Clinical and Genomic Characteristics of Patients with Hormone Receptor–Positive, Human Epidermal Growth Factor Receptor 2–Negative Metastatic Breast Cancer Following Progression on Cyclin-Dependent Kinase 4 and 6 Inhibitors

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ABSTRACT

Purpose: We explored the clinical and genomic characteristics of hormone receptor–positive (HR+), HER2-negative (HER2−) metastatic breast cancer (MBC) after progression on cyclin-dependent kinase 4 and 6 inhibitors (CDK4 and 6i) ± endocrine therapy (ET) to understand potential resistance mechanisms that may aid in identifying treatment options.

Experimental Design: Patients in the United States with HR+, HER2− MBC had tumor biopsies collected from a metastatic site during routine care following progression on a CDK4 and 6i ± ET (CohortPost) or prior to initiating CDK4 and 6i treatment (CohortPre) and analyzed using a targeted mutation panel and RNA-sequencing. Clinical and genomic characteristics were described.

Results: The mean age at MBC diagnosis was 59 years in CohortPre ($n = 133$) and 56 years in CohortPost ($n = 223$); 14% and 45% of patients had prior chemotherapy/ET, and 35% and 26% had *de novo* stage IV MBC, respectively. The most common biopsy site was liver (CohortPre, 23%; CohortPost, 56%). CohortPost had significantly higher tumor mutational burden (TMB; median 3.16 vs. 1.67 Mut/Mb, $P < 0.0001$), *ESR1* alteration frequency (mutations: 37% vs. 10%, FDR < 0.0001; fusions: 9% vs. 2%, $P = 0.0176$), and higher copy-number amplification of genes on chr12q15, including *MDM2*, *FRS2*, and *YEATS4* versus patients in the CohortPre group. In addition, *CDK4* copy-number gain on chr12q13 was significantly higher in CohortPost versus CohortPre (27% vs. 11%, $P = 0.0005$).

Conclusions: Distinct mechanisms potentially associated with resistance to CDK4 and 6i ± ET, including alterations in *ESR1* and amplification of chr12q15 and *CDK4* copy-number gain, were identified.

Introduction

Breast cancer is the most common noncutaneous cancer among women in the United States and is now the most diagnosed cancer globally (1). The majority of patients in the United State are initially diagnosed with early-stage disease, with 6% of breast cancers diagnosed with metastatic disease (2). It is estimated that 30% of patients who present with early-stage disease will eventually experience relapse with metastatic disease (3).

The hormone receptor–positive (HR+), HER2-negative (HER2−) subtype accounts for over 70% of diagnoses (4), and as such, endocrine therapy (ET) remains the most effective therapy backbone for this subgroup (5). The introduction of cyclin-dependent kinase 4 and 6 inhibitors (CDK4 and 6i) has further advanced the therapeutic landscape (5, 6), with three FDA- and European Medicine Agency (EMA)–approved agents: abemaciclib (US FDA approved September 2017), palbociclib (US FDA approved February 2015), and ribociclib (US FDA approved March 2017) indicated for the treatment of HR+, HER2− locally advanced or MBC in combination with ET (7). Abemaciclib is also approved as monotherapy in the United States for patients with advanced or MBC with disease progression following ET and prior chemotherapy (8) and for certain patients with HR+, HER2− node-positive early breast cancer (9) in combination with ET (10). The addition of CDK4 and 6i to ET has led to significant improvement in progression-free survival (PFS) and overall survival (OS) for patients with MBC (11–15); however, despite these improvements, ET and CDK4 and 6i resistance can affect the sustained longer-term efficacy of these therapies for patients and represents a major challenge in breast cancer treatment (16).

Previous genomic studies have revealed several mechanisms potentially implicated in the development of CDK4 and 6i resistance (17–19). Next-generation sequencing (NGS) of circulating tumor DNA (ctDNA) from patients with disease progression on ribociclib and ET identified *FGFR1* amplification or activating mutations in 41% of specimens in a limited number of patients (20). DNA sequencing of ctDNA from patients enrolled in the PALOMA-3 trial receiving palbociclib with fulvestrant showed enrichment of *RB1* mutations compared with fulvestrant alone (21). There is also evidence that the overexpression of CDK4 or CDK6 may be a driver of CDK4 and 6i resistance *in vitro* (22). Genetic alterations and/or change in expression of genes such as *FGF3, CCND1, ERBB2, IGF1R, NF1, AKT1, AKT2, AKT3, TP53, RB1, CCNE1* as well as targets within the RAS/RAF/MEK/ERK pathway are also linked to CDK4 and 6i resistance (23). Characterizing the tumor genomic landscape of patients

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Translational Relevance

This was a large-scale genomic study of tumor biopsies collected from patients after progression on a CDK4 and 6i ± ET, which identified mechanisms of CDK4 and 6i resistance in HR+, HER2− MBC. We found that patients with disease progression on a CDK4 and 6i ± ET had significantly higher TMB. We identified potential mechanisms of resistance, including genomic aberrations in *ESR1*, copy-number amplification of chr12q15, and *CDK4* copy-number gain. These findings may aid in the development of approaches to overcome CDK4 and 6i resistance in patients with HR+, HER2− MBC.

with HR+, HER2− MBC who experienced progression on a CDK4 and 6i is critical to identify mechanisms of acquired resistance to CDK4 and 6i and may aid in the development of treatment strategies following disease progression on a CDK4 and 6i.

In this study, we describe the clinical and demographic characteristics of patients at initial and/or MBC diagnosis and CDK4 and 6i initiation. We add to the body of literature by describing the most frequent molecular alterations in tumor samples from patients with HR+, HER2− MBC treated in routine clinical practice and who experienced disease progression on a CDK4 and 6i ± ET.

Materials and Methods

Patient selection and study design

This was a retrospective observational study that analyzed demographic, clinical, and molecular data provided by Tempus Labs, Inc. (www.tempus.com). Tempus licensed its proprietary, deidentified, real-world health data ("Tempus Data") to Eli Lilly, and Tempus understands that such Tempus Data were analyzed by Eli Lilly to generate results that are the subject of a manuscript for which publication is sought. Tempus Data are real-world data collected in accordance with applicable law and not generally obtained pursuant to a prospective clinical study conducted based on study-specific informed written consent and supervised by an Institutional Review Board. All patient-level data were deidentified in accordance with the Health Insurance Portability and Accountability Act (HIPAA). The Tempus Data originate from protected health information that Tempus receives in the course of providing various services, including: (i) as a covered entity (within the meaning of federal HIPAA regulation), for example, when Tempus sequences a patient based on a doctor's order, (ii) as business associate (within the meaning of federal HIPAA regulation), for example, when Tempus ingests data from health care providers to facilitate clinical trial matching, or (iii) under patient informed consent and authorization, for example, when a patient participates in an observational study conducted by Tempus. Tempus ingests protected health information pursuant to such avenues and deidentifies it in accordance with federal HIPAA regulation so that it is proprietary, deidentified, real-world Tempus Data that is no longer considered protected health information under HIPAA and can be used to facilitate research and development to benefit the next generation of patients.

All samples were taken after the patients' metastatic diagnosis date, from a metastatic site and not from the primary tumor. Patients who met all the eligibility criteria were included regardless of date of HR+, HER2− MBC diagnosis: adult and ≥18 years of age at initial breast cancer diagnosis, tumor biopsy taken after metastatic diagnosis date, and NGS data from tumor tissue and matched normal blood. Patients were excluded if records contained evidence of any primary malignancy other than breast cancer for which the patient was actively receiving systemic treatment in the 3 years prior to the MBC diagnosis date and within 3 years after the MBC diagnosis date, except for nonmelanoma skin cancer or other benign *in situ* neoplasms. Patients were assigned to CohortPre if they received a CDK4 and 6i as part of their MBC treatment, and their biopsy was taken *before* receiving CDK4 and 6i treatment, or if they had not received a CDK4 and 6i as part of their treatment during the study period. Patients were assigned to CohortPost if they had a biopsy taken following their disease progression on a CDK4 and 6i (**Fig. 1**).

Patient demographics and clinical characteristics

Demographic and clinical characteristics were derived from data obtained from patient charts (Tempus Labs Inc.). Demographic variables described for patients included sex, race, and age at initial breast cancer and MBC diagnosis. The clinical characteristics of interest included stage of initial diagnosis, number of metastatic sites, biopsy site (liver or nonliver), number of biopsies obtained, prior ET, and/or chemotherapy (prior to biopsy for CohortPre and prior to CDK4 and 6i initiation for CohortPost), and duration of follow-up since MBC diagnosis. Median time from initial MBC diagnosis to tumor biopsy was determined for patents in CohortPre, whereas median time from MBC diagnosis to CDK4 and 6i initiation, from CDK4 and 6i initiation to progression, and from progression to tumor biopsy were assessed for patients in CohortPost.

Genomic assessment

Tumor biopsy and matched normal samples (blood and saliva) were sequenced using the Tempus xT assay (Tempus Labs Inc.), an NGS-based cancer gene panel test that analyzes 648 cancer-related genes in tumor tissue with a matched normal sample as a reference (xT.version2/xT.version3/xT.version4). Sample processing, library construction, and sequencing were performed by Tempus as described previously (24). Germline and somatic short variants, including single-nucleotide variants (SNV) and insertion-deletion variants (indels), were detected, filtered, and annotated according to previously published methods (see Supplementary Materials and Methods for detailed analyses). On the basis of recommendations from the AMP/ASCO/CAP (25) and ACMG guidelines (26), the variants were reported into one of the following categories: pathogenic, likely pathogenic, variants of unknown significance (VUS), benign, and likely benign. Copy-number variations (CNV) were assessed using the Tempus copy-number algorithm (24), which utilizes sequencing coverage and variation in heterozygous germline SNVs between tumor and normal samples.

Pathogenic and likely pathogenic short variants (both somatic and germline), copy-number deletion (copy number = 0) and copy-number amplification (copy number ≥6) were included for alteration frequency comparison between CohortPre and CohortPost. Genes with alteration frequency ≥5% in either cohort were assessed, in addition to those related to breast cancer. The genomic alterations were visualized using OncoPrint (27) and plotted with R package ComplexHeatmap. Copy-number gain (copy number ≥3 and <6) of specified genes (*CDK4*, *MDM2*, *FRS2*, *YEATS4*) were also compared between cohorts.

Tumor mutational burden

As described previously (24), tumor mutational burden (TMB) was calculated by dividing the number of nonsynonymous mutations by

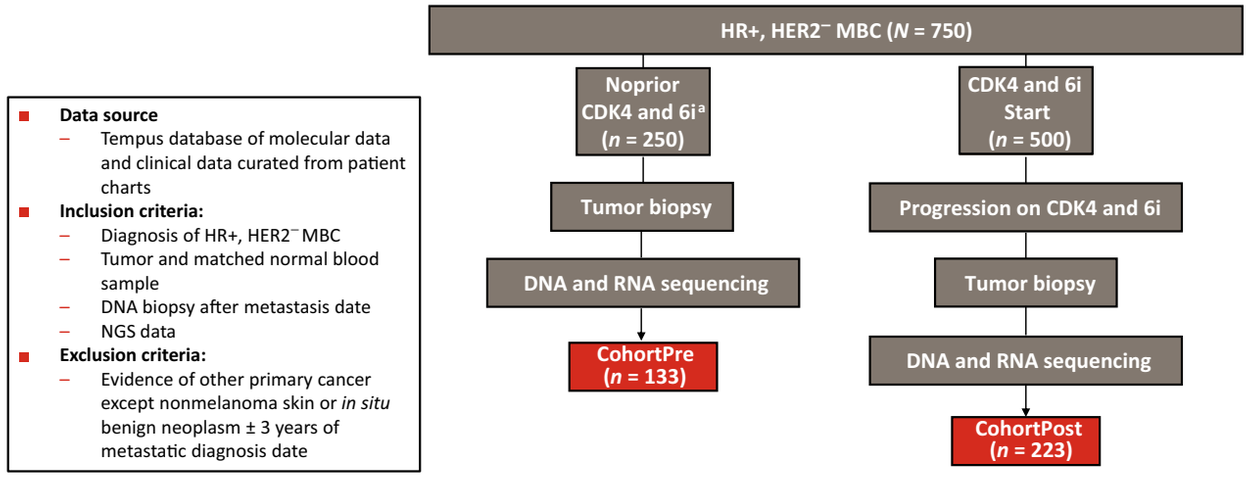

Figure 1.
Study design. A percentage of patients in CohortPre initiated CDK4 and 6i treatment during the study period. However, tumor biopsies for those patients occurred prior to initiating CDK4 and 6i.

the megabase size of the panel (2.4Mb). All nonsilent somatic coding mutations, including missense, indel and stop-loss variants with coverage greater than 100× and a variant allele frequency (VAF) >5% were included as nonsynonymous mutations.

Gene expression data collection and normalization

The Tempus RNA whole-transcriptome assay uses the IDT xGen Exome Research Panel v1.0, which spans a 39-Mb target region of the human genome and covers 19,396 genes. Sample processing, library construction, and sequencing with a minimum depth of 30 million reads per sample were performed by Tempus as described previously (24). Transcript level pseudoalignment and quantification to the Ensembl GRCh37 Release 97 (July 2019) reference was performed using Kallisto (version 0.44). The gene level counts were summarized using *tximport* (28) R package. The raw counts were normalized using the method of median of ratios and then subjected to variance stabilizing transformations (vst) to obtain log2-transformed gene expression using the *DESeq2* R package. Patients with *CDK4* copy number equal to 2 were used to determine baseline *CDK4* expression. *CDK4* expression over 1.3-fold of baseline expression was considered overexpression.

Detection of *ESR1* fusion

The Tempus RS bioinformatics fusion calling pipeline was used for detection of transcript fusions from RNA-seq data (GRCh38 reference, see Supplementary Materials and Methods for detailed analyses; ref. 29). A fusion was called if ≥5 high-quality spanning junction reads (a significant fraction of the read is present on both the 5′ and 3′ sides of the breakpoint) were detected. Tandem duplications and inversions were also included.

Statistical analysis

Mann–Whitney *U* test was used to compare the continuous variable TMB between CohortPre and CohortPost with significance at $P < 0.05$. χ^2 or Fisher exact test was used as appropriate for alteration frequency comparison, with significance at FDR <0.2. The frequency difference of *ESR1* fusion was assessed by Fisher exact test with significance at $P < 0.05$. Student *t* test was used to compare normalized gene expression with significance $P < 0.05$. Data were analyzed using R statistical software version 4.0.3 (R Project for Statistical Computing). Generalized linear regression was used to assess the difference of TMB, *ESR1* expression, as well as gene expression of *CDK4*, *MDM2*, *YEATS4*, and *FRS2* between cohorts (see Supplementary Materials for detailed analyses).

Data availability

The data analyzed in this study are available from Tempus. Restrictions apply to the availability of these data, which were used under license for this study. Tempus cannot make datasets publicly available because study data are used under license from source practices and other data providers. For data inquiries, please contact Tempus at https://www.tempus.com/contact-us/. Processed data are available in the article and supplementary files and can be provided by the corresponding author upon request.

Results

Patient characteristics

Overall, 750 patients with a diagnosis of HR+, HER2− MBC were considered for this analysis. Of these, 356 patients met eligibility criteria for this analysis and were assigned into CohortPre ($n = 133$) and CohortPost ($n = 223$; Supplementary Fig. S1).

Overall, the mean age at the time of MBC diagnosis was 59 and 56 years in CohortPre and CohortPost, respectively; 4% and 32% had prior ET, 11% and 14% had prior chemotherapy; 35% and 26% were diagnosed with *de novo* stage IV MBC; 19% and 43% had ≥3 metastatic sites. The most common biopsy site was liver, occurring in 44% of patients overall (CohortPre, 23%; CohortPost, 56%). The median (interquartile range) duration of follow-up was 17.4 (4.9–30.2) months and 39.2 (23.9–62.4) months for CohortPre and CohortPost, respectively. The majority of CohortPost patients were treated with palbociclib (87.0%), followed by ribociclib (7.2%) and abemaciclib (5.8%; **Table 1**).

For patients in the CohortPre, the median time from initial diagnosis of MBC to tumor biopsy was 0.5 months (IQR, 0–1.5 months). For patients in the CohortPost, three time intervals were included: from diagnosis of MBC to CDK4 and 6i initiation, from CDK4 and 6i initiation to disease progression, and from

Table 1. Demographics and clinical characteristics.

	CohortPre ($n = 133$)	CohortPost ($n = 223$)
Age, mean (26)		
Primary diagnosis	55 (12.7)	53 (11.7)
MBC diagnosis	59 (12.9)	56 (11.2)
Biopsy	60 (12.8)	60 (11.4)
Sex, *n* (%)		
Female	130 (97.7)	221 (99.1)
Race, *n* (%)		
Asian	4 (3.0)	5 (2.2)
Black or African American	21 (15.8)	11 (4.9)
White	68 (51.1)	125 (56.1)
Other	2 (1.5)	38 (28.6)
Unknown/missing	5 (2.2)	77 (34.5)
Follow-up, median (IQR)	17.4 (4.9–30.2)	39.2 (24.0–62.4)
Recurrent status, *n* (%)		
De novo MBC	46 (34.6)	58 (26.0)
Recurrent MBC	87 (65.4)	165 (74.0)
Biopsy site, *n* (%)		
Liver	31 (23.3)	124 (55.6)
Nonliver	102 (76.7)	100 (44.8)
No. of metastasis sites at biopsy, *n* (%)		
≥3	25 (18.8)	73 (43.2)
Prior treatment, *n* (%)		
Chemotherapy	14 (10.5)	31 (13.9)
Endocrine therapy	5 (3.8)	71 (31.8)
CDK4 and 6i treatment, *n* (%)		
Abemaciclib	—	13 (5.8)
Palbociclib	—	194 (87.0)
Ribociclib	—	16 (7.2)

Abbreviation: CCI, Charlson Comorbidity Index.

progression to tumor biopsy. The median time from diagnosis of MBC to CDK4 and 6i treatment was 2.6 months (IQR, 1.0–19.8 months), and the median time from CDK4 and 6i initiation to documented progression was 17.5 months (IQR, 9.6–29.1 months). After progression, half of the patients in CohortPost had tumor biopsy within 2.6 months (IQR, 0.5–10.7 months; Supplementary Fig. S2A). The tumor purity was slightly different between CohortPre and CohortPost (mean, 58.3% vs. 63.7%, $P = 0.009$; Supplementary Fig. S2B).

TMB

TMB analysis indicated that patients in CohortPost had significantly higher TMB compared with patients in CohortPre (median, 3.16 vs. 1.67 Mut/Mb, $P < 0.0001$; **Fig. 2A**). A significantly ($P = 0.005$) higher proportion of patients had TMB ≥10 mut/Mb in CohortPost than CohortPre [11.2% ($n = 25$) vs. 3.0% ($n = 4$); Supplementary Table S1]. Because CohortPost had more patients with recurrent MBC at initial diagnosis and more biopsies from liver metastases, we examined whether the increased TMB was associated with these differences. As shown in Supplementary Fig. S3A, patients with recurrent MBC tended to have higher TMB compared with *de novo* MBC in both cohorts. However, the differences were not statistically significant. Patients in CohortPost consistently had significantly higher TMB than CohortPre, regardless of *de novo* status; patients with recurrent MBC in CohortPost had the highest TMB (median, 3.16; Supplementary Fig. S3A). Similar results were observed when stratifying patients based on biopsy site or prior treatment, with no significant difference in TMB within the cohorts (Supplementary Fig. S3B), but patients in CohortPost consistently had higher TMB than those in the CohortPre group (Supplementary Fig. S3C). A multivariable analysis confirmed that high TMB is significantly associated with CohortPost when controlling other demographic/clinical variables, as well as the age at metastatic diagnosis (Supplementary Fig. S3D).

Genomic alterations following progression on a CDK4 and 6i ± ET

In both cohorts, the most frequently altered gene was *PIK3CA*, including 41% of patients in CohortPre and 45% in the CohortPost group (**Fig. 2B**). The frequency of double *PIK3CA* mutations was similar in both cohorts [CohortPre, 8/133 (6.0%); CohortPost, 14/223 (6.3%)]. The majority of alterations were found in three known hotspots (30): E542, E545, and H1047 (Supplementary Fig. S4). *TP53* was the next most frequently altered gene, including 40% of patients in CohortPre and 30% in CohortPost (**Fig. 2B**). However, significant differences in alteration frequency were not observed between cohorts for *PIK3CA*, *TP53*, or for other frequently altered genes in MBC, such as *CCND1*, *GATA3*, and *FGFR1* (Supplementary Table S2).

Enrichment analysis identified genes ($n = 16$) significantly altered (Fisher exact test or χ^2 test FDR < 0.2) between CohortPre versus CohortPost (**Fig. 2B**; Supplementary Table S2). Among these genes, *ESR1* (10% vs. 37%), *UGT1A1* (2% vs. 9%), *FRS2* (2% vs. 8%), *YEATS4* (2% vs. 8%), *NRG1* (2% vs. 8%), *MDM2* (2% vs. 7%), *ABCC3* (1% vs. 6%), *SPOP* (1% vs. 5%), *TSC2* (1% vs. 5%), and *NFKBIA* amplification (copy number ≥6, 0% vs. 4%) were more frequently mutated in the CohortPost versus the CohortPre group. Fusion calls from whole transcriptome RNA sequencing data indicated that CohortPost also had a significantly higher frequency of *ESR1* fusions compared with CohortPre (9% vs. 2%, $P = 0.02$; **Fig. 2B**; Supplementary Table S2). The higher alteration frequency for *ESR1*, *YEATS4, MDM2, FRS2*, and *UGT1A1* in CohortPost was further confirmed in the multivariable analysis, while controlling other demographic/clinical variables (Supplementary Table S3), suggesting their association with resistance to CDK4 and 6i ± ET.

In contrast, we observed limited *RB1* loss of function (homozygous deletion/point mutation) in both cohorts (CohortPost 5% vs. CohortPre 2%), but a significantly higher frequency of heterozygous *RB1* loss in CohortPost (47.5% vs. 36.1%, $P = 0.04$, Supplementary Table S4). Point mutation in combination with heterozygous *RB1* loss was rare and was not significantly different between the cohorts (CohortPost, 2.2% vs. CohortPre, 0.8%). Similarly, amplification of *CCNE1* was rarely observed (CohortPost 1% vs. CohortPre 2%). There were no short variants in *CDK4* and *CDK6* in either cohort, but there was a slightly higher frequency of *CDK4* amplification (copy number ≥6) in CohortPost (2%) than CohortPre (0%; **Fig. 2B**). Among the above four genes, *CCNE1* and *CDK4* had significantly higher expression in CohortPost (Supplementary Table S5; Supplementary Fig. S5). We further examined whether individual postprogression genomic alterations were associated with CDK4 and 6i treatment duration and found no significant association. (Supplementary Table S6; Supplementary Fig. S6).

ESR1 alteration is associated with progression on a CDK4 and 6i ± ET

According to the above results (**Fig. 2B**), *ESR1* is the most frequently mutated gene associated with resistance to CDK4 and 6i ± ET (37% in CohortPost vs. 10% in CohortPre). Except for amplification in five

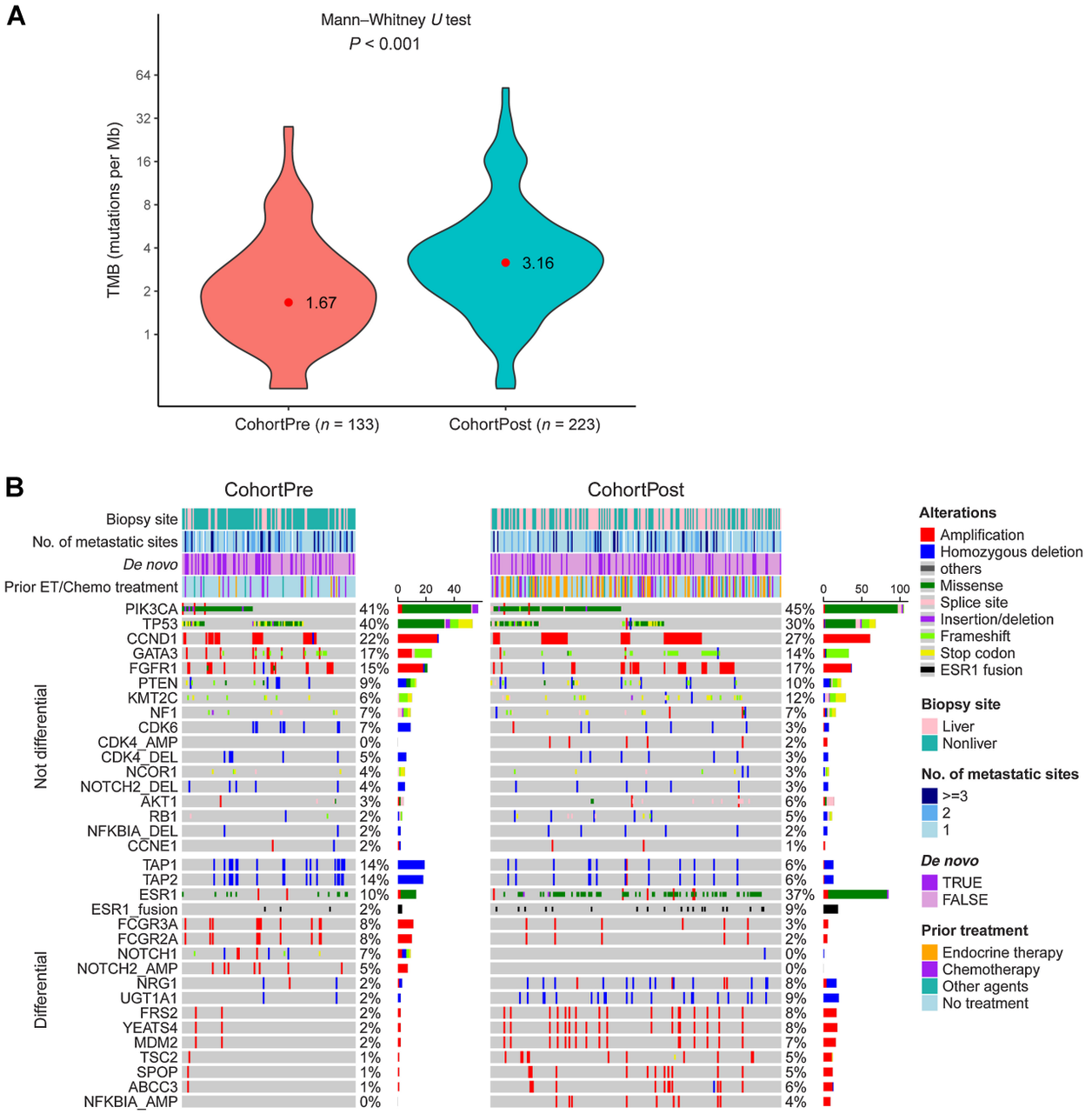

Figure 2.
TMB in CohortPre and CohortPost (**A**) and OncoPrint showing the distribution of genomic alterations in CohortPre and CohortPost (**B**).

patients, all *ESR1* alterations occurred in the ligand binding domain (LBD). In CohortPre, the alterations were located at D538 ($n = 6$), followed by E380 ($n = 3$) and Y537 ($n = 2$), whereas the alterations in CohortPost were located mostly at Y537 ($n = 37$) and D538 ($n = 32$), in addition to other locations with lower frequency (**Fig. 3A**). Among the 19 patients with *ESR1* fusions in CohortPost, nine had out-of-frame fusion or fusion from 5′UTR of ESR1 with CCDC170, and 10 had in-frame fusion with *CRCT3*, *IMPG1*, *N48BP2*, *PLEKHG1*, *SMAD3*, *SNAP91*, *ESRRG*, and *UST* due to tandem duplication or interchromosomal translocation (**Fig. 3A**).

The above multivariable analysis (Supplementary Table S3) indicated that *ESR1* alteration frequency was not associated with initial MBC diagnosis status (*de novo* vs. recurrent) or age, but was significantly associated with biopsy site, number of metastasis sites, and prior treatment, in addition to cohorts. We further examined the *ESR1* alteration frequency in patients stratified by these clinical variables. As shown in **Fig. 3B**, in CohortPre, 2% of patients with *de novo* and 14% of patients with recurrent MBC had *ESR1* alterations. Whereas, in CohortPost, patients initially diagnosed with *de novo* MBC or with recurrent metastatic cancer had similar *ESR1* alteration frequencies

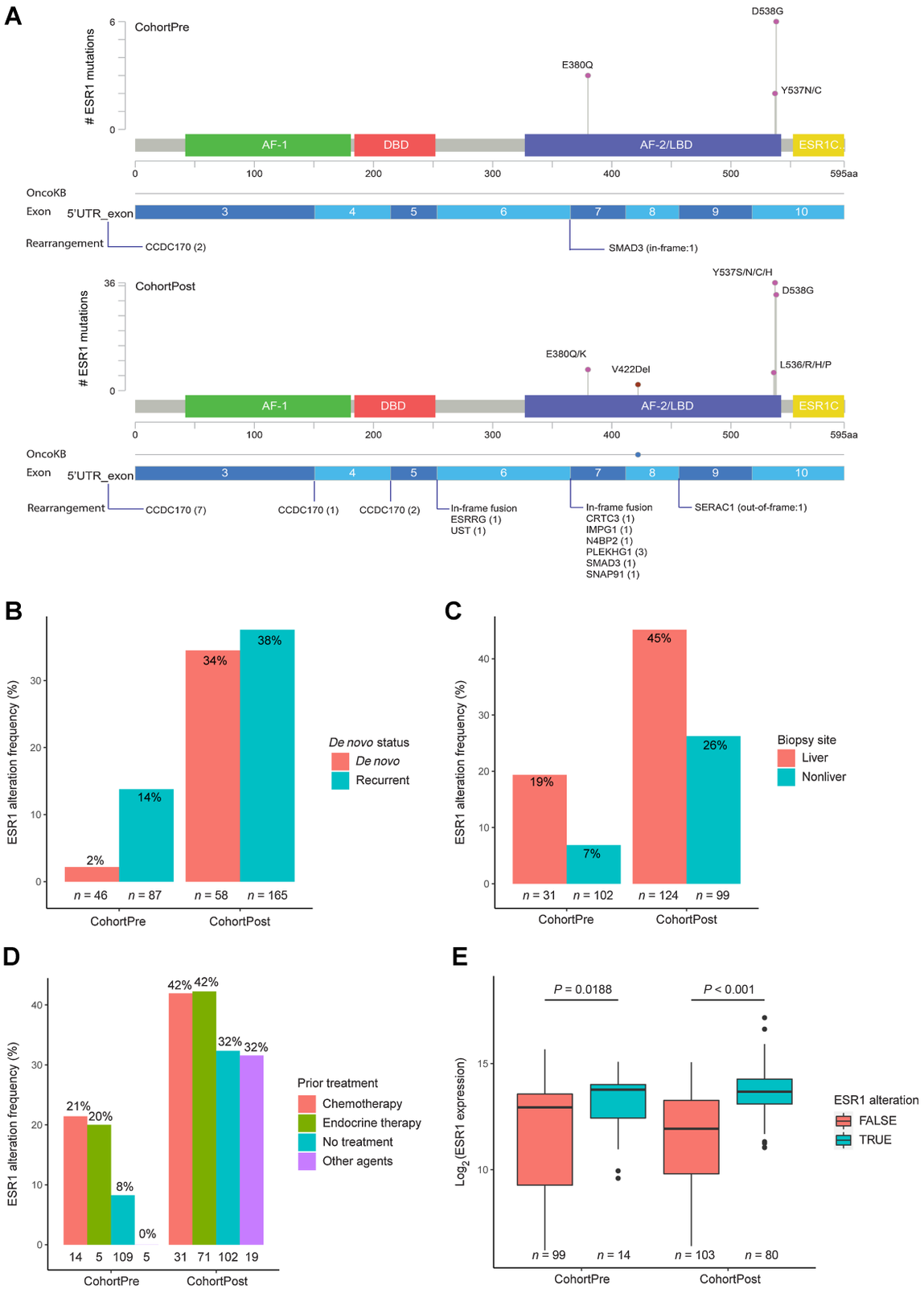

Figure 3.

Summary of *ESR1* alterations and fusions (**A**), *ESR1* expression in patients with *de novo* and recurrent disease (**B**), *ESR1* alteration frequency between biopsy sites (**C**), *ESR1* alteration frequency by prior treatment (**D**), and *ESR1* expression in patients with *ESR1* alteration vs. no *ESR1* alteration (**E**).

(34% vs. 38%), indicating no correlation between the diagnosis status and *ESR1* alteration. However, when patients were stratified by biopsy from liver or from other metastasis sites, those with liver biopsy in both cohorts showed a higher *ESR1* alteration frequency compared with nonliver biopsies (CohortPre, 19% vs. 7%; CohortPost, 45% vs. 26%; **Fig. 3C**). Similarly, patients with prior ET/chemotherapy had higher *ESR1* alteration frequency in both cohorts (CohortPre, 20% for prior ET and 21% for prior chemotherapy; CohortPost, 42% for both prior ET and prior chemotherapy) compared with patients without prior ET/chemotherapy (CohortPre, 8%; CohortPost, 32%; **Fig. 3D**). Despite these varieties within the cohorts, overall CohortPost had a higher *ESR1* alteration frequency compared with CohortPre (Supplementary Table S3).

Overall, *ESR1* expression was similar between cohorts (Supplementary Fig. S7A). However, in both cohorts, tumors with altered *ESR1* demonstrated significantly higher *ESR1* expression, compared with tumors without *ESR1* alterations (mean of Log2 of expression: CohortPre 13.1 vs. 11.7; CohortPost 13.6 vs. 11.5; **Fig. 3E**); these differences were not related to biopsy site and were slightly related to tumor purity (Supplementary Fig. S7B–S7D), as confirmed by a multivariable analysis (Supplementary Fig. S7E).

We further explored whether *ESR1* mutations or fusions co-occurred with *TP53* alterations or high TMB. *ESR1* mutations or fusions were mutually exclusive with *TP53* alterations in the overall cohort and CohortPost. Patients with wild-type *ESR1* were more likely to have mutations in *TP53*, compared with patients with altered *ESR1* (39.8% vs. 20.9%, $P = 0.0004$, Supplementary Table S7). In the overall cohort, *ESR1* alterations were not significantly associated with higher TMB, compared with wild-type *ESR1* (3.6% vs. 10.2%, $P = 0.0375$, Supplementary Table S8). In addition, a significantly higher frequency of *ESR1* p.D538G mutations was observed in liver metastases in the overall cohort, but there was no significant difference in CohortPre or CohortPost (Supplementary Table S9).

Chr12q15 amplicon harbors genes potentially associated with resistance to CDK4 and 6i ± ET

Amplifications of *MDM2*, *YEATS4*, and *FRS2* were also associated with progression on a CDK4 and 6i ± ET (*FRS2* and *YEATS4*, CohortPost 8% vs. CohortPre 2%; *MDM2*, CohortPost 7% vs. CohortPre 2%; **Fig. 2B** and **D**). These three genes are located on chromosome 12q15 (Chr12q15) with copy number highly correlated across the overall cohort (**Fig. 4A–C**), suggesting a focal amplification of the region. Besides amplification (copy number ≥6), 12q15 also harbored more prevalent copy-number gain (3 ≤ copy number < 6), with higher frequency in the CohortPost group versus those in the CohortPre group (*MDM2*, 27% vs. 14%, $P = 0.0056$; *YEATS4*, 26% vs. 15%, $P = 0.0121$; and *FRS2*, 27% vs. 15%, $P = 0.0073$; **Fig. 4D**; Supplementary Table S10), which was confirmed by a multivariable analysis when controlling other demographic/clinical variables (Supplementary Table S11). Increased copy number of *MDM2*, *FRS2*, and *YEATS4* was associated with increased mRNA expression, as expected, in both cohorts (**Fig. 4E–G**). Furthermore, when copy numbers were ≥3, patients in CohortPost had significantly higher *MDM2* and *FRS2* expression compared with patients in CohortPre, with a similar but nonsignificant trend for *YEATS4* (**Fig. 4E–G**). However, only *FRS2* showed a trend of higher expression in CohortPost while controlling copy number and other clinical variables (Supplementary Table S12; Supplementary Fig. S8). Among patients with 12q15 amplification ($n = 20$), 17 (85%) tumors were *TP53* wild-type. A trend was observed in which 12q15 amplification was mutually exclusive with *TP53* mutations; however, it was not statistically significant (Supplementary Table S13).

CDK4 is located on chromosome 12q13, close to the 12q15 amplicon. *CDK4* amplification was infrequent within the overall cohort (CohortPre, $n = 0$; CohortPost, $n = 5$). Although there was no significant correlation between copy number of *CDK4* and *MDM2* (**Fig. 5A**), a subset of patients with 12q15 gain/amplification also had *CDK4* copy-number gain (**Fig. 4D**), suggesting a chromosomal gain on the large region of 12q13–15, in addition to the focal amplification of 12q15. Similar to *MDM2*, a significantly greater proportion of patients in CohortPost than in CohortPre had CDK4 copy-number gain/amplification (27% vs. 11%, $P = 0.0005$, **Fig. 4D**), higher *CDK4* expression when copy numbers were ≥3 ($P = 0.0007$; **Fig. 5B**), and *CDK4* overexpression when *CDK4* gain/amplification was present (54% vs. 7%, $P = 0.0039$, **Fig. 5C**). Multivariable analyses confirmed the significantly increased frequency of *CDK4* gain/amplification in CohortPost (Supplementary Table S11) and significantly increased *CDK4* expression in CohortPost when controlling copy number and other clinical variables (Supplementary Table S12; Supplementary Fig. S8).

Discussion

CDK4 and 6i in combination with ET is the standard of care for patients with HR+, HER2− MBC; however, clinical benefit eventually becomes limited by drug resistance. Thus, the development of optimal therapeutic strategies for patients who experience disease progression on CDK4 and 6i is an unmet need. Understanding the mechanisms involved in resistance can enable development of treatment options following disease progression and CDK4 and 6i resistance. Prior work has revealed potential mechanisms driving resistance to CDK4 and 6i including loss of estrogen receptor (ER) expression, *RB1* disruption, and activation of *AKT1, RAS, ERBB2, FGFR2*, *AURKA*, and *CCNE2* (22). This study further contributes to the understanding of the genomic landscape of resistance by demonstrating additional gene alterations that may be mediators of resistance to CDK4 and 6i ± ET, including *ESR1* mutation and fusion, amplification of *FRS2*, *MDM2*, and *YEATS4*, as well as alteration in *UGT1A1*, *NRG1*, *ABCC3*, *SPOP*, *TSC2*, and *NFKBIA* (**Fig. 2B**). Furthermore, we show that TMB was significantly increased in patients with disease progression on a CDK4 and 6i ± ET.

TMB is emerging as a predictive marker for immunotherapy (31), and its use in breast cancer clinical practice is being explored (32). TMB was significantly higher in biopsies obtained postprogression on CDK4 and 6i ± ET than in biopsies from patients who had not yet received a CDK4 and 6i (3.16 Mut/Mb vs. 1.67 Mut/Mb). Multivariable analysis confirmed that progression on CDK4 and 6i ± ET was a significant risk factor for high TMB. Although TMB was higher following progression on CDK4 and 6i ± ET, a TMB ≥10 mut/Mb is commonly used to define patients who may benefit from immunotherapy (33). A significantly higher proportion of patients with disease progression on CDK4 and 6i ± ET had TMB ≥10 mut/Mb (11.2% vs. 3.0%), suggesting that a subset of patients with disease progression on CDK4 and 6i ± ET may benefit from immunotherapy. However, as only 5% of breast cancer cases are considered TMB-high with a large degree of variability between metastatic breast cancers and primary cancers (34), further research is needed to determine the impact of immunotherapy across TMB levels in the MBC setting. The PACE trial (NCT03147287) recently evaluated fulvestrant with palbociclib and avelumab in patients with HR+, HER2− MBC who experienced disease progression despite prior CDK4 and 6i and

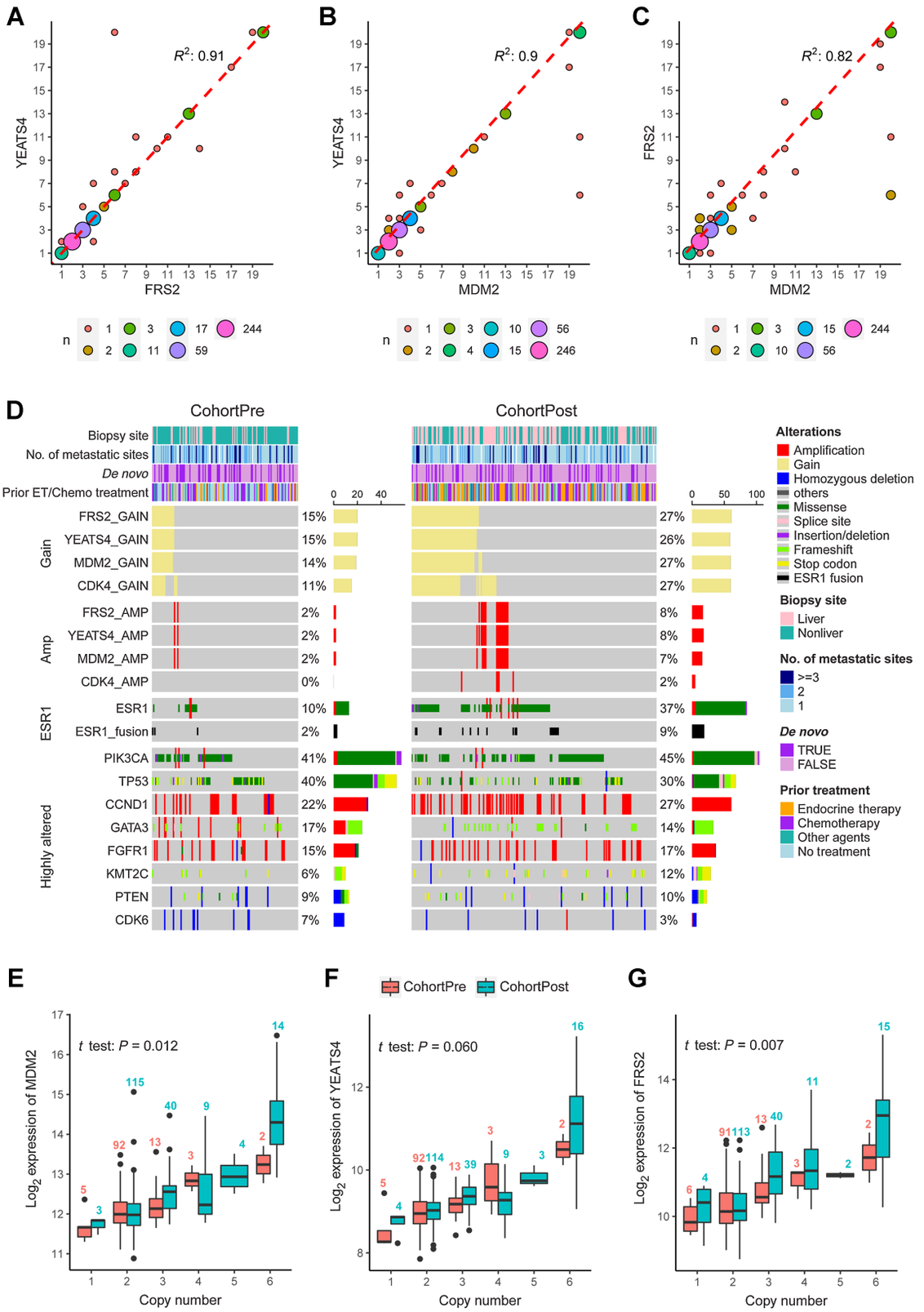

Figure 4.

Correlation of *MDM2*, *FRS2*, *YEATS4*, and *CDK4* amplified on Chr12q15 (**A–C**), OncoPrint showing the distribution of genomic alterations in CohortPre and CohortPost (**D**) and expression (copy number ≥3) of *MDM2*, *FRS2*, *YEATS4*, and *CDK4* amplified on Chr12q15 (**E–G**) (59).

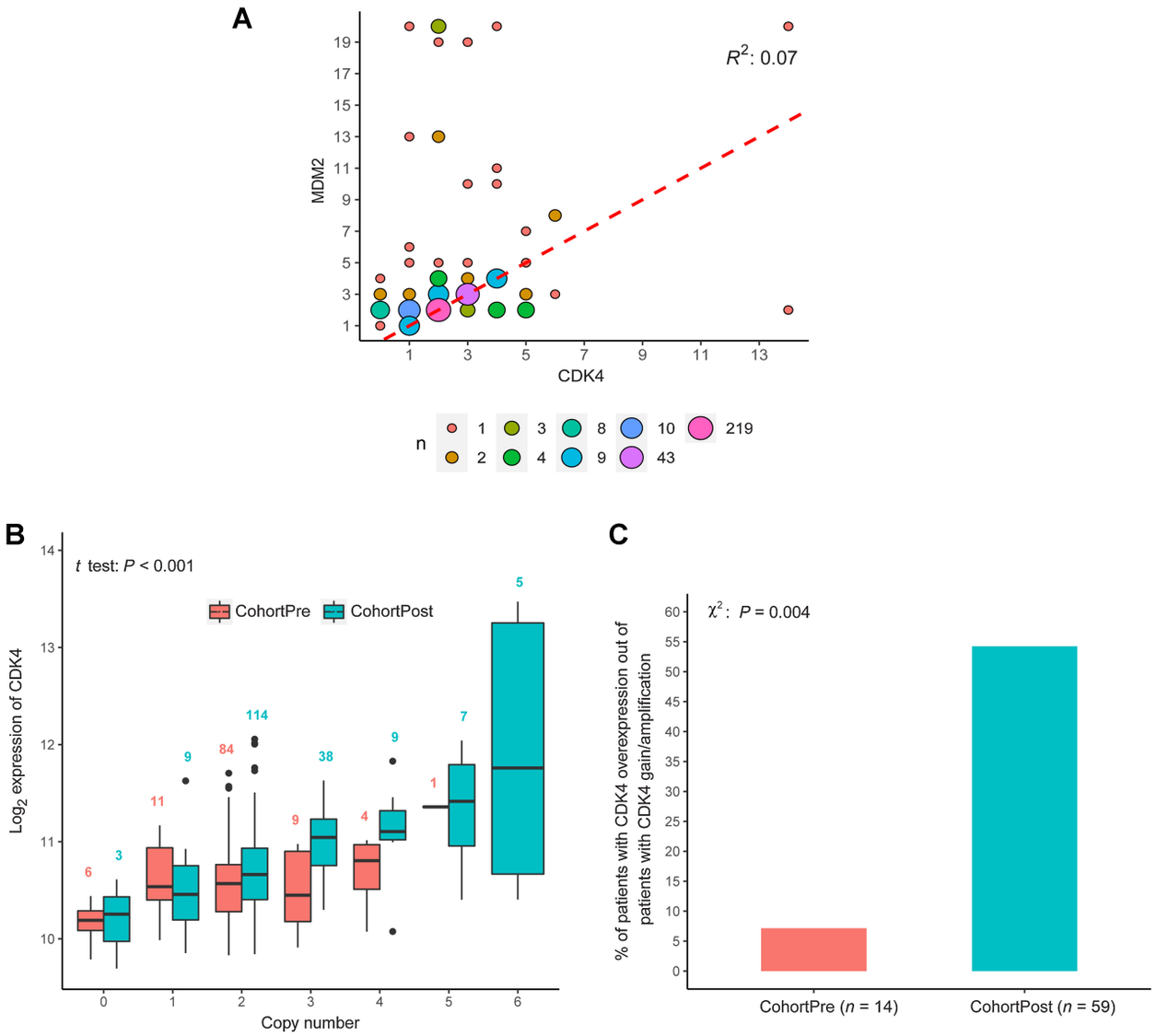

Figure 5.

Correlation between copy number of *CDK4* and *MDM2* (**A**), frequency of *CDK4* copy number gain/amplification (copy number ≥3) in CohortPost vs. CohortPre (**B**), and proportion of patients with *CDK4* overexpression in those with *CDK4* gain/amplification in CohortPost vs. CohortPre (**C**).

ET (35). Older age at metastatic diagnosis was also a significant risk factor for high TMB, which is consistent with previous findings that TMB increases significantly with age (36). Further analysis is required to fully understand the role of TMB as a biomarker in resistance to CDK4 and 6i ± ET.

Several previous studies highlight *ESR1* alterations as a potential mechanism of endocrine resistance in a substantial proportion of patients with MBC (37). Acquired *ESR1* mutations were also reported in patients with MBC treated with palbociclib and letrozole (38). In our study, the frequency of *ESR1* alterations in patients with disease progression on a CDK4 and 6i ± ET was 37% compared with 10% in patients who had not yet received a CDK4 and 6i. Increased *ESR1* alterations including mutations and amplifications are extensively linked with ET resistance (39); therefore, the increased *ESR1* alterations observed in this study are most likely due to selective pressure conferred by ET. *ESR1* mutation status was associated with metastatic site, prior chemotherapy, and progression on a CDK4 and 6i + ET, whereas *ESR1* expression was associated with *ESR1* mutation. These data are consistent and complementary to previous findings in which *ESR1* mutations were enriched during metastasis and associated with progression (40). *ESR1* gene fusions have also been shown to trigger endocrine resistance and metastatic progression (41). The same study found that *ESR1* fusions were suppressed by CDK4 and 6i (41). Interestingly, our findings show that *ESR1* fusions were significantly higher in biopsies following progression on a CDK4 and 6i ± ET (9% vs. 2%). We speculate that these differences may be attributed to endocrine resistance mediating progression on the combination therapy; therefore, patients experiencing progression on a CDK4 and 6i in combination with ET may benefit from CDK4 and 6i combined with an alternative ET, such as an oral selective estrogen receptor degrader

(SERD). The mutual exclusivity of *ESR1* and *TP53* mutation observed in our study is consistent with a previous report in endocrine-resistant MBC (42), suggesting similar molecular interplay of these two pivotal genes in treatment with CDK4 and 6i ± ET. A significantly higher frequency of *ESR1* p.D538G mutations was observed in liver metastases in the overall cohort, consistent with a previous report (43).

The frequencies of *FRS2*, *MDM2*, and *YEATS4* alterations were also significantly increased in biopsies postprogression. A pooled analysis of ctDNA assay results from patients enrolled in the MONALEESA-2/3/7 trials ($N = 1503$) revealed potential biomarkers for resistance to ribociclib treatment, which included alterations in *FRS2* and *MDM2* (44). Although *YEATS4* has been implicated as a driver of drug resistance in other cancers (45), to our knowledge, it has not been explicitly linked with drug resistance in breast cancer. Experimental validation supporting a causative role of *FRS2*, *MDM2*, and *YEATS4* alterations in resistance to CDK4 and 6i in patients with MBC is warranted. *FRS2*, *MDM2*, and *YEATS4* amplifications occurred on the chromosomal region 12q15, a significant genome-wide region implicated in several cancers, including breast cancer, that harbors candidate genes as therapeutic targets (46). Although amplifications on 12q15 may be associated with ET exposure, it is also plausible that 12q15 amplification may represent a mediator of CDK4 and 6i resistance. In addition, a subset of postprogression tumors with 12q15 gain/amplification had increased *CDK4* copy numbers and higher expression spanning a larger region of 12q13–15. These findings suggested that *CDK4* may be mediating tumor growth in a subset of these patients, and may benefit from a CDK4 and 6i, which preferentially inhibits *CDK4* (47) or using a combination treatment with an *MDM2* inhibitor (48). Further genomic analyses are required to confirm the role of 12q15 amplification and *CDK4* as a mediator of resistance to CDK4 and 6i in patients with MBC.

Several other genomic alterations were observed in postprogression tumors. *ABCC3* is known to be frequently amplified and overexpressed in HER2-positive breast cancer (49), and genomic analysis revealed *ABCC3* as a mediator of taxane resistance in patients with HER2-positive breast cancer (50). In our study, mutations in *ABCC3* were significantly enriched in biopsies of patients with disease progression on CDK4 and 6i ± ET, implicating this as a potential mechanism of resistance in patients with HER2- breast cancer. *NRG1* alterations were also significantly enriched in biopsies postprogression compared with those from patients not treated with CDK4 and 6i ± ET. Although data on the association of *NRG1* and progression on CDK4 and 6i or ET are lacking, *NRG1* was shown to mediate the activation of HER3, which induced primary resistance to trastuzumab in HER2-overexpressing breast cancer cell lines (51). *SPOP* is frequently mutated in many cancers including breast cancer and has been identified as a potential therapeutic marker (52). Furthermore, *SPOP* has been associated with resistance to BET inhibitors in *SPOP*-mutant prostate cancer, indicating a promising biomarker for drug response (53). In this study, *SPOP* alterations were significantly enriched in postprogression biopsies, highlighting, for the first time, a potential link to CDK4 and 6i or ET resistance. *TSC2* and *NFKBIA* were also significantly enriched postprogression. The role of *TSC2* and *NFKBIA* in drug resistance is not clear; however, increased expression of *TSC2* was shown to stimulate invasiveness and was associated with increased metastasis and reduced survival (54); whereas *NFKBIA* has been proposed as a prognostic marker for triple-negative breast cancer (55).

Some alterations previously associated with CDK4 and 6i resistance were not found in this study. For example, no significant differences in *FGFR1* alterations were observed, which has been reported as a mechanism of resistance to ribociclib in the MONALEESA-2 study (20). In addition, the frequency of *FGFR1* alterations was lower in patients with disease progression on CDK4 and 6i in this study (17%) than with progression on ribociclib (41% ref. 20). These inconsistencies may be attributed to the differences in sample type (tumor biopsy vs. ctDNA), copy number cut-off, and sample size ($n = 223$ vs. $n = 34$). *FAT1* loss was previously implicated as a resistance mechanism to CDK4 and 6i, with loss-of-function alteration in *FAT1* observed in ∼6% of metastatic tumors and resulting in poor outcomes with a median PFS of 2.4 months (56). In this real-world data set, a similar rare occurrence of *FAT1* loss was observed in biopsies obtained prior to progression on CDK4 and 6i and following progression on a CDK4 and 6i (1.5% vs. 2.2%), suggesting this resistance mechanism only occurred in a small group of patients treated with CDK4 and 6i ± ET. Heterozygous *RB1* loss was reported as a biomarker of acquired resistance to CDK4 and 6i and was associated with poor outcomes (57). We observed higher frequency of heterozygous *RB1* loss in CohortPost (47.5% vs. 36.1%). However, point mutation in combination with heterozygous *RB1* loss was very rare (CohortPost, 2.2% vs. CohortPre, 0.8%). Our results suggested that acquisition of subclonal *RB1* mutations in tumors with *RB1* heterozygous loss may mediate resistance to CDK4 and 6i ± ET in a small subset of patients. High levels of *CCNE1* were identified as a biomarker of resistance to CDK4 and 6i and were associated with attenuated benefit (58). We found significantly higher expression levels of *CCNE1* in postprogression tumors, supporting *CCNE1* as a biomarker of resistance. Alterations in *NOTCH1*, *NOTCH2*, *TAP1*, *TAP2, FCGR2A*, and *FCGR2B* were significantly enriched in biopsies from patients prior to receiving CDK4 and 6i (**Fig. 2B**). Further analysis is required to determine the role of these genes in disease progression on CDK4 and 6i.

Some limitations should be considered when interpreting these results. Because of limitations associated with data collection, it is possible that patients were treated with regimens that were not fully characterized, which may also have an influence on the genomic profiles observed in this study. There was a large difference in the frequency of patients who received prior ET in both cohorts, which could be due in part to missing data. It was not possible to include paired biopsy samples in this study, which would strengthen these results and the understanding of CDK4 and 6i resistance mechanisms. Finally, this study only included patients in the United States, which limits the generalizability of these findings.

This study identified potential mechanisms of resistance to CDK4 and 6i ± ET, including alterations in *ESR1* and amplification of chr12q15 in patients with HR+, HER2− MBC. These findings should be further explored to determine implications in the development of systemic treatments to overcome resistance for patients with HR+, HER2− MBC following progression on a CDK4 and 6i ± ET.

Authors' Disclosures

J. Beyrer reports other support from Eli Lilly and Company during the conduct of the study. E. Nash Smyth reports other support from Eli Lilly and Company during the conduct of the study, employment with Eli Lilly and Company, and ownership of Eli Lilly and Company stock. C. Morato Guimaraes reports other support from Eli Lilly and Company during the conduct of the study and other support from Eli Lilly and Company outside the submitted work. L.M. Litchfield reports personal fees from Eli Lilly and Company during the conduct of the study, personal fees from Eli Lilly and Company outside the submitted work, and ownership of Eli Lilly and Company stock. L. Bowman reports other support from Eli Lilly and Company during the conduct of the study and other support from Eli Lilly and Company outside the submitted work. G.W. Lawrence reports other support from Eli Lilly and Company outside the submitted work. A. Aggarwal reports personal fees from Eli Lilly during the conduct of the study and personal fees from Daiichi Sankyo outside the submitted work. F.

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Authors' Contributions

X. Rao: Conceptualization, data curation, formal analysis, visualization, methodology, writing–original draft, writing–review and editing. **Y. Chen:** Conceptualization, data curation, formal analysis, visualization, methodology, writing–original draft, writing–review and editing. **J. Beyrer:** Conceptualization, methodology, writing–original draft, writing–review and editing. **E. Nash Smyth:** Conceptualization, methodology, writing–original draft, writing–review and editing. **C. Morato Guimaraes:** Conceptualization, methodology, writing–original draft, writing–review and editing. **L.M. Litchfield:** Conceptualization, methodology, writing–original draft, writing–review and editing. **L. Bowman:** Conceptualization, methodology, writing–original draft, writing–review and editing. **G.W. Lawrence:** Visualization, writing–original draft, project administration, writing–review and editing. **A. Aggarwal:** Conceptualization, methodology, writing–original draft, writing–review and editing. **F. Andre:** Conceptualization, methodology, writing–review and editing.

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Note

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