



Somatic and Germline Genomic Alterations in Very Young Women with Breast Cancer

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ABSTRACT

Purpose: Young age at breast cancer diagnosis correlates with unfavorable clinicopathologic features and worse outcomes compared with older women. Understanding biological differences between breast tumors in young versus older women may lead to better therapeutic approaches for younger patients.

Experimental Design: We identified 100 patients ≤ 35 years old at nonmetastatic breast cancer diagnosis who participated in the prospective Young Women's Breast Cancer Study cohort. Tumors were assigned a surrogate intrinsic subtype based on receptor status and grade. Whole-exome sequencing of tumor and germline samples was performed. Genomic alterations were compared with older women (≥ 45 years old) in The Cancer Genome Atlas, according to intrinsic subtype.

Results: Ninety-three tumors from 92 patients were successfully sequenced. Median age was 32.5 years; 52.7% of tumors were

hormone receptor-positive/HER2-negative, 28.0% HER2-positive, and 16.1% triple-negative. Comparison of young to older women (median age 61 years) with luminal A tumors ($N = 28$ young women) revealed three significant differences: *PIK3CA* alterations were more common in older patients, whereas *GATA3* and *ARID1A* alterations were more common in young patients. No significant genomic differences were found comparing age groups in other intrinsic subtypes. Twenty-two patients (23.9%) in the Young Women's Study cohort carried a pathogenic germline variant, most commonly (13 patients, 14.1%) in *BRCA1/2*.

Conclusions: Somatic alterations in three genes (*PIK3CA*, *GATA3*, and *ARID1A*) occur at different frequencies in young versus older women with luminal A breast cancer. Additional investigation of these genes and associated pathways could delineate biological susceptibilities and improve treatment options for young patients with breast cancer.

Introduction

Approximately 4% of breast cancers are diagnosed among women younger than 40 years old (1). It has long been recognized that young age at breast cancer diagnosis correlates with unfavorable clinicopathologic disease features and, by extension, worse outcomes compared with disease in older women (2). Many of these differences are

explained by the fact that distribution of breast cancer subtypes differs between young and older women, with more biologically aggressive intrinsic subtypes (basal-like and HER2-enriched) significantly overrepresented in the young. Early investigations of the biological differences between breast tumors in young versus older women showed that those differences were largely eliminated after accounting for intrinsic subtype (3). However, the availability of more comprehensive approaches for genomic analysis provides the opportunity to re-evaluate the biological landscape of breast cancers across age groups.

Moreover, examination of subtype-specific breast cancer outcomes by age at diagnosis has revealed an age-related prognostic difference among women with luminal A breast cancer. Specifically, younger women with luminal A tumors appear to experience worse outcomes than older women with luminal A tumors (4–6). The potential importance of very young age for outcomes and choice of therapy in luminal tumors was underscored in the SOFT/TEXT trials, where women under 35 years old were found to have a particularly high risk of recurrence and to benefit significantly from the addition of ovarian suppression to standard oral hormonal therapy (7). A clearer understanding of the biology underlying these observations could have important implications for optimizing therapy in young women with luminal breast cancer.

In this study, we performed whole-exome sequencing (WES)—comprising the coding sequence of $\sim 20,000$ genes—of both tumor and germline samples from a cohort of women age 35 years and younger at breast cancer diagnosis. We sought to define the genomic landscape of primary breast tumors in this very young cohort; to compare it to the landscape of tumors diagnosed in older women from The Cancer Genome Atlas (TCGA) in a subtype-specific manner; and to

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Translational Relevance

We performed whole-exome sequencing of tumor and germline for 92 women diagnosed with breast cancer at ≤ 35 years old, compared with older women (≥ 45 years old; median 61 years) in TCGA. In luminal A tumors, *PIK3CA* alterations were more common in older women, whereas *GATA3* and *ARID1A* alterations were more common in younger women; 23.9% of young women carried a pathogenic germline variant (14.1% in *BRCA1/2*), which in several cases had not been covered in patients' original clinical germline testing. These results point to biological differences that may underlie some of the clinically distinct behavior of luminal A tumors in younger versus older women (e.g., differential chemosensitivity); suggest chromatin and transcriptional profiling as an important focus for follow-up investigations of distinct therapeutic targets among young patients; and underscore the importance of repeat germline testing as clinical methodologies improve and panels expand, particularly for groups at high germline risk.

comprehensively identify all pathogenic germline variants within the cohort.

Materials and Methods

Patient cohort

This is a subcohort derived from the Young Women's Breast Cancer Study ($N = 1,302$), a larger prospective cohort study of women aged 40 years or younger at breast cancer diagnosis who sought care at one of 13 sites in the United States or Canada between 2006 and 2016. Eligible patients for the overall cohort were < 6 months from initial breast cancer diagnosis. Participants were identified by review of pathology records and clinic visit lists, then sent a formal invitation to participate by mail. Participation entails medical record review for clinical variables including breast cancer stage and subtype, and germline mutation status; collection of blood and tissue biospecimens; and completion of baseline and follow-up questionnaires (mailed every 6 months for three years following breast cancer diagnosis, then annually thereafter) on which patients self-report data including personal demographics, breast cancer treatments received, family history, pregnancy history, and disease status at follow-up. Categories of pregnancy-associated breast cancer were defined based on evolving standards (8). The study was approved by the institutional review board at Dana-Farber/Harvard Cancer Center and all participating centers, and all patients provided written informed consent prior to any study activities. The study was conducted in accordance with the Declaration of Helsinki. Additional study details have been described previously (9–11).

Participants in this genomic sequencing subcohort were ≤ 35 years old at breast cancer diagnosis and represent the first 100 patients enrolled into the larger cohort who met this age cut-off (though sequencing did not succeed in all selected patients). All patients in this subcohort were diagnosed between 2006 and 2013. Patient and disease characteristics were obtained through medical record review and patient surveys. Central pathology review for grade and histology was conducted for all participants, and biomarkers including estrogen receptor (ER), progesterone receptor (PR), and HER2 status were abstracted from pathology reports and designated based on standard clinical guidelines as described previously (9). Receptor status and

grade were used as a surrogate for intrinsic subtype, also as previously described, as follows: luminal A [ER-positive (ER+) and/or PR-positive (PR+), HER2–, and grade 1–2]; luminal B (ER+ and/or PR+ and HER2+, or ER+ and/or PR+ and grade 3); HER2 enriched (ER–, PR–, and HER2+); or basal-like (ER, PR, and HER2–; ref. 12).

WES

Tumor or germline DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue and peripheral blood mononuclear cells, respectively, and libraries for massively parallel sequencing were constructed as previously described, and are essentially the same as all protocols followed by TCGA (13). Sequencing data were generated via the Illumina HiSeq platform. The following pipeline was designed to match the software used to derive the TCGA-breast genomic data (14). Germline and tumor FASTQ files were aligned to the GRCh37 human reference assembly using BWA version 0.5.9 (15). The resulting BAM files were fed into Picard version 2.6–14 to filter out duplicate reads (MarkDuplicates) and flag OxoG artifacts (CollectOxoGMetrics; Picard Toolkit, Broad Institute, GitHub repository 2019). GATK version 3.1–1 was then used to fix misaligned short indels (IndelRealigner), adjust base quality scores (BaseRecalibrator), and in the case for germline samples, to call variants (HaplotypeCaller in GVCF mode; ref. 16). Somatic SNVs were called using MuTect version 1.1.6, and short indels were called using Strelka version 1.0.11, using default settings [LOD score > 6.3 for MuTect, read depth > 2 , combined tumor/normal variant allele frequency (VAF) $> 10\%$ for < 5 bp indels, VAF $> 2\%$ for > 5 bp indels, and downstream post-call filtration for Strelka; ref. 17). False-positive somatic calls were controlled using a panel of normals (PoN) derived from TCGA samples, and with candidate read realignment with BLAT & NovoAlign (<http://www.novocraft.com>; ref. 18). GATK FilterByOrientationBias was used to control FFPE artifacts. Visual verification of somatic mutations via IGV was performed for mutations in SLC22A2. TCGA-breast somatic mutations were downloaded from the Broad Institute TCGA Genome Data Analysis Center (GDAC; Broad Institute TCGA Genome Data Analysis Center: Firehose version 2016_01_28. doi:10.7908/C11G0KM9, 2016; accessed September 13, 2018) and clinical variables were downloaded from the Genomic Data Commons (GDC) portal (accessed September 13, 2018; refs. 14, 19).

Somatic copy-number variants (CNV) were called with ReCapSeg version 1.5.0, and ABSOLUTE version 1.06 was used to estimate tumor purity and ploidy, to detect subclonal copy-number alterations (SCNA), and to estimate mutation multiplicity (20). GISTIC 2.0 was used to detect significant areas of focal and arm-level amplifications and deletions (21). Significantly altered genes in the cohort were identified using MutSig2CV (22). Oncotator version 1.9.0 paired with the April 5, 2016, datasource corpus was used to annotate somatic SNVs and short indels (23). Mutational signatures were identified using SignatureAnalyzer-CPU (24). Fisher exact test (with Benjamini–Hochberg correction) was used to find Cancer Gene Census (CGC) genes (accessed May 27, 2020; ref. 25) significantly enriched or depleted in somatic mutations between cohorts (faceted by intrinsic subtype). Both somatic and germline statistical analyses were performed in Python using the Pandas, SciPy, and statsmodels modules. The oncoprint heatmaps depicting somatic alterations were made using ComplexHeatmap package (26). Somatic SNVs in *PIK3CA* were visualized as lollipop plots generated using the MutationMapper tool in cBioPortal (27).

An ordinary least squares (OLS) regression model was used to examine tumor mutational burden (TMB) as a function of diagnosis age and study cohort, faceted by intrinsic subtype. TCGA TMB data

were taken from a prior study on hypermutated breast cancer (28). The Python seaborn package was used to generate the visualization.

Pathogenic germline TCGA-breast cancer variants were derived from the TCGA PanCanAtlas germline working group Supplementary Materials (29). CharGer was used to isolate clinically relevant germline variants in 152 familial cancer-related genes, and gnomAD was used as an additional filter against low-quality ultrarare variants (30). Fisher exact test was used to compare the proportion of patients impacted by a pathogenic germline variant between cohorts.

Data availability statement

The data generated in this study are available within the article and its Supplementary Data files.

Results

Patient/tumor characteristics and breast cancer outcomes in young women

Patient and tumor characteristics for the overall cohort (93 tumors from 92 patients; one patient had bilateral disease) are shown in **Table 1**. Median age in the Young Women's Study cohort was 32.5 years (range 23–35 years). The majority of patients had hormone receptor-positive (HR+; meaning, ER+ and/or PR+)/HER2– breast cancer (52.7%), and 64.5% of patients had grade 3 breast cancer. Of note, 13 patients (14.0%) had received some neoadjuvant therapy prior to their sequenced tissue specimen. Given the very young age of women in the cohort overall, we assume that all or nearly all participants were premenopausal at breast cancer diagnosis. With 9.2 years of median follow-up, 4 patients had experienced locoregional breast cancer recurrence (without subsequent distant recurrence or death), 17 patients had experienced distant breast cancer recurrence, and 10 patients had died from breast cancer, for median distant recurrence-free survival of 9.5 years and median overall survival of 10.0 years.

Somatic genomic landscape in very young women

Analysis of somatic SNVs and short indels in the tumor samples demonstrated that the most common gene to contain SNVs and indels across the cohort was *TP53* (41% of patients), followed by *GATA3* (16%) and *PIK3CA* (14%; Supplementary Fig. S1). Full sequencing files are available in the data supplement (Supplementary Table S1; Supplementary Tables S2 and S3 provide associated clinical information by patient). Somatic CNV analysis revealed that *ERBB2* was the most commonly affected gene, with 22% of patients showing *ERBB2* amplification (Supplementary Fig. S2A). Analysis of significantly recurrently altered genomic alterations using MutSig2CV (**Fig. 1**) revealed 6 genes to be mutated more than expected by chance (in decreasing order of frequency): *TP53*, *GATA3*, *PIK3CA*, AT-rich interaction domain 1A (*ARID1A*), *MAP3K1*, and *SLC22A2*. Of these, *ARID1A* and *SLC22A2* were not identified in a previous landmark analysis of nonmetastatic primary breast tumors across all age groups (31). *SLC22A2* is a cation transporter with a role in cellular uptake of platinum chemotherapy agents (32); two tumors contained missense mutations in *SLC22A2* at the exact same locus, although of unknown functional consequence (Supplementary Fig. S3). *ARID1A*, a component of the SWI/SNF chromatin remodeling complex, was altered in 8% of patients in our cohort, nearly all with loss of function mutations (nonsense or frameshift).

We evaluated whether differences in somatic mutational profile (SNVs and short indels) were observed on the basis of parity at diagnosis or pregnancy-associated breast cancer status within the sequenced cohort. We did not find any significant genomic differences

between women who were parous versus nulliparous at breast cancer diagnosis (Supplementary Fig. S4). For women with pregnancy-associated breast cancer ($N = 37$; defined as breast cancer diagnosed during pregnancy or ≤ 5 years post-partum), there was a statistically significant enrichment in *TP53* and *GATA3* alterations, compared with women with nonpregnancy-associated breast cancer (Supplementary Fig. S5).

Homologous recombination deficiency (HRD), APOBEC, and microsatellite instability (MSI) were the three mutational signatures detectable within the cohort (**Fig. 1**). The presence of the HRD signature was associated with basal-like subtype (Wilcoxon rank-sum $P < 7e-6$). Copy-number changes were analyzed and visualized in Supplementary Figs. S2A and S2B; full results of CNV analysis are included in the data supplement (Supplementary Table S4).

Comparison of somatic landscapes in very young and older women

We compared all SNVs and short indels identified in our cohort with all alterations identified in older women (age ≥ 45 years) in TCGA for breast tumors. Median age in the older TCGA cohort was 61 years (range 45–90 years; **Table 1**). To avoid confounding by intrinsic subtype or histology (both of which are known to differ significantly based on age at breast cancer diagnosis; refs. 3, 33, 34), we excluded women with pure lobular histology from these analyses to focus entirely on ductal histology, and performed each comparison within the four intrinsic subtypes (luminal A, luminal B, HER2 enriched, and basal-like; **Fig. 2A–D**; ref. 35). Patient and tumor characteristics within luminal A and luminal B intrinsic subtype patients from the very young women's cohort versus TCGA older women's cohort are shown in Supplementary Table S5. Among women with nonlobular luminal A tumors ($N = 28$), *PIK3CA* alterations were significantly enriched in older women (14% vs. 38% in younger vs. older women, respectively, $q < 0.05$), whereas *GATA3* and *ARID1A* alterations were significantly more common in younger women (*GATA3*: 43% vs. 12% in younger vs. older women, respectively, $q < 0.05$; *ARID1A*: 18% vs. 2% in younger vs. older women, respectively, $q < 0.05$; **Figure 2B**). Lollipop plot representations did not reveal any obvious difference between the localization of *PIK3CA* mutations in very young versus older women, which were primarily canonical hotspot mutations in the helical and kinase domains (Supplementary Figs. S6A and S6B). Correlation of specific alterations with invasive disease-free survival (iDFS) events in young women was not evaluated due to insufficient power (only two iDFS events in young women with luminal A tumors). No significant differences between young and older women were identified with the same analysis among nonlobular luminal B ($N = 41$; **Fig. 2C**), HER2 enriched ($N = 4$), and basal-like tumors ($N = 17$; **Fig. 2D**). When the same analysis was run without consideration of intrinsic subtype or histology, *PIK3CA* alterations were still detected to be enriched in older women, and *CDH1* was found to be enriched in older women (reflecting the known increased prevalence of lobular histology), but other significant differences were not found. Analysis of pure lobular histology tumors was not performed due to small numbers of this histology in the Young Women's Study cohort.

Comparative analysis of mutational signatures between the very young women's cohort and older women (≥ 45 years old) in TCGA revealed no statistically significant differences between the two age groups for the signatures represented among very young women (APOBEC, HRD, and MSI). TMB was also compared across age groups, and appeared overall similar. In the young women's cohort, median TMB was 1.55 mutations/megabase (mut/Mb; range 0.13–11.6) and one sample (1.1%) was hypermutated (defined

Table 1. Patient and tumor characteristics, treatments received, and breast cancer outcomes for the sequenced young women's cohort versus TCGA ≥ 45 -yo cohort.

Parameter	YWBC (≤ 35 yo), N = 92 patients No. of patients (%)	TCGA (≥ 45 yo), N = 925 patients No. of patients (%)
Age at diagnosis (years)	Median 32.5 (range 23-35)	Median 61 (range 45-90)
Race		
Caucasian	79 (85.9%)	649 (70.2%)
Black or African-American	0 (0%)	146 (15.8%)
Asian	7 (7.6%)	48 (5.2%)
Multi-racial	3 (3.3%)	Not collected
Other/unknown	3 (3.3%)	82 (8.9%)
Ethnicity		
Hispanic or Latina	6 (6.5%)	31 (3.4%)
Non-Hispanic or Latina	81 (88.0%)	744 (80.4%)
Unknown/did not answer	5 (5.4%)	150 (16.2%)
Anatomic stage at diagnosis		
0 (DCIS)	3 (3.3%)	0 (0%)
I	32 (34.4%)	160 (17.3%)
II	46 (49.5%)	525 (56.8%)
III	12 (12.9%)	201 (21.7%)
IV	0 (0%)	18 (1.9%)
Unknown	0 (0%)	21 (2.3%)
T stage		
Tis	3 (3.3%)	0 (0%)
T1	45 (48.9%)	241 (26.1%)
T2	39 (42.4%)	535 (57.8%)
T3	5 (5.4%)	112 (12.1%)
T4	0 (0%)	34 (3.7%)
TX	0 (0%)	3 (0.3%)
N stage		
N0	47 (51.1%)	455 (49.2%)
N1	35 (38.0%)	289 (31.2%)
N2-3	8 (8.7%)	162 (17.5%)
NX	2 (2.2%)	19 (2.1%)
Tumor grade		
1	5 (5.4%)	
2	28 (30.1%)	
3	60 (64.5%)	
Receptor status		
ER+ or PR+/HER2- HER2+	49 (52.7%)	
ER- /PR- /HER2- Other (HER2 indeterminate or unknown)	26 (28.0%) 15 (16.1%) 3 (3.2%)	
Histology		
Ductal	70 (75.3%)	643 (69.5%)
Lobular	2 (2.2%)	190 (20.5%)
Mixed ductal and lobular	8 (8.6%)	Not collected
Other/unknown	13 (14.0%)	92 (9.9%)
Intrinsic subtype ^a		
Luminal A	28 (30.1%)	429 (46.4%)
Luminal B	43 (46.2%)	169 (18.3%)
HER2	4 (4.3%)	68 (7.4%)
Basal-like	17 (18.3%)	139 (15.0%)
Normal	0 (0%)	29 (3.1%)
Unknown	1 (1.1%)	91 (9.8%)

(Continued on the following column)

Table 1. Patient and tumor characteristics, treatments received, and breast cancer outcomes for the sequenced young women's cohort versus TCGA ≥ 45 -yo cohort. (Cont'd)

Parameter	YWBC (≤ 35 yo), N = 92 patients No. of patients (%)	TCGA (≥ 45 yo), N = 925 patients No. of patients (%)
Surgery at original diagnosis		
Lumpectomy	32 (34.8%)	
Unilateral mastectomy	20 (21.7%)	
Bilateral mastectomy	40 (43.5%)	
Chemotherapy receipt		
Yes	79 (85.9%)	
No	13 (14.1%)	
Hormonal therapy receipt ^b		
Yes	70 (76.1%)	
No	22 (23.9%)	
Received neoadjuvant therapy prior to sequenced tissue specimen		
Yes	13 (14.0%)	
No	80 (86.0%)	
Parity at breast cancer diagnosis		
Nulliparous	43 (46.7%)	
Parous	49 (53.3%)	
Number of prior pregnancies	Median 2 (range 1-7)	
Pregnancy-associated breast cancer ^c		
Breast cancer during pregnancy	5 (5.4%)	
Breast cancer ≤ 1 year post-partum	7 (7.6%)	
Breast cancer > 1 year and ≤ 5 years post-partum	25 (27.2%)	
Invasive disease-free survival events ^c (median follow-up 9.2 years)		
None	71 (77.2%)	
Ipsilateral locoregional recurrence	4 (4.3%)	
Distant recurrence	17 (18.5%)	
Contralateral new breast primary invasive tumor	0	
Breast cancer death	10 (10.9%)	

Abbreviations: DCIS, ductal carcinoma *in situ*; TCGA, The Cancer Genome Atlas; yo, years old; YWBC, young women's breast cancer cohort.

Note: Ninety-two patients were included in the young women's cohort, comprising 93 specimens (one patient with bilateral breast cancers). Per patient parameters are listed with N = 92, per specimen parameters are listed with N = 93. Treatments received are for original diagnosis of nonmetastatic disease. Nine hundred twenty-five patients were in the older TCGA cohort. Parameters from the TCGA cohort that were not collected or not collected in the same manner as the YWBC cohort are left blank in the TCGA column.

^aSee intrinsic subtype definitions in Materials and Methods section. One patient with ER+/PR+/HER2 unknown and grade 2 disease was categorized as luminal A.^bHormonal therapy receipt was categorized as "yes" if a patient received any hormonal treatment approach (including ovarian suppression, tamoxifen, aromatase inhibitor, or other) through 5 years from study enrollment. The cohort included 72 patients with ER+ or PR+ tumor (either HER2+ or HER2-), of whom 70 received some hormonal therapy.^cBreast cancer during pregnancy was defined as breast cancer diagnosis date within 40 weeks prior to a child's reported birth date.^dIpsilateral locoregional recurrence indicates patients with isolated locoregional recurrence only, without concurrent distant recurrence (there were no patients with locoregional recurrence and subsequent distant recurrence). Breast cancer death is not mutually exclusive of other categories.

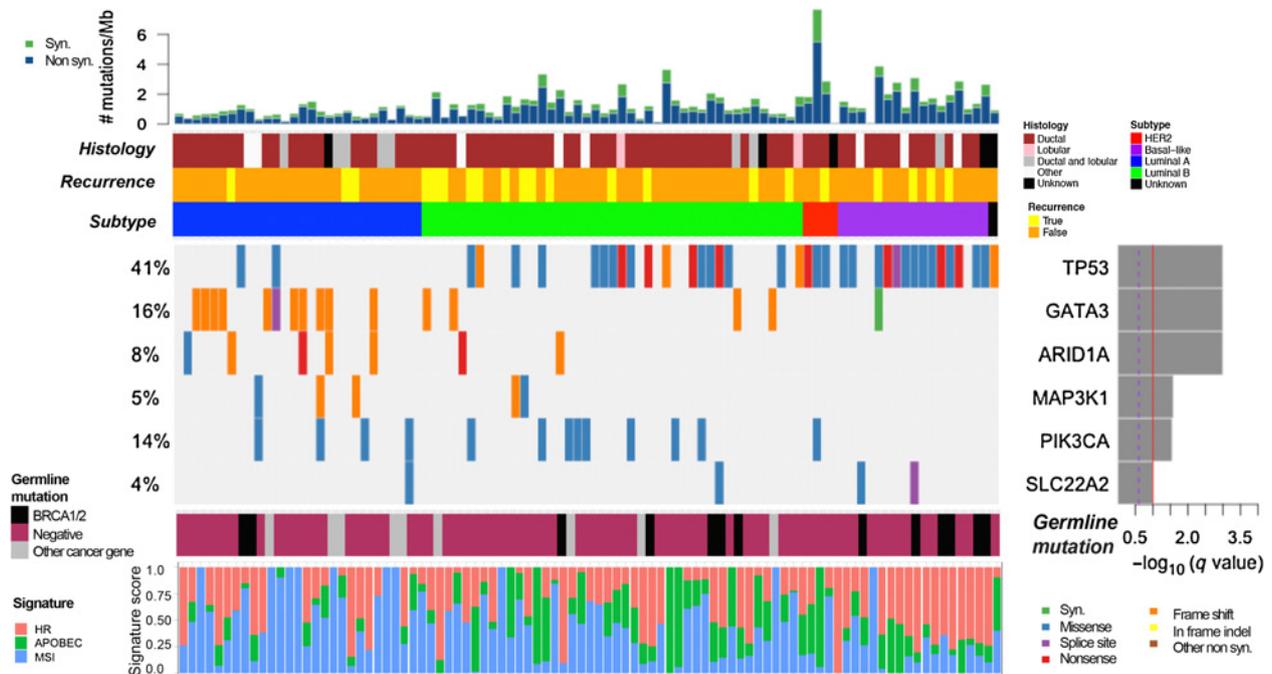


Figure 1.

Significant SNVs, short indels, and signature analysis. Computation plot showing recurrent somatic alterations in significantly mutated genes across the cohort ($N = 93$) as analyzed by MutSig2CV. *TP53*, *GATA3*, *ARID1A*, *MAP3K1*, *PIK3CA*, and *SLC22A2* are significantly mutated. The P values were computed using the Fisher method and truncated product method. FDR (q values) were generated using the Benjamini-Hochberg method to correct for multiple hypotheses. Genes that have a $-\log_{10} q$ -value ≥ 1 (red line) are considered significant. Bar graph (top) depicts the TMB (mutations/megabase) of each patient's tumor samples, followed by clinical annotations depicting histology, disease recurrence, and breast cancer subtype (key to the right of panel). Bottom panel annotations show cancer-specific pathogenic germline variants, and somatic mutational signatures of homologous recombination (HR) deficiency, APOBEC activity, and microsatellite instability (MSI).

as ≥ 10 mut/Mb). Among older women in TCGA, median TMB was 1.13 mut/Mb (range 0.025–142.6) and 10 samples (1.21%) were hypermutated. Incorporating both the young women's cohort patients and TCGA patients of all ages, we analyzed the correlation of TMB with age according to intrinsic subtype, and found no statistically significant correlation between TMB and age except in the HER2-enriched subpopulation, where there was a positive correlation between increasing age and increasing TMB (Supplementary Fig. S7).

Germline genomic landscape of very young women

We examined the frequency and identities of pathogenic variants in germline DNA from our cohort of very young women and from older women (age ≥ 45 years) in TCGA. Median age in the older TCGA cohort was 61 years (range 45–90 years). Pathogenic germline variants were defined by the TCGA PanCanAtlas germline working group as described previously, with CharGer used to isolate clinically relevant variants (29, 30). Of the 92 women in our cohort, 22 (23.9%) carried a pathogenic germline variant, of which 13 (14.1%) had alterations in *BRCA1* or *BRCA2*, and 10 (10.9%) had an alteration in another cancer-related gene. These frequencies were similar to the youngest women (≤ 35 years old; median 32.5 years) in TCGA ($N = 34$). By comparison, among older women in TCGA ($N = 925$), 8.8% carried a pathogenic germline variant, of which 3.0% had alterations in *BRCA1/2*, and 5.7% had an alteration in another cancer-related gene (Table 2). All genes with pathogenic cancer-related germline hits by cohort are shown in Table 2. Supplementary Table S6 shows all pathogenic hits in any germline gene, cancer-related and noncancer-related.

All patients in whom WES identified a pathogenic germline variant had also undergone some clinical germline genetic testing in the past, though because all patients in this cohort were diagnosed with breast cancer between 2006 and 2013, the clinical testing these patients received typically involved only a small number of genes as opposed to the broader germline panels used today. Across all patients in our cohort, *BRCA1/2* had been clinically evaluated in all patients; *TP53* had been evaluated in 11% of patients; and additional genes had been evaluated in only two patients. Table 3 shows results from clinical germline genetic testing compared with germline WES performed in this study. WES identified all pathogenic variants found through clinical testing. For four additional patients, WES identified pathogenic variants in commonly described breast cancer susceptibility genes (*PALB2* in three patients; *TP53* in one patient) that had not been performed in the prior clinical genetic testing. For six more patients, WES identified pathogenic variants in cancer susceptibility genes never or rarely linked to breast cancer, which also had not been included in these patients' prior clinical genetic testing (*BUB1B* in two patients; *COL7A1*, *GJB2*, and *PRSSI* in one patient each; one final patient with germline pathogenic *BRCA2* mutation had a *PRSSI* mutation as well; Table 3; refs. 36, 37).

Discussion

In this work, we compared the somatic and germline landscape of very young women to older women with breast cancer. By focusing on a particularly young patient subset (≤ 35 years old, whereas many other investigations have used a cut off of ≤ 40 years old) and segregating

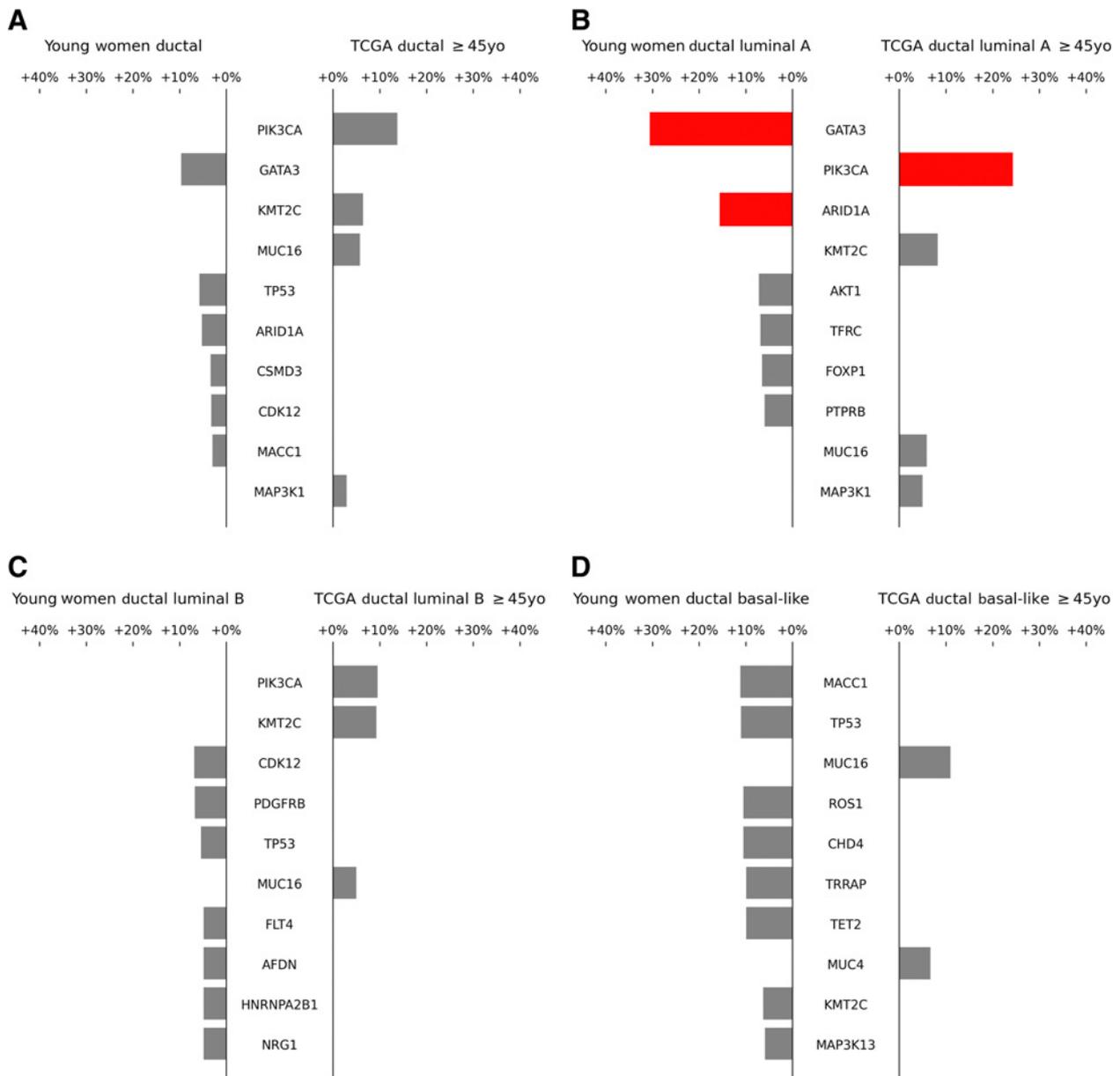


Figure 2. Comparison of single nucleotide and short indel prevalence between Young Women's Breast Cancer Study cohort (≤35 years old) and TCGA patients ≥45 years old. **A**, All intrinsic subtypes; **B**, Luminal A; **C**, Luminal B; **D**, Basal-like. HER2-enriched subtype is not shown as there were only 4 Young Women's Breast Cancer Study cohort samples in this subtype. Analysis excluded patients with pure lobular tumor histology. Forty genes identified from the 2016 METABRIC study (35), in addition to one gene found to be significant by MutSig, were included for analysis. Only differences in the frequencies of alteration of each gene between the two cohorts, as opposed to the absolute frequency of alterations within each cohort, are depicted by the bars. Statistically significant differences (FDR < 5%) are highlighted in red.

analyses by tumor histology and intrinsic subtype, we sought to specifically identify genomic alterations that may have clinical importance for young women with breast cancer. Our focus on women ≤35 years old was also driven by the differential treatment implications around this age cut-off for premenopausal women participating in the SOFT/TEXT trials (7).

In the somatic genomic landscape of very young women with luminal A tumors, we identified fewer *PIK3CA* alterations and more alterations in *GATA3* and *ARID1A* compared with older women. Previous literature demonstrates that younger women with luminal A

tumors experience worse breast cancer outcomes, and a number of reasons for this have been suggested including a lower chance of permanent chemotherapy-associated amenorrhea among younger women, decreased adherence to endocrine therapy among younger women, and inherent biology (4–6). *PIK3CA* mutations affect the catalytic subunit of a kinase in the PI3K pathway, which is overactive in a number of cancers including breast cancer. In early-stage breast cancer, the presence of a *PIK3CA* mutation correlates with improved long-term outcome (38), which is congruent with our observation that *PIK3CA* mutations are more common in luminal A tumors from older

Table 2. Comparison of pathogenic germline findings by WES from Young Women’s Breast Cancer Study cohort vs. TCGA.

Patient cohort (age group)	Pathogenic germline variant in any cancer gene no. (%)	Pathogenic germline variant in <i>BRCA1/2</i> no. (%)	Pathogenic germline variant in non- <i>BRCA1/2</i> cancer gene no. (%)
Young Women’s Breast Cancer Study (≤35 yo), N = 92	22 (23.9%) ^a	13 (14.1%) <i>BRCA1</i> (10 patients), <i>BRCA2</i> (3 patients)	10 (10.9%) <i>PALB2</i> (3 patients), <i>BUB1B</i> (2 patients), <i>TP53</i> (1 patient), <i>PRSS1</i> (2 patients), <i>GJB2</i> (1 patient), <i>COL7A1</i> (1 patient)
TCGA (≤35 yo), N = 34	9 (26.5%) ^a	6 (17.6%) <i>BRCA2</i> (4 patients), <i>BRCA1</i> (2 patients)	3 (8.8%) <i>TP53</i> (1 patient), <i>ATR</i> (1 patient), <i>ATM</i> (1 patient), <i>BRIPI</i> (1 patient)
TCGA (≥45 yo), N = 925	81 (8.8%) ^a	28 (3.0%) <i>BRCA1</i> (14 patients), <i>BRCA2</i> (14 patients)	53 (5.7%) <i>ATM</i> (8 patients), <i>ATR</i> (3 patients), <i>BRIPI</i> (3 patients), <i>CHEK2</i> (3 patients), <i>PALB2</i> (3 patients), Other (34 patients)
Comparison between YWBCS ≤35 yo and TCGA ≥45 yo	P = 4.0e−5	P = 2.5e−5	P = 0.066
Comparison between TCGA ≤35 yo and TCGA ≥45 yo	P = 2.8e−3	P = 8.3e−4	P = 0.44

Abbreviations: TCGA, The Cancer Genome Atlas; yo, years old; YWBCS, Young Women’s Breast Cancer Study.

^aTwo TCGA patients (one ≤35 years, one ≥45 years) had two pathogenic germline variants. One YWBCS patient had two pathogenic germline variants.

women, in whom prognosis is more favorable. Whether the level of PI3K pathway activity plays a causative role in the differential prognosis bears further investigation, which could shed light on the biological drivers of poorer prognosis in younger women.

Increased *GATA3* alterations observed in very young women with luminal A tumors is of interest, but with unclear biological implications. *GATA3*, a transcription factor with important roles in ER-regulated transcription and maintenance of luminal differentiation, is commonly mutated in breast tumors (39, 40). Two other groups have also reported a significantly higher percentage of *GATA3* alterations in younger patients with breast cancer (33, 41). Lower *GATA3* expression correlates with worse prognosis, thus its increased alterations in younger patients could possibly explain some of their less favorable outcomes. However, the biological effects of *GATA3* in breast cancer in general are uncertain: evidence has suggested both oncogenic and tumor suppressor roles, and different mutations may be gain-of-function or loss-of-function (33, 40).

ARID1A is a component of the chromatin-regulating SWI/SNF complex and regulates ER-dependent transcriptional programs. Loss or depletion of *ARID1A* causes a switch in cell identities from luminal to basal-like (39). Alterations in *ARID1A* are well documented to correlate with worsened clinical outcomes among patients with breast cancer (39, 42), consistent with the increased frequency of *ARID1A* alterations in very young women with luminal A tumors in our cohort. Decreased *ARID1A* causes resistance to both tamoxifen and fulvestrant in breast cancer cell line models. In contrast, preclinical experiments suggest that *ARID1A*-mutated breast cancers may be sensitive to BET inhibitors (39, 42). The biological implications of increased *ARID1A* alterations among young patients with breast cancer should be further explored, with the hypothesis that *ARID1A* alteration may cause a more ER-independent transcriptome even in tumors that appear to be “luminal A” by histopathology. This in turn could clarify a mechanism behind poorer prognosis in young women with luminal A tumors, and suggest chromatin regulatory elements as a therapeutic target.

Our findings build on previous findings in breast cancer genomics of young women in multiple important ways, and broaden the clinical implications. In a previous paper by Azim and colleagues (33) examining tumor genomics by patient age within the TCGA breast

cancer cohort (≤45 years old vs. 46–69 years old vs. ≥70 years old), *GATA3* was the only gene found to be mutated more frequently in younger patients. *PIK3CA* was numerically more likely to be mutated in older patients but this did not reach statistical significance (33). More recently, Kan and colleagues (41) showed a higher prevalence of *GATA3* alterations in a cohort of Korean women that was enriched for, but not exclusively composed of, younger women, compared with the TCGA breast cancer cohort (this analysis did not control for intrinsic subtype). *PIK3CA* and *ARID1A* were not identified as differentially altered between younger and older patients in the Kan and colleagues analyses (41). Here, potentially due to upfront stratification by subtypes and focus on a particularly young patient subset (≤35 years old), we were able to replicate the *GATA3* findings, demonstrate statistical significance of increased *PIK3CA* mutations in older patients, and newly identify *ARID1A* alterations as more likely to occur in younger patients.

In the Azim and colleagues analysis, intrinsic subtype (by 50-gene signature PAM50) was incorporated as a covariate in a logistic regression model to assess genomic differences (33). In contrast, our analysis stratified by intrinsic subtype (approximated from histologic parameters) ahead of time, allowing us to demonstrate that significant genomic differences are seen specifically in luminal A patients (despite the fact that this was not the most prevalent, and therefore not the highest powered, subgroup within the Young Women’s Study cohort). The identification of genomic differences specific to luminal A tumors is notable because multiple groups have shown that luminal A is the only breast cancer subtype in which prognosis differs between young and older women—and therefore it is the subtype where biological differences may drive distinct clinical outcomes (4–6). Whether true biological differences exist between hormone receptor-positive younger and older patients with breast cancer, and if or how we should use that to guide different therapeutic decisions based on age, is an issue of enormous clinical importance. The large, prospective TAILORx and RxPONDER trials both suggest differential chemotherapy benefit between younger and older women with HR+/HER2– breast cancer (43, 44). It remains unclear what proportion, if any, of chemotherapy benefit seen in younger women may be due to a chemoendocrine effect versus an inherent biological difference that produces differential chemosensitivity. Findings, like those we present

Table 3. Comparison of WES and clinical genetic testing among very young women with pathogenic germline variants.

Patient	Pathogenic germline variant identified by WES (alteration type)	Somatic hit identified by WES? (if yes, alteration type)	Gene ID from WES tested clinically?	Pathogenic/likely pathogenic alteration identified in clinical genetic testing
26	<i>BRCA1</i> (frameshift_ins)	Yes (LOH)	Yes	<i>BRCA1</i> (positive, clinically actionable)
309	<i>BRCA1</i> (frameshift_del)	Yes (LOH)	Yes	<i>BRCA1</i> (positive, clinically actionable)
315	<i>BRCA1</i> (frameshift_del)	No	Yes	<i>BRCA1</i> (positive, clinically actionable)
663	<i>BRCA1</i> (nonsense mut)	Yes (LOH)	Yes	<i>BRCA1</i> (positive, clinically actionable)
774	<i>BRCA1</i> (nonsense mut)	Yes (LOH)	Yes	<i>BRCA1</i> (positive, clinically actionable)
786	<i>BRCA1</i> (nonsense mut)	Yes (LOH)	Yes	<i>BRCA1</i> (positive, clinically actionable)
844	<i>BRCA1</i> (frameshift_ins)	Yes (LOH)	Yes	<i>BRCA1</i> (positive, clinically actionable)
879	<i>BRCA1</i> (frameshift_del)	Yes (LOH)	Yes	<i>BRCA1</i> (positive, clinically actionable)
953	<i>BRCA1</i> (frameshift_del)	Yes (LOH)	Yes	<i>BRCA1</i> (positive, clinically actionable)
1063	<i>BRCA1</i> (nonsense mut)	No	Yes	<i>BRCA1</i> (positive, clinically actionable)
228	<i>BRCA2</i> (frameshift_ins)	No	Yes	<i>BRCA2</i> (positive, clinically actionable)
491	<i>BRCA2</i> (frameshift_del)	No	Yes	<i>BRCA2</i> (positive, clinically actionable)
857	<i>BRCA2</i> (nonsense mut)	Yes (LOH)	Yes	<i>BRCA2</i> (positive, clinically actionable)
29	<i>PALB2</i> (frameshift_del)	Yes (frameshift_del and splice site)	No	None
1033	<i>PALB2</i> (frameshift_del)	Yes (nonsense mut)	Unknown ^a	None
1074	<i>PALB2</i> (splice site)	No	No	None
406	<i>BUB1B</i> (splice site)	No	No	None
915	<i>BUB1B</i> (missense mut)	No	No	None
653	<i>TP53</i> (frameshift_del)_	No	Unknown ^a	None
493	<i>COL7A1</i> (splice site)	No	No	None ^b
614	<i>GJB2</i> (frameshift_del)	No	No	None
491	<i>PRSS1</i> (frameshift_del)	No	No	None
686	<i>PRSS1</i> (splice site)	No	No	None

Abbreviation: LOH, loss of heterozygosity.

Note: All patients had some clinical genetic testing performed. For the “Gene ID from WES tested clinically” column: *Yes* indicates the gene was confirmed as evaluated in clinical testing; *No* indicates the gene was confirmed as not evaluated in clinical testing; *Unknown* indicates it is unknown whether the gene was evaluated in clinical testing (i.e., clinical genetic testing was performed, but it is unknown whether the specific gene of interest was evaluated).

^aClinical genetic testing for the gene of interest in these patients was highly unlikely, but its absence could not be entirely confirmed based on the records available.

^bPatient 493 had a *BRCA2* VUS identified on clinical testing.

here, of potential true biological differences between tumors from younger versus older women may ultimately help to address this important clinical question.

Our germline WES of very young women with breast cancer revealed a number of findings with clinical implications. Germline mutations in *BRCA1/2* are unsurprisingly enriched in this very young cohort, with 14.1% of patients harboring pathogenic mutations. This is congruent with germline findings in the Prospective Outcomes in Sporadic versus Hereditary breast cancer (POSH) cohort of nearly 3,000 patients with breast cancer diagnosed at age ≤40 years old, in which 12% of patients carried a pathogenic *BRCA1/2* mutation (45). The frequency of germline susceptibility decreases substantially with increasing age, as demonstrated by our germline findings in older women from TCGA and, for example, the very large CARRIERS study cohort in which over 19,000 patients with breast cancer diagnosed at age ≤50 years old had a 7.3% chance of carrying a pathogenic variant in a cancer predisposition gene (46). With comprehensive sequencing we identified four patients with clinically important pathogenic germline variants (in *PALB2* and *TP53*) not identified on their original clinical genetic testing because the altered gene was not clinically evaluated. The most important clinical lesson of our germline sequencing results is to underscore the importance of repeat germline testing as clinical methodologies improve and panels expand, particularly for groups at high germline risk such as young patients.

Beyond expected alterations in *BRCA1/2*, *PALB2*, and *TP53*, germline WES identified variants in four cancer susceptibility genes (*BUB1B*, *PRSS1*, *COL7A1*, and *GJB2*) that historically have not been

linked or are only weakly linked to breast cancer. Specifically, germline mutations in *BUB1B* and *PRSS1* have been previously linked to heritable gastrointestinal and pancreatic cancers, respectively. Germline mutations in *COL7A1* and *GJB2* have been implicated as potentially pathogenic in patients with breast and ovarian cancer (36, 37, 47). Of note, a somatic second hit was not detected in any of the patients with germline first hits in these less common genes. Though it is not clear whether these germline variants played a causative role in breast cancer pathogenesis for our patients, these data and similar data obtained from clinical expanded germline panel testing will be instrumental for establishing connections between germline alterations and specific cancer risks. At the same time, the fact that only 23.9% of these very young women had pathogenic germline variants found on WES suggests that there may be much yet to discover in this population.

Our analysis has several limitations. When considering the comparisons between luminal A tumors in young versus older women, it is important to note that intrinsic subtype in our cohort was defined according to pathologic features, and was defined according to gene expression microarrays in TCGA. However, pathology-based intrinsic subtype was previously shown to be an adequate surrogate for expression-based intrinsic subtype classification (12). In addition, the genomic differences we identified between luminal A patients were not observed when luminal A and B patients were grouped together (Supplementary Fig. S8), suggesting that the findings were not simply a result of differential classification between luminal subtypes in young versus older patients. Our failure to find significant differences in somatic alterations between young and older women with breast cancer in HER2 enriched and basal-like subtypes may be due to

underpowering given the small number of patients in each subgroup of young women. Although grade and histology were centrally reviewed in all patients, receptor status was centrally reviewed in only some cases. The receipt of neoadjuvant chemotherapy predating the sequenced tumor specimen may have impacted somatic alterations, although this was only an issue for a small minority of patients. Our results would be strengthened by analysis of a separate validation cohort, however we were not able to identify a cohort with a sufficient number of similarly young women and intrinsic subtype information available for each tumor, pointing to the novelty of our cohort as well as the need for further investigation in this area.

In summary, we have used WES of both tumor and germline specimens to investigate the unique pathogenesis of breast cancer in very young women. In germline sequencing, we identified a 14.1% prevalence of pathogenic *BRCA1/2* alterations, and found several patients with actionable germline findings that were missed on older clinical testing platforms, serving as a reminder of the importance of updating germline testing in any patient at high risk for a familial cancer syndrome. Through somatic sequencing, we demonstrated an enrichment for *ARID1A* alterations in young women's breast tumors and identified three genes (*PIK3CA*, *GATA3*, *ARID1A*) in which somatic alterations could plausibly contribute to less favorable biology for young patients with luminal A breast cancer. The suggestion of distinct biological features for luminal tumors in young versus older women will be important to follow-up in order to understand whether these features could explain any of the age-related difference in chemosensitivity that was observed in the TAILORx and RxPONDER trials. Given the roles of *GATA3* as a transcription factor and *ARID1A* as a chromatin regulator, examination of transcriptional profiles among young women's luminal A tumors will be an important next step. The ongoing study of larger cohorts of young women, with genomic analysis by tumor subtype, may help to delineate biological susceptibilities and improve treatment options for young patients with breast cancer.

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Authors' Contributions

A.G. Waks: Conceptualization, data curation, methodology, writing—original draft, writing—review and editing. **D. Kim:** Conceptualization, data curation, software, formal analysis, validation, visualization, methodology, writing—original draft, writing—review and editing. **E. Jain:** Visualization, writing—review and editing. **C. Snow:** Data curation, writing—review and editing. **G.J. Kirkner:** Formal analysis, writing—review and editing. **S.M. Rosenberg:** Writing—review and editing. **C. Oh:** Formal analysis, writing—review and editing. **P.D. Poorvu:** Writing—review and editing. **K.J. Ruddy:** Writing—review and editing. **R.M. Tamimi:** Writing—review and editing. **J. Peppercorn:** Writing—review and editing. **L. Schapira:** Writing—review and editing. **V.F. Borges:** Writing—review and editing. **S.E. Come:** Writing—review and editing. **E.F. Brachtel:** Writing—review and editing. **E. Warner:** Writing—review and editing. **L.C. Collins:** Writing—review and editing. **A.H. Partridge:** Conceptualization, resources, supervision, funding acquisition, methodology, writing—review and editing. **N. Wagle:** Conceptualization, resources, supervision, funding acquisition, methodology, writing—review and editing.

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