic genetic alterations in male breast cancers using a platform containing the genes most frequently mutated in female breast cancers as well as genes involved in DNA repair, given the reported enrichment for *BRCA2* and *PALB2* mutations in male breast cancers, and (ii) to compare the mutational and CNA profiles of male breast cancers with female breast cancers and postmenopausal female breast cancers using this platform, given the similarities in the hormonal milieu and age of presentation between male breast cancers and postmenopausal female breast cancers.

Materials and Methods

Cases

Fifty-nine male breast cancers were retrieved from the pathology archives of Memorial Sloan Kettering Cancer Center (MSKCC, New York, NY). Only patients diagnosed and managed at MSKCC, whose tumors were >1 cm in size and for which representative histologic slides and blocks were available for review were included. Patients for whom not all histologic slides were available for review were excluded, as well as samples from patients who received neoadjuvant therapy. Samples were anonymized prior to analysis and the study was approved by the Institutional Review Board of MSKCC. All cases were independently reviewed by four pathologists (M.P. Murray, E. Guerini-Rocco, N. Fusco, and J.S. Reis-Filho) who classified and graded the tumors following the World Health Organization criteria (21) and the Nottingham grading system (22), respectively. All clinicopathologic features are summarized in Table 1 and Supplementary Table S1.

Immunohistochemistry and intrinsic subtyping

Immunohistochemical profile of the included cases ($n = 59$) was assessed on 4- μ m-thick sections, using antibodies against ER, progesterone receptor (PR), HER2, and Ki-67 as described previously (23). Positive and negative controls were included in each experiment. The ER, PR, and HER2 immunohistochemical results were evaluated by three pathologists (M.P. Murray, E. Guerini-Rocco, and N. Fusco) according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines (24, 25). Ki-67 score was defined as the percentage of cells with nuclear staining in at least 1,000 invasive neoplastic cells randomly selected from the periphery of the tumor over 10 high-power fields (magnification, 400X; ref. 26). Immunohistochemistry (IHC) with antibodies against E-cadherin and p120 catenin was performed in selected cases and interpreted by two of the authors (F.C. Geyer and J.S. Reis-Filho). Antibody clones and dilutions are described in Supplementary Table S2. The intrinsic (molecular) subtype of male breast cancers were defined using the immunohistochemical surrogate according to the St. Gallen criteria for female breast cancers (27).

Microdissection and DNA extraction

Eight- μ m-thick sections from representative formalin-fixed paraffin-embedded histologic blocks of male breast cancers were stained with Nuclear Fast Red in RNase-free condition and subjected to microdissection with a sterile needle under a

Table 1. Clinicopathologic features and immunohistochemical subtypes of the 59 male breast cancers included in this study

Clinicopathologic features	Male breast cancers (n = 59)
Median age at diagnosis (y)	66 (range, 33–88)
Histologic grade ^a	
1	4 (7%)
2	29 (49%)
3	26 (44%)
Median size (cm)	2.0 (range, 0.6–4.7)
Histologic type	
IDC-NST	54 (91%)
IPC	2 (3%)
IMC	1 (2%)
IMuC	1 (2%)
Mixed ductal lobular carcinoma	1 (2%)
ER status	
Negative	0 (0%)
Positive	59 (100%)
PR status	
Negative	7 (12%)
Positive	52 (88%)
HER2 status	
Negative	57 (97%)
Positive	2 (3%)
Ki-67 labeling index	
High ($\geq 14\%$)	39 (66%)
Low ($< 14\%$)	20 (34%)
Intrinsic subtype ^b	
Luminal A-like	17 (29%)
Luminal B-like	42 (71%)

Abbreviations: IDC-NST, invasive ductal carcinoma of no special type; IMC, invasive micropapillary carcinoma; IMuC, invasive mucinous carcinoma; IPC, invasive papillary carcinoma; PR, progesterone receptor.

^aAccording to the Nottingham grading system (22).

^bDefined by immunohistochemical analysis according to St. Gallen criteria (27).

stereomicroscope (Olympus) to ensure a percentage of tumor cells >90%, as described previously (28). Microdissection was also performed for matched normal tissue to ensure that normal samples were devoid of neoplastic or atypical cells. DNA was extracted from microdissected tissue using the DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's guidelines, and quantified using the Qubit Fluorometer assay (Life Technologies). All samples yielded DNA of sufficient quantity and quality for sequencing analysis.

Targeted capture massively parallel sequencing

Tumor and germline DNA of 59 male breast cancers were subjected to targeted capture massively parallel sequencing (MPS) using a platform containing baits targeting all exons of 241 genes that are either recurrently mutated in breast cancer or involved in DNA repair pathways (28). Custom oligonucleotides (NimblegenSeqCap) were designed for hybridization capture of the 241 genes (Supplementary Table S3; ref. 28). Barcoded sequencing libraries were prepared (New England Biolabs) using 50 to 250 ng DNA and pooled at equimolar concentrations into a single capture reaction as described previously (23, 28). Paired-end 76 bp reads were generated on the Illumina HiSeq2000. Sequence reads were aligned to the human reference genome GRCh37 using the Burrows-Wheeler Aligner (BWA, v0.7.10; ref. 29). Local realignment, duplicate removal, and base quality recalibration were performed using the Genome Analysis Toolkit (GATK, v3.1.1; ref. 30). Sequencing data have been deposited in the NCBI Sequence Read Archive under the accession SRP055003.

Somatic single-nucleotide variants (SNV) were identified using MuTect (v1.0; ref. 31), Strelka (v2.0.15; ref. 32), and VarScan 2 (v2.3.7; ref. 33) using a consensus approach, retaining those detected by at least two mutation callers. Small insertions and deletions (indels) were detected using Strelka (v2.0.15; ref. 32) and VarScan 2 (v2.3.7; ref. 33). We filtered out SNVs and indels outside of the target regions, those with mutant allelic fraction (MAF) of <1% and/or those supported by ≤5 reads (34). We further excluded SNVs and indels for which the tumor MAF was <5 times that of the matched normal MAF, as well as SNVs and indels found at >5% global minor allele frequency of dbSNP (build 137). All candidate mutations were subsequently reviewed manually using the Integrative Genomics Viewer (IGV; ref. 35).

Copy number alterations (CNA) were identified using FACETS (ref. 36; Supplementary Methods). A combination of Mutation Taster (37), CHASM (breast; ref. 38), and FATHMM (39) was used to define the potential functional effect of each missense SNV. Missense SNVs defined as nondeleterious/pasenger by both MutationTaster (37) and CHASM (breast; ref. 38), a combination of mutation function predictors shown to have a high negative predictive value (40), were considered passenger alterations. The missense SNVs considered not to be passengers using this combination of mutation function predictors were defined as likely pathogenic if predicted by CHASM (breast; ref. 38) and/or FATHMM (39) as "driver" and/or "cancer" alterations, respectively. For in-frame indels, those defined as "deleterious" by MutationTaster or PROVEAN (41), and targeted by loss of heterozygosity (LOH) of the wild-type allele or affected cancer genes included in the cancer gene lists described by Kandoth and colleagues (127 significantly mutated genes; ref. 42), the Cancer Gene Census (43), or Lawrence and colleagues (Cancer5000-S gene set; ref. 44) were considered to be likely pathogenic. In addition, frameshift, splice-site, and truncating mutations in the presence of LOH of the wild-type allele or affected genes of at least one of the three cancer gene datasets were also considered likely pathogenic.

The cancer cell fraction (CCF) of each mutation was inferred using the number of reads supporting the reference and the alternate alleles and the segmented Log₂ ratio from MPS as input for ABSOLUTE (v1.0.6; ref. 45). Solutions from ABSOLUTE were manually reviewed as recommended (45, 46). A mutation was classified as clonal if its clonal probability, as defined by ABSOLUTE, was >50% (46) or if the lower bound of the 95% confidence interval of its CCF was >90%. Mutations that did not meet the above criteria were considered subclonal.

Sequenom MassARRAY

The 59 male breast cancers included in this study were also subjected to hotspot mutation screening of eight known cancer genes (i.e., PIK3CA, GATA3, CTNNB1, KRAS, NRAS, CDH1, AKT1, and ERBB2) using a validated screening panel (Sequenom; ref. 47). The experiments were carried out as described previously in duplicate (47).

Sanger sequencing

PCR amplification of 10 ng of genomic DNA was performed using the AmpliTaq 360 Master Mix Kit (Life Technologies) on a Veriti Thermal Cycler (Life Technologies) as described previously (ref. 48; Supplementary Methods; for primers see Supplementary Table S2).

Pathway analysis

Genes included in the targeted sequencing assay were mapped to KEGG pathways to identify pathways recurrently altered. Significantly regulated pathways and networks were determined using the Ingenuity Pathway Analysis (IPA) program as previously described (49). For the analysis, genes harboring likely pathogenic mutations in male breast cancers, and not mutated in female breast cancers, were mapped to networks available in the Ingenuity database and ranked by a score indicating the likelihood of finding those genes together by chance. Using a 99% confidence level, scores of ≥ 3 were considered significant.

Statistical analysis

Genes significantly mutated in male breast cancers were defined using Mutational Significance in Cancer (MutSigCV) version 1.4 as described previously (46). Genes of $q < 0.1$ were considered significantly mutated. Mutational frequencies and copy number data for the ER-positive/HER2-negative, ER-positive/HER2-positive, and luminal A/B female breast cancers from TCGA were obtained from the TCGA Data Portal (13). Comparisons of the number of nonsynonymous mutations between male breast cancer and female breast cancer/postmenopausal female breast cancer were performed using the Wilcoxon rank-sum test. Fisher exact tests were employed to compare the frequency of specific nonsynonymous mutations or the frequency of a given gene being affected by nonsynonymous mutations in male breast cancer versus female breast cancer. Two-tailed P values were adopted for these analyses. For the statistical analysis, comparing the copy number profiles between subtypes of male breast cancer, copy number states were collapsed on a per gene basis and compared using Fisher exact tests corrected for multiple comparisons using the Benjamini–Hochberg method. P values <0.05 were considered statistically significant. Univariate survival analysis (disease-free and overall survival) was performed using the Kaplan–Meier method and analyzed using the log-rank test. Disease-free and overall survival were expressed as the number of months from diagnosis to the occurrence of an event (local recurrence/metastasis and disease-related death, respectively). All tests were two-sided. P values <0.05 were considered statistically significant. The above statistical analyses were performed with the software suites GraphPad Prism 6 (GraphPad Software Inc.), SPSS 20.0 (IBM), and R v3.0.2.

Results

Clinicopathologic features of male breast cancers

The median age at diagnosis of the 59 patients with male breast cancer was 66 years (range, 33–88 years). The median tumor size was 2.0 cm (range, 0.6–4.7 cm). Ten of the patients consented to germline genetic analysis and three were found to carry *BRCA2* germline mutations.

All 59 male breast cancers included in this study were subjected to central histologic review and review of the immunohistochemical results, which revealed that most male breast cancers were IDC-NSTs (91%) and that 7%, 49%, and 44% of cases were of histologic grades 1, 2, and 3, respectively (Table 1 and Supplementary Table S1). Fifty-seven male breast cancers were ER-positive/HER2-negative and two cases were ER-positive/HER2-positive.

The surrogate IHC definitions according to the St. Gallen criteria (27) for female breast cancers were used to define the intrinsic subtypes of the male breast cancers included in this

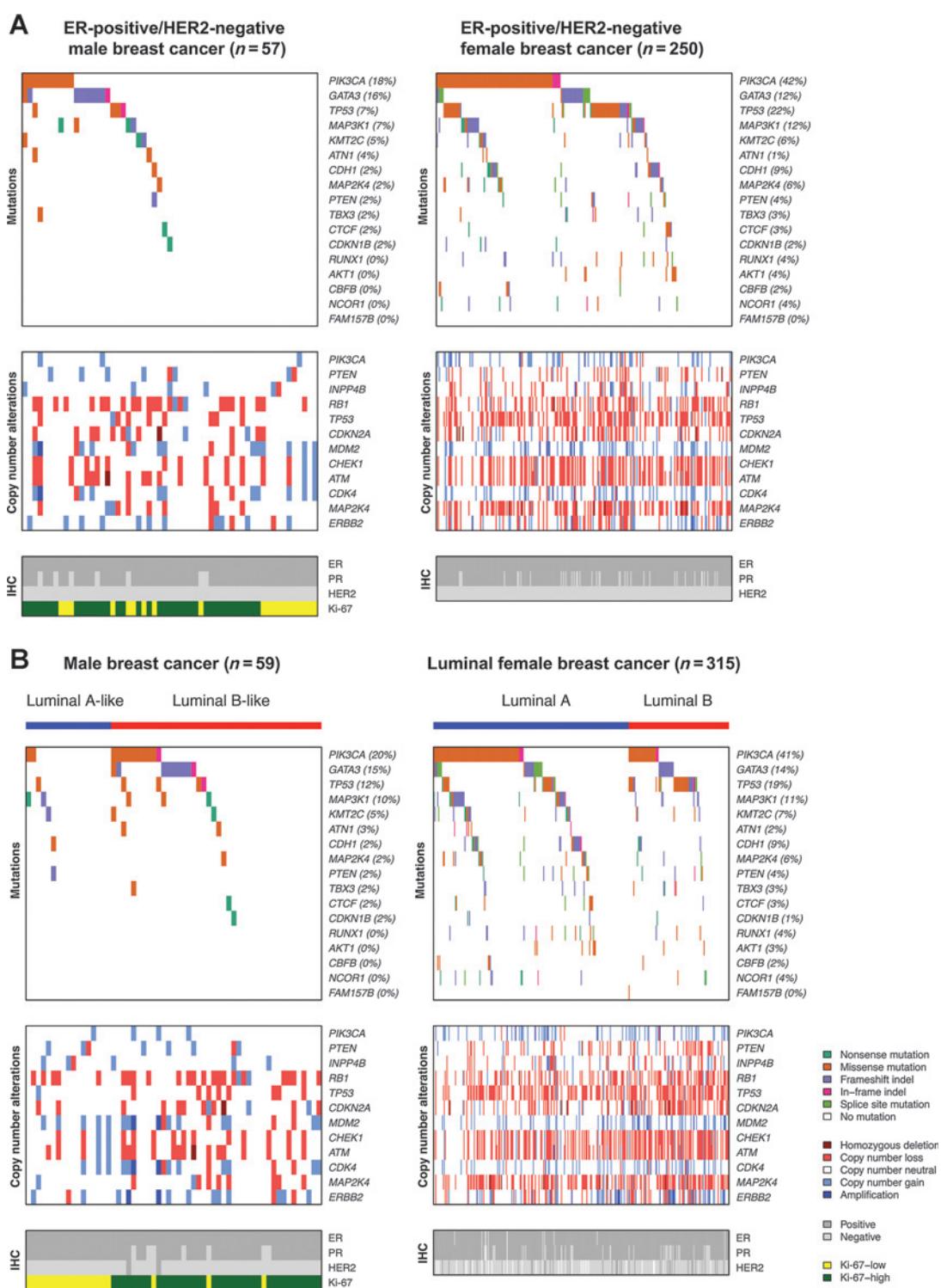
study. Briefly, luminal A-like tumors were defined as ER-positive ($>1\%$), PR-positive ($>20\%$), HER2-negative, and Ki-67-low ($<14\%$), whereas the remaining ER-positive tumors were classified as luminal B-like. Using this approach, we classified our cohort of male breast cancers into luminal A-like ($n = 17$; 29%) and luminal B-like ($n = 42$; 71%; Table 1 and Supplementary Table S1). The higher prevalence of luminal B-like male breast cancers is in contrast with the higher frequency of the luminal A subtype among female breast cancers. It should be noted, however, that the differences observed in the intrinsic subtype frequencies between male breast cancers and female breast cancers may in part be due to the moderate correlation between IHC and gene expression for intrinsic molecular subtyping.

Somatic genetic alterations in male breast cancers

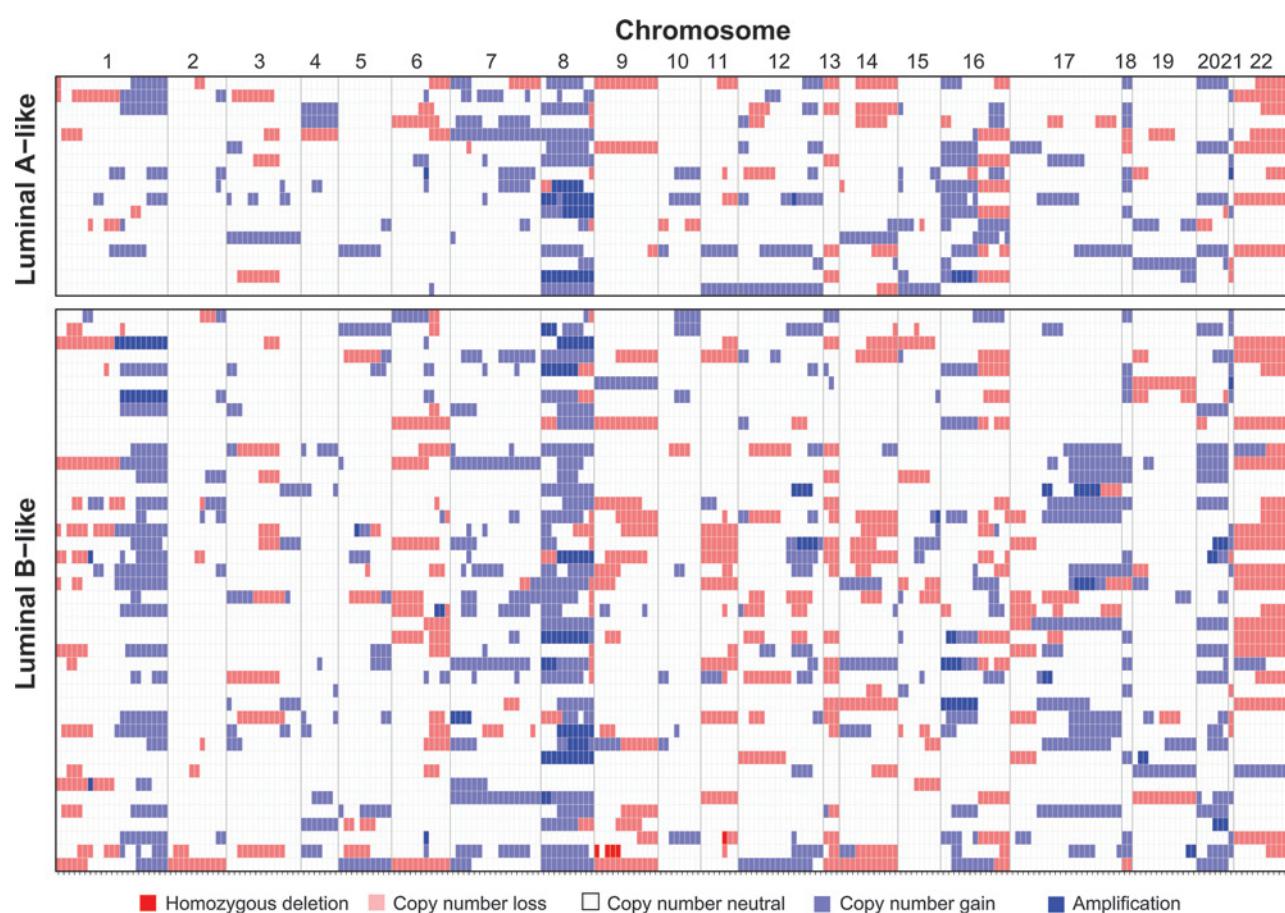
To define the repertoire of somatic genetic alterations in male breast cancers, we subjected the series of 59 cases to MPS of the coding regions of 241 genes (Supplementary Table S3), including the genes most frequently mutated in female breast cancers (13) and genes associated with DNA repair (28). Our MPS analysis (median depth of 626x; range, 135x–1881x; Supplementary Table S1) revealed somatic mutations in 102 of the 241 genes investigated, with a median of three nonsynonymous mutations per case (range, 0–24; Supplementary Table S4). As a validation, all samples were subjected to hotspot mutation screening of eight known cancer genes using Sequenom MassARRAY, which confirmed the genotype of all SNVs tested ($n = 26$, Supplementary Fig. S1A), demonstrating the accuracy of the MPS performed. In addition, all mutations selected for Sanger sequencing were validated (Supplementary Fig. S1B).

The genes most frequently mutated in male breast cancers were *PIK3CA* (20%) and *GATA3* (15%, Fig. 1 and Supplementary Fig. S2; Supplementary Tables S4 and S5). All but one *PIK3CA* mutation affected the hotspots E542, E545, or H1047 (Supplementary Tables S4). Of the 241 genes sequenced, MutSigCV demonstrated that *PIK3CA*, *GATA3*, *TP53*, and *MAP3K1* were significantly mutated in male breast cancers ($q < 0.1$; Supplementary Table S6). Cancer genes belonging to the Cancer Gene Census (43), or the 127 genes significantly mutated in cancer (42) and the Cancer5000-S pan-TCGA datasets (44), including *PIK3CA*, *GATA3*, *KMT2C*, and *TP53*, were also mutated in male breast cancers (Supplementary Table S4). All *PIK3CA* (12/12) and all but two *GATA3* (7/9) mutations were found to be clonal by ABSOLUTE analysis. In contrast, only two of five *TP53* mutations were considered to be clonal using the same methodology (Supplementary Fig. S3). One case displayed a *CDH1* missense mutation coupled with LOH of the wild-type allele; this case was found to be a mixed ductal lobular carcinoma. Immunohistochemical analysis revealed that in this case, the ductal areas displayed strong E-cadherin and p120 catenin membranous expression, whereas the lobular carcinoma areas either lacked or displayed discontinuous E-cadherin membranous expression and displayed cytoplasmic expression of p120 catenin (Supplementary Fig. S4).

Analysis of the CNAs revealed that male breast cancers harbored recurrent gains of 1q, 8q, and 16p, and losses of 1p, 16q, and 17p (Fig. 2). High-level amplifications of 8p11.23 (14%, including *FGFR1* and *ZNF703*), 8q24.21 (17%, encompassing *MYC*), 17q23.2 (3%, including *PPM1D*), and 20q13.2 (3%, encompassing *AURKA*) were observed, as were homozygous deletions in *CDKN2A* (2%) and *ATM* (2%; Fig. 2).

**Figure 1.**

Repertoires of mutations and copy number alterations in male breast cancer, ER-positive/HER2-negative female breast cancer and female breast cancer of luminal subtype. Somatic mutations in the 241 genes, either recurrently mutated in female breast cancer or related to DNA repair, included in the targeted capture massively parallel sequencing assay employed in this study. The results for ER-positive/HER2-negative male breast cancer and ER-positive/HER2-negative female breast cancer (**A**) and male breast cancer and female breast cancer of luminal subtype (**B**) are ordered from top to bottom in decreasing order of mutational frequency in male breast cancers (top). Patterns of copy number alterations in male breast cancers and female breast cancers in selected genes (middle). Expression of ER, PR, HER2, and Ki-67 (male breast cancer only) as defined by immunohistochemistry (IHC) in the samples analyzed (bottom). In **B**, male breast cancers were classified into luminal A-like and luminal B-like subtypes defined based on their immunohistochemical profiles following the St Gallen's criteria (27), and female breast cancers are classified into luminal A and luminal B molecular subtypes based on PAM50. Sequencing data, copy number data, and intrinsic subtype information of the female breast cancers were retrieved from The Cancer Genome Atlas (13).

**Figure 2.**

Patterns of copy number alterations in male breast cancer. Repertoire of copy number alterations as defined by targeted capture massively parallel sequencing in male breast cancers classified into luminal A-like and luminal B-like subtypes. Samples are distributed along the y-axis; the 232 genes mapping to autosomes included in the targeted capture massively parallel sequencing assay are distributed according to their genomic position on the x-axis. Dark blue, amplification; light blue, copy number gain; white, neutral; light red, copy number loss; dark red, homozygous deletion.

As an exploratory, hypothesis-generating analysis, we sought to define whether the presence of mutations affecting highly recurrently mutated genes or affecting pathways whose genes were recurrently affected by somatic mutations would be associated with the disease-free or overall survival of patients with male breast cancer. Univariate survival analysis revealed that mutations in *GATA3* were found to be associated with worse disease-free survival in patients with male breast cancer ($P = 0.038$, Supplementary Fig. S5) and mutations affecting DNA repair pathway-related genes were associated with worse disease-free survival and overall survival ($P = 0.036$ and $P = 0.041$, respectively; Supplementary Fig. S5). None of these associations were found to be statistically significant on multivariate models including age, size, and lymph node status (data not shown).

Genomic landscape of luminal A-like and luminal B-like male breast cancers

As an exploratory, hypothesis-generating analysis, we compared the repertoires of somatic genetic alterations between luminal A-like ($n = 17$; 29%) and luminal B-like ($n = 42$; 71%) male breast cancers. The most frequently mutated genes in luminal A-like male breast cancers included *PIK3CA*, *HERC2*,

and *MAP3K1* (all at 12%), whereas the most frequently mutated genes in luminal B-like male breast cancers were *PIK3CA* and *GATA3* (24% and 21%, respectively, Supplementary Table S7). *GATA3* mutations were found only in luminal B-like male breast cancers (21% vs. 0% in luminal A-like male breast cancers, Fisher exact test $P = 0.0482$, Supplementary Table S7; Fig. 1). Interestingly, the pattern of mutations found in *GATA3* did not resemble that of female breast cancers by TCGA (Supplementary Fig. S5; ref. 13), in which hotspot *GATA3* mutations differed according to luminal subtype (at residues S308 and S407 in luminal A and luminal B subtypes, respectively). In fact, seven of nine *GATA3* mutations found in male breast cancers were frameshift mutations and none of these affected the aforementioned hotspots.

We next mapped the mutated genes to KEGG pathways, including DNA repair, homologous recombination, PI3K/AKT/mTOR, and classical MAPK pathways and compared the number of luminal A-like and luminal B-like male breast cancers harboring mutations in these pathways/networks (Supplementary Table S8). We found that DNA repair-related genes were more frequently mutated in luminal B-like (33%) than in luminal A-like male breast cancers (6%, Fisher exact test $P = 0.04$; Supplementary Table S8).

In contrast with the distinct repertoires of somatic mutations among luminal A-like and B-like male breast cancers, the two cohorts exhibited remarkably similar CNA profiles, both subtypes displaying recurrent gains of 1q, 8q, and 16p and losses of 1p and 16q (Fig. 3).

Male breast cancer is different from female breast cancer and postmenopausal female breast cancer

We next compared the mutational landscape of male breast cancers with that of female breast cancers from TCGA (13). Given that the male breast cancers analyzed here were consistently ER-positive, we specifically compared their repertoire of somatic genetic alterations to that of ER-positive female breast cancers and to that of postmenopausal ER-positive female breast cancers (13). The latter comparison was performed given the similarities between male breast cancers and postmenopausal female breast cancers in terms of hormonal exposure and age at presentation. Given that only two of the male breast cancers were ER-positive/HER2-positive, we have restricted the comparisons to ER-positive/HER2-negative male breast cancers and ER-positive/HER2-negative female breast cancers and postmenopausal ER-positive/HER2-negative female breast cancers. We observed that the number of somatic nonsynonymous mutations in the 241 genes profiled in ER-positive/HER2-negative male breast cancers (mean 3.4 per case; range, 0–24) was similar to that of both sets of ER-positive/HER2-negative female breast cancers/postmenopausal female breast cancers (means 2.9 and 3.0, respectively; Wilcoxon

test $P > 0.05$; Supplementary Table S9; ref. 13). Genes reported to be recurrently mutated in ER-positive/HER2-negative female breast cancers and postmenopausal ER-positive/HER2-negative female breast cancers, including PIK3CA, GATA3, MAP3K1, and TP53, were also altered in ER-positive/HER2-negative male breast cancers, but at significantly different frequencies. In fact, we found a lower frequency of PIK3CA mutations in ER-positive/HER2-negative male breast cancers compared with ER-positive/HER2-negative female breast cancers/postmenopausal female breast cancers (18% vs. 42% and 42%, respectively; Fisher exact tests, $P = 0.0005$ and $P = 0.0014$, respectively, Fig. 1; Supplementary Table S5), as well as a higher frequency of mutations affecting the E3 ubiquitin-protein ligase gene HERC2 (7.0% vs. 0.8% and 0.8%, respectively; Fisher exact tests, $P = 0.0121$ and $P = 0.0296$, respectively; Supplementary Table S5). Lower frequencies of mutations in HUWE1 and TP53 were detected when ER-positive/HER2-negative male breast cancers were compared with germline BRCA2-mutant ER-positive/HER2-negative female breast cancers (HUWE1: 0% vs. 25%, TP53: 7% vs. 38%, Fisher exact tests, $P = 0.0135$ and $P = 0.0348$, respectively; Supplementary Table S5). Of note, additional genes identified as significantly mutated genes in female breast cancers, including RUNX1, AKT1, and NCOR1, were not found to be mutated in this cohort of male breast cancers (Supplementary Table S5). Given that none of the male breast cancers included in this study were classified as invasive lobular carcinomas, we repeated the analyses comparing the

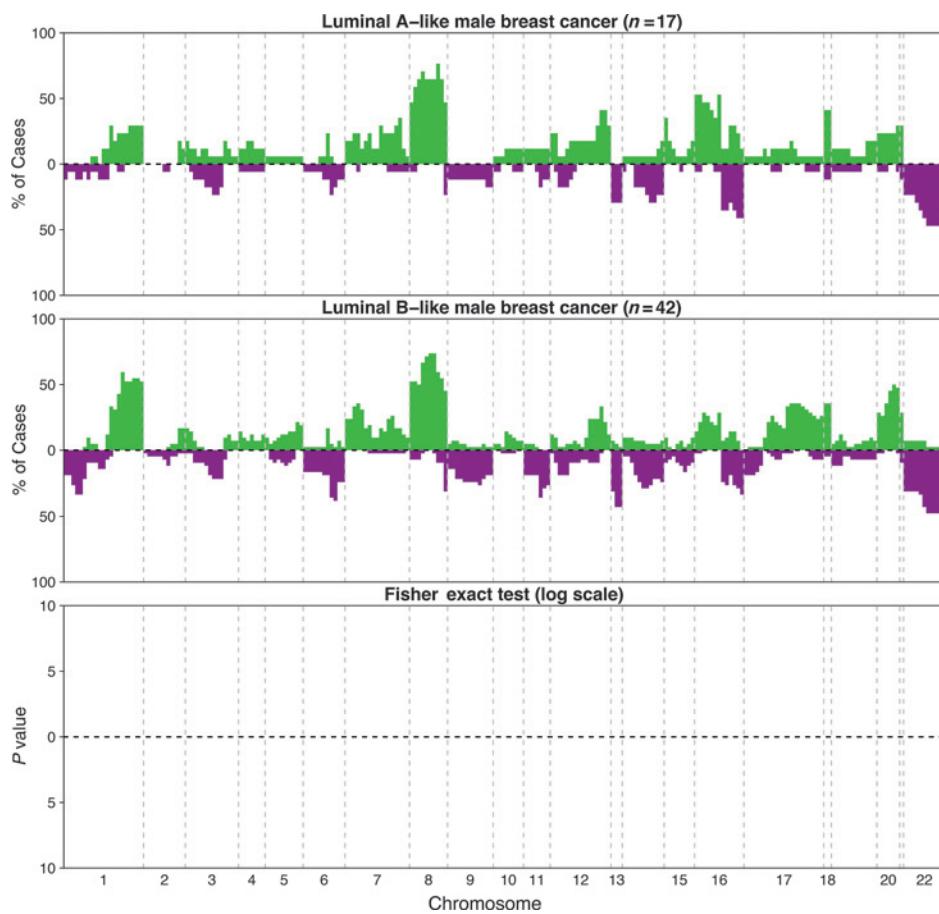
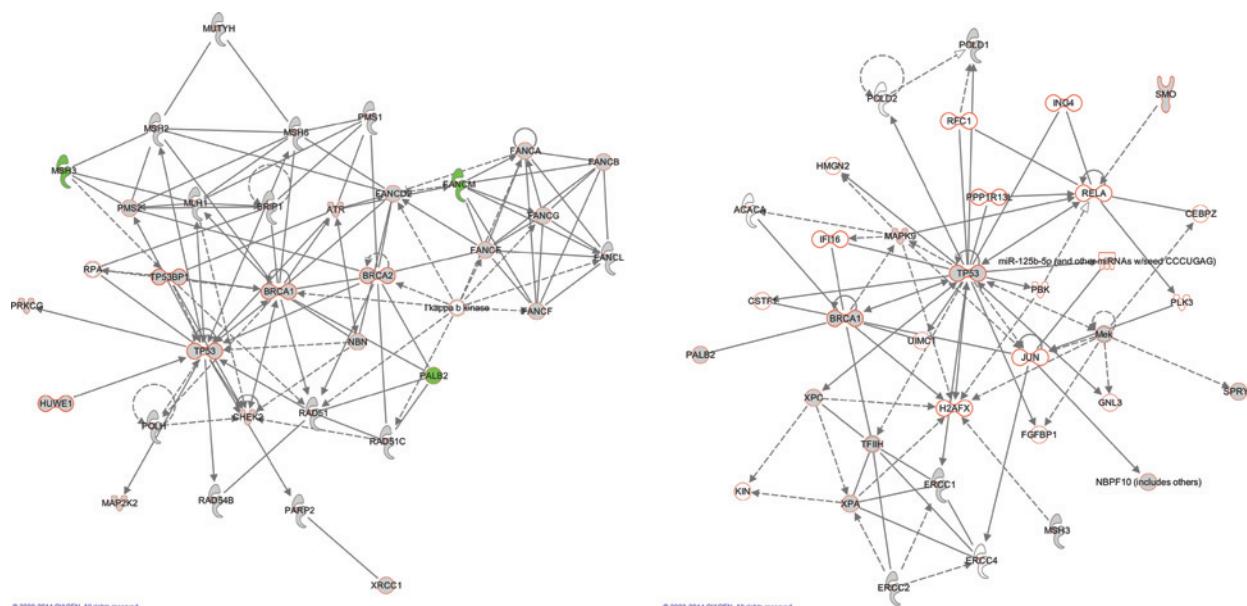


Figure 3.

Comparative genomic profiling of luminal A-like and luminal B-like male breast cancers. Frequency plots and multi-Fisher exact test comparisons of chromosomal gains and losses in luminal A-like (top) and luminal B-like (middle) male breast cancers. The frequency of gains (green bars) or losses (purple bars) for each gene is plotted on the y-axis, according to their genomic position on the x-axis. Inverse Log₁₀ values of the Fisher exact test P values are plotted according to genomic location (x-axis) (bottom). Note the lack of any significant differences in the copy number profiles between luminal A-like and luminal B-like male breast cancers.

**Figure 4.**

Ingenuity Pathway Analysis of 25 genes mutated in ER-positive/HER2-negative male breast cancers but not in ER-positive/HER2-negative female breast cancers from The Cancer Genome Atlas study. Likely pathogenic mutations present in ER-positive/HER2-negative male breast cancers but not targeted by likely pathogenic mutations in ER-positive/HER2-negative female breast cancers from The Cancer Genome Atlas (TCGA) study were annotated using Ingenuity Pathway Analysis (IPA). The "DNA replication, recombination and repair" network was significantly enriched for the 25 genes affected by likely pathogenic mutations present in ER-positive/HER2-negative male breast cancers but not in ER-positive/HER2-negative female breast cancers from TCGA (IPA score 17). Two views of the network are shown.

repertoire of somatic mutations in ER-positive/HER2-negative male breast cancers with that of ER-positive/HER2-negative female breast cancers/postmenopausal female breast cancers, after the exclusion of all cases classified as "infiltrating lobular carcinoma" or "breast lobular adenocarcinoma" in the TCGA dataset. The differences reported above were confirmed (Supplementary Table S5).

Next, we compared the proportion of cases harboring mutations in selected pathways. This analysis revealed an enrichment of ER-positive/HER2-negative male breast cancers with mutations in genes affecting DNA repair pathway when compared with female breast cancers/postmenopausal female breast cancers of ER-positive/HER2-negative subtype (30% vs. 16% and 15%, respectively; Fisher exact tests, $P = 0.0211$ and $P = 0.0274$, respectively; Supplementary Table S8) but lower mutation rates in genes affecting the PI3K/AKT/mTOR pathway (37% vs. 58% in ER-positive/HER2-negative female breast cancer and 58% in postmenopausal ER-positive/HER2-negative female breast cancer; Fisher exact tests, $P = 0.0049$ and $P = 0.0073$, respectively; Supplementary Table S8).

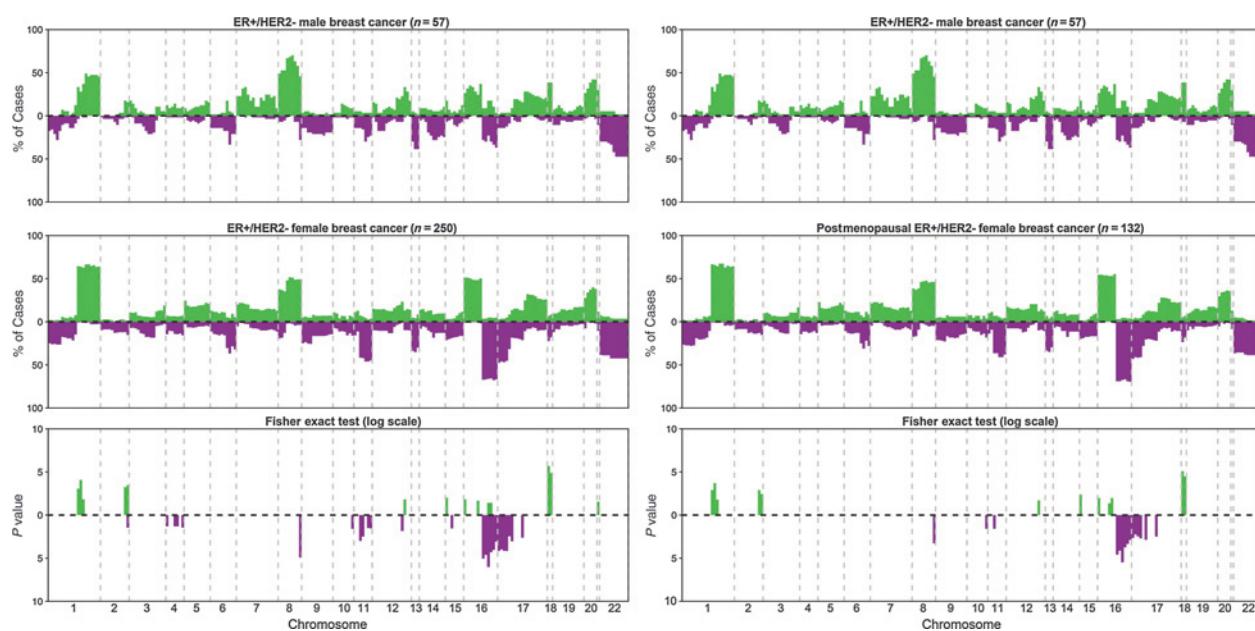
Of the 241 genes sequenced, 27 genes were found to be mutated exclusively in ER-positive/HER2-negative male breast cancers but not in female breast cancers of ER-positive/HER2-negative phenotype. A pathway and networks analysis of these genes using the IPA software revealed an enrichment of genes involved in DNA repair mechanisms (network score=17, Fig. 4). Of note, the genes enriched for DNA repair mechanisms, such as FANCM and PALB2, were also associated with LOH in the ER-positive/HER2-negative male breast cancers harboring mutations in these genes (Supplementary Table S4). Furthermore, using The Drug Gene Interaction Database (50), we investigated whether

any of these 27 genes exclusively mutated in ER-positive/HER2-negative male breast cancers would be actionable. This analysis revealed that ten genes, including PRKCA and RICTOR, may be actionable by a variety of antineoplastic drugs (Supplementary Table S10).

In terms of CNAs, ER-positive/HER2-negative male breast cancers and female breast cancers/postmenopausal female breast cancers of ER-positive/HER2-negative subtype shared many of the recurrent amplifications, gains, and losses, including gains of 1q, 8q, and 16p, losses of 1p and 16q, and amplifications of 8q. Losses of 16q and 17p were significantly less frequent in ER-positive/HER2-negative male breast cancers than in ER-positive/HER2-negative female breast cancers/postmenopausal female breast cancers (Fisher exact test $P < 0.05$; Fig. 5 and Supplementary Fig. S6). Given the high frequency of 16q losses and 16p gains in lobular carcinomas and that none of the male breast cancers included in this study were of invasive lobular carcinoma histologic type, we compared the repertoire of CNAs in male breast cancers and female breast cancers/postmenopausal female breast cancers, after the exclusion of all cases classified as "infiltrating lobular carcinoma" or "breast lobular adenocarcinoma" in the TCGA dataset. The differences reported above were confirmed (Supplementary Fig. S7).

Luminal A-like and luminal B-like male breast cancers are distinct from luminal A and luminal B female breast cancers/postmenopausal female breast cancers

As another exploratory, hypothesis-generating analysis, we compared male breast cancers stratified into luminal A-like and luminal B-like by IHC with female breast cancers/postmenopausal female breast cancers stratified into luminal A and B intrinsic

**Figure 5.**

Patterns of copy number alterations in male breast cancers and ER-positive/HER2-negative female breast cancers/postmenopausal ER-positive/HER2-negative female breast cancers from The Cancer Genome Atlas study. Frequency plots of chromosomal gains and losses in 57 ER-positive/HER2-negative male breast cancers compared with 250 ER-positive/HER2-negative female breast cancers (left) and 132 postmenopausal ER-positive/HER2-negative female breast cancers (right) from The Cancer Genome Atlas study. The frequency of gains (green bars) or losses (purple bars) for each gene is plotted on the y-axis, according to their genomic position on the x-axis. Inverse Log₁₀ values of the Fisher exact test *P* values are plotted according to genomic location (x-axis). Copy number data of female breast cancers were retrieved from The Cancer Genome Atlas (13).

subtypes by PAM50 (13). Similar to the previous analysis, luminal A-like male breast cancers displayed significantly fewer PIK3CA mutations than luminal A female breast cancer/postmenopausal female breast cancers (12% vs. 46% and 50%, respectively, Fisher exact tests, $P = 0.005$ and $P = 0.003$, respectively; Supplementary Table S11). Accordingly, within the luminal A subtype, the number of male breast cancers harboring mutations in the PI3K/AKT/mTOR pathway was significantly lower than in both female breast cancers and postmenopausal female breast cancers (29% vs. 61% and 65%, respectively, Fisher exact tests, $P = 0.02$ and $P = 0.01$, respectively; Supplementary Table S8). An enrichment of mutations in genes involved in the classical MAPK pathway was observed in luminal A-like male breast cancers as compared with female breast cancers of luminal A molecular subtype (24% vs. 7%, Fisher exact test, $P = 0.04$; Supplementary Table S8). Within the luminal B subtype, male breast cancers had a significantly lower frequency of TP53 mutations than female breast cancers/postmenopausal female breast cancers (10% vs. 34% and 43%, respectively; Fisher exact tests, $P = 0.002$ and $P = 0.0004$, respectively; Supplementary Table S12). Furthermore, genes related to DNA repair pathways were more frequently mutated in luminal B-like male breast cancers than in luminal B female breast cancers (36% vs. 18%, Fisher exact test, $P = 0.03$; Supplementary Table S8).

CNA analysis within luminal A and luminal B subtypes evaluated separately demonstrated similar copy number profiles between male breast cancers of luminal A-like or luminal B-like subtype and female breast cancers/postmenopausal female breast cancers of luminal A or B subtype, respectively, with notable differences in the frequency of 16q and 17p losses (Fisher exact test $P < 0.05$; Supplementary Fig. S8). These differences remained

significant after the exclusion of invasive lobular carcinomas from the TCGA dataset (Supplementary Fig. S9). No differences were observed in the pattern of gene amplifications upon stratification according to luminal subtypes (Supplementary Fig. S10).

Discussion

MPS approaches have provided evidence that breast cancers have complex and heterogeneous genomes and have offered means to study specific subsets of rare breast cancers (12). Here we characterized a series of 59 male breast cancers, which were found to display a luminal phenotype and to be largely of intermediate to high grade. Of the 10 patients who consented to genetic testing, three harbored germline *BRCA2* mutations and none carried a germline *PALB2* mutation. Germline genetic analyses were not performed in the remaining 49 patients. Our targeted sequencing analysis of somatic genetic alterations affecting 241 genes either frequently mutated in female breast cancer or directly related to DNA repair revealed that overall male breast cancers are heterogeneous at the mutational and CNA levels, and bear resemblance to ER-positive/HER2-negative female breast cancers and postmenopausal ER-positive/HER2-negative female breast cancers.

Despite the similarities between ER-positive/HER2-negative male breast cancers and ER-positive/HER2-negative female breast cancers, important differences were observed in their repertoire of somatic genetic alterations. Similar to female breast cancers, we demonstrated that male breast cancers constitute a heterogeneous disease, with a small number of significantly recurrently mutated genes, namely, PIK3CA, GATA3, TP53, and MAP3K1, as well as numerous genes harboring potentially pathogenic mutations at

lower frequencies. Compared with ER-positive/HER2-negative female breast cancers, male breast cancers less frequently harbored mutations in *PIK3CA* and genes involved in the PI3K/AKT/mTOR pathway. Male breast cancers also less frequently harbored *TP53* mutations, but more frequently displayed mutations in genes associated with DNA repair pathways. Further studies are warranted to investigate whether the lower frequencies of *TP53* mutations in male breast cancers may be compensated by the increased frequency of mutations in DNA repair-related genes in these tumors.

The landscape of CNAs found in male breast cancers closely recapitulates that of ER-positive/HER2-negative female breast cancers, with recurrent copy number gains of 1q, 8q, and 16p, and losses of 1p and 16q, as well as recurrent amplifications of the 8p11.23 and 8q24.21 loci. Male breast cancers, however, displayed 16q and 17p losses significantly less frequently than ER-positive/HER2-negative female breast cancers. Of note, the infrequent 17p losses, encompassing the *TP53* gene locus, coupled with infrequent mutations in *TP53* suggest that complete inactivation of *TP53* may not be as prevalent in male breast cancers compared to female breast cancers. These findings in conjunction with the lower prevalence of 16q loss and *PIK3CA* mutations in male breast cancers than in ER-positive/HER2-negative female breast cancers suggest that at least a subset of male breast cancers may be driven by a distinct repertoire of genetic alterations, and most likely, follow a different evolutionary pathway. Given that the adult male breast, outside of systemic hormonal imbalance, is not as fully developed as the adult female breast, and may be composed of cell populations distinct from those of the adult female breast, it could be posited that the genetic differences in female breast cancer and male breast cancer may be in part related to differences in their cell of origin or the differentiation status of the cell of origin of these tumors.

Actionable somatic genetic alterations, although present in male breast cancers, are limited compared with female breast cancers. For instance, *PIK3CA* mutations, which are present in approximately 35% of female breast cancers, were found in only 20% of the male breast cancers analyzed here. Importantly, however, we have identified potentially pathogenic somatic mutations affecting DNA repair-related genes, including *PALB2* and *FANCM*, which may also be exploited therapeutically. Given that the majority of patients with breast cancer enrolled in breast cancer-specific clinical trials are female and that the DNA repair-related genes mutated in male breast cancers are rarely altered in female breast cancers, studies investigating the potential use of therapeutic agents targeting DNA repair defects caused by the loss of function of these genes are warranted.

The survival analysis performed in this retrospectively accrued cohort of male breast cancers revealed that mutations affecting *GATA3* and DNA repair pathway-related genes were associated with disease-free and both disease-free and overall survival, respectively. These differences were not significant when tested in a multivariate survival model including known prognostic parameters (e.g. age, size, and nodal status). Further studies investigating the prognostic impact of mutations affecting *GATA3* and DNA repair pathway-related genes in larger cohorts of male breast cancers are warranted.

Our study has important limitations, including the retrospective nature of the study design, its relatively small sample size, and the fact that only 17% of the patients consented for germline

genetic analysis. Although we cannot exclude that the differences between male breast cancers and female breast cancers may have stemmed from differences in the prevalence of *BRCA2* mutation carriers in the cohort of male breast cancers analyzed here, a previous study (15) demonstrated that male breast cancers in *BRCA2* mutation carriers have a low frequency of *PIK3CA* mutations (6%), a gene whose mutation frequency was relatively high in our cohort (20%). Given that *BRCA2* mutations have been reported in 2% to 8% of all male breast cancers (1), the overall frequency of 5% of *BRCA2* germline mutations in the cases included in this study suggests that it is unlikely that a substantial number of *BRCA2* germline mutations were missed in the cohort included in this study. Moreover, the exploratory, hypothesis-generating analysis male breast cancers stratified into luminal A-like and luminal B-like was performed using a validated immunohistochemical surrogate for luminal A and luminal B breast cancers as defined by PAM50 (27); albeit validated, the agreement between this immunohistochemical surrogate and the PAM50 results for the classification of the same cases is by no means perfect. Despite these limitations, our data suggest that although overall male breast cancers and female breast cancers appear to share similar patterns of genetic alterations, their mutational landscapes differ, with notable differences in the distribution and frequency of the most frequently mutated genes. In particular, a significant enrichment for mutations in DNA repair-related genes was observed in male breast cancers. Given these important differences, further investigation to define potentially targetable genetic alterations and the optimal clinical management of male breast cancer is warranted. On the basis of this work, we would contend that caution should be exercised in the extrapolation of biologic findings and potential targeted therapies for male breast cancers from prior studies performed in female breast cancers.

Disclosure of Potential Conflicts of Interest

C.A. Eberle is an employee of Genomic Health, Inc. J.B. Hicks is a consultant/advisory board member for Celmatix, Inc. and Curis Inc. No potential conflicts of interest were disclosed by the other authors.

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