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ORIGINAL RESEARCH

Genomic and clinical landscape of metastatic hormone receptors-positive breast cancers carrying *ESR1* alterations

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Background: Somatic genetic alterations of the estrogen receptor 1 gene (*ESR1)* are enriched in endocrine therapy-resistant, estrogen receptor-positive (ER+) metastatic breast cancer (mBC). Herein, we investigated and compared the clinical and genomic landscape of *ESR1*-mutant ($ESR1^{MUT}$) and *ESR1* wild type ($ESR1^{WT}$) ER+/ human epidermal growth factor receptor 2 (HER2)− mBCs.

Methods: Clinical and genomic data were retrieved from cBioPortal using the publicly-available MSK MetTropism dataset. Metastatic, ER+/HER2− mBC samples were included in the analysis. Only oncogenic and likely oncogenic alterations according to OncoKB were included. Statistical analyses were carried out using alpha level of 0.05, with a false discovery rate threshold of 10% for multiple comparisons using the Benjamini−Hochberg method.

Results: Among 679 samples, 136 $ESR1^{MUT}$ among 131 tumors were found (19.2%). The frequency of $ESR1^{MUT}$ was higher in ductal versus lobular mBC (21.2% versus 13.8%, $P = 0.052$) and enriched in liver metastasis compared with other sites (22.5% versus 12.7%; $q = 0.02$). Compared with $ESR1^{WT}$ mBC, $ESR1^{MUT}$ tumors showed higher fraction of genome altered (FGA) {[0.28 interquartile range (IQR), 0.15-0.43] versus 0.22 (0.11-0.38); $P = 0.04$} and tumor mutational burden (TMB) [4.89 (IQR 3.46-6.85) versus 3.92 (2.59-6.05) mut/Mb; $P = 0.001$]. Tumors harboring p.E380X alterations showed higher TMB compared with those with H11-12 alterations [8.24 (IQR 5.06-15.3) versus 4.89 (IQR 3.46-6.75) mut/Mb; $P = 0.01$]. Genetic alterations of *TP53* were enriched in $ESR1^{WT}$ tumors (36% versus 14%) [odds ratio (OR) 3.17, 95% confidence interval (CI) 1.88-5.64, $q = 0.001$]. Considering signaling pathways, $ESR1^{MUT}$ tumors showed a lower occurrence of *TP53* (OR 0.48, 95% CI 0.30-0.74; $q = 0.003$) and *MAPK* (OR 0.29, 95% CI 0.11-0.65; $q = 0.009$) alterations. *TP53* ($q < 0.001$), *CDH1* ($q < 0.001$), and *ERBB2* ($q < 0.001$) demonstrated mutual exclusivity with $ESR1^{MUT}$.

Conclusions: ER+/HER2− mBCs carrying $ESR1^{MUT}$ exhibit a divergent genomic background, characterized by a lower prevalence of *TP53* and *MAPK* pathway alterations. Less common *ESR1* alterations falling outside the H11-H12 region seem to occur in tumors with higher TMB, deserving further investigation to understand their potential actionability.

Key words: ESR1, breast cancer, genomic analysis, precision medicine, next-generation sequencing

INTRODUCTION

Estrogen receptor-positive (ER+) and human epidermal growth factor receptor 2 (HER2)-negative breast cancer (BC) represents the most common BC subtype, accounting for ∼70% of diagnosed cases.[1] Endocrine therapy (ET) represents the therapeutic cornerstone for ER+/HER2− BC, having been shown to result in improved survival in all settings of disease.[1] ER signaling can be manipulated through different pharmacological strategies, including direct antagonism to estrogen receptor 1 (ESR1),[2] by means of reduction of expression,[3] or inhibition of estrogen production from the ovaries[4] or from androgen aromatization into estrogens.[5]

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Most of ER+ metastatic breast cancer (mBC) will ultimately develop biological resistance to ET, which may be promoted by several factors. Among them, the emergence of mutations in estrogen receptor 1 (*ESR1*^{MUT}) represents one of the most well-characterized mechanisms, occurring in ∼30% of cases displaying ET resistance after prior exposure to antiestrogen therapy.[1,6] Most genomic variants in *ESR1* occur within the ligand-binding domain, promoting a conformational change of the protein resulting in an estrogen-independent constitutive transcription of ER-regulated genes.[7-9]

The impact of *ESR1*^{MUT} on the activity of ET agents varies across different classes, being an established mechanism of resistance to aromatase inhibitors (AIs), while generally preserving variable sensitivity to selective estrogen receptor degrader (SERD).[6,7,10-12] More recently, a novel generation of oral SERDs have been developed, demonstrating more favorable pharmacokinetic properties than fulvestrant and the ability to target a larger spectrum of hotspot *ESR1*-*MUT*.[10,13] Following preclinical studies, randomized clinical trials demonstrated the superior efficacy of oral SERD to AI and fulvestrant, and particularly among ET-resistant, *ESR1*-*MUT* ER+ mBC, thus leading to the validation of *ESR1*^{MUT} as a predictive biomarker for which diagnostic testing is routinely undertaken in clinical practice.[14-17]

Nevertheless, other genomic mechanisms may account for ET resistance, whose actionability yielded likewise expanded therapeutic options, with most represented by agents targeting the phosphoinositide 3-kinase-protein kinase B-mammalian target of rapamycin (*PI3K-AKT-mTOR*) pathway.[18,19] Accordingly, in light of the increasing genomically-driven therapeutic strategies for ER+ mBC, a critical task consists of elucidating the principal pathway promoting ET resistance, which is essential for tailoring treatments to restore sensitivity to ET and potentially delaying time to chemotherapy.

In this setting, limited data exist to examine the genomic background of *ESR1*^{MUT} ER+/HER2− mBC, and particularly to determine the presence of additional potentially actionable pathways, and to assess whether distinct molecular mechanisms may account for ET resistance compared with ESR1 wild-type (*ESR1*^{WT}) tumors. Within this context, the present study aimed to assess whether the presence of *ESR1*^{MUT} dictates or is dictated by a distinct genomic background, as well as to examine further potential biomarkers suitable for clinical actionability among ER+ mBC carrying or not *ESR1*^{MUT}.

METHODS

Data retrieval

Clinical and genomic data were retrieved from cBioPortal[20,21] using the *cBioPortalR* package.[22] For our analysis, publicly available data were queried from the MSK MetTropism dataset, an integrated pan-cancer cohort study of >25 000 patients affected by different primary tumors, including about 2500 BC.[23] Metastatic samples from patients affected by ER+/HER2−, which were previously exposed to ET, were considered for the analysis. All samples were subjected to genomic profiling by the FDA-cleared, tumor-normal MSK-IMPACT assay.[24]

Genomic analysis

Oncogenic and likely oncogenic alterations by OncoKB annotation[25] were included for downstream analysis. In the *ESR1*^{MUT} group, we excluded tumors carrying *ESR1* copy number gains while we considered for inclusion single nucleotide variants (SNV), small insertion/deletions (indels), rearrangement, or fusion within the *ESR1* gene, having copy number positive changes of the ESR1 gene a less clarified pathogenetic significance and not deemed as responsive to SERDs.[26] We selected genes to include in the pathway-level analysis according to those included in The Cancer Genome Atlas Program (TCGA) project.[27] *ESR1*^{MUT} were categorized according to the functional protein domain affected, as previously reported.[28] Tumor mutational burden (TMB) was calculated as the proportion of nonsynonymous mutations to the total number of base pairs sequenced per sample. Fraction of genome altered (FGA) was defined as the fraction of genome analyzed for copy number changes presenting with $\log_2$ copy number gain >0.2 or loss less than −0.2. The MSIsensor score with a threshold of ≥10 was used to define microsatellite instability (MSI-high).[29]

Statistical analysis

Categorical variables were reported as absolute numbers and proportions, and continuous variables as median and interquartile range (IQR). Associations of categorical variables were carried out using the Fisher's exact test, or logistic regression model, as appropriate. The Bartlett test and Shapiro−Wilk test were used to assess variances and normal distributions, respectively. Nonparametric tests for group comparisons for continuous variables included the Wilcoxon rank sum test and Kruskal−Wallis test. Dunn's test was used for multiple pairwise comparisons after a significant Kruskal−Wallis test. Statistical tests were carried out using a two-sided significance level of <0.05. The Benjamini and Hochberg method was used for multiple comparisons, whenever appropriate, using a false discovery rate threshold of 10%. Statistical analyses were carried out using R Software version 4.3.2.[30] Genomic co-occurrence and mutual exclusivity analyses were carried out using the DISCOVER[31] and Rediscover[32] R packages.

RESULTS

Clinical characteristics

A total of 679 ET-pre-treated ER+/HER2− mBC cases were retrieved. Among them, 99.26% (674 of 679) were females, with a median age of 58.8 years (IQR 50.6-67.0 years). One-hundred thirty-one (19.29%) samples exhibited *ESR1*^{MUT}, while the *ESR1*^{WT} group included 80.71% (548 of 679) of cases. Patients whose tumors had *ESR1*^{MUT} exhibited a significantly higher median age [62.5 years (IQR 54.5-67.8 years) versus 57.9 years (IQR 49.6-66.8 years); $P = 0.004$],

Table 1. Clinical and pathological characteristics among ESR1 mutant and ESR1 wild-type tumors

Characteristic	$ESR1^{MUT}$, N = 131	$ESR1^{WT}$, N = 548	P value
Sex			0.6
Female	131 (100%)	543 (99%)	
Male	0 (0%)	5 (0.9%)	
Age, years	63 (IQR 55-68)	58 (IQR 50-67)	**0.004**
Self-reported ethnicity			0.3
Asian	3 (2.4%)	28 (5.5%)	
Black or African American	10 (8.1%)	45 (8.9%)	
White	111 (90%)	433 (86%)	
Unknown	7	42	
Histology subtype			**0.026**
Ductal	110 (84%)	410 (75%)	
Lobular	21 (16%)	138 (25%)	

Significant *P* values ($P < 0.05$) are indicated in bold.
IIQR, interquartile range; MUT, mutant; WT, wild-type.

while a numerically lower proportion of $ESR1^{MUT}$ was observed among Asian patients (Asian 3 of 31, 9.68%; versus black 10 of 55, 18.2%; $n = 55$; white 111 of 544, 20.4%; $P = 0.329$) (Table 1).

Invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) represented 76.58% (520 of 679) and 23.42% (159 of 679) of cases, respectively. IDC exhibited a numerically higher proportion of $ESR1^{MUT}$ compared with ILC (21.2% versus 13.8%, $P = 0.052$).

Liver represented the most commonly sampled site on which comprehensive genomic profiling (CGP) had been carried out (27.69%, 188 of 679), followed by bone (17.53%, 119 of 679), and lymph nodes (15.32%, 104 of 679). Considering the proportion of $ESR1^{MUT}$ according to the anatomical site sampled, an enrichment of $ESR1^{MUT}$ was observed in liver lesions compared with non-liver sites (22.5% versus 12.7%; $q < 0.001$) (Figure 1A).

Finally, in the multivariable binary logistic regression model, older age ($P < 0.001$), presence of liver metastasis ($P = 0.001$), and IDC histology ($P = 0.05$) were all associated with the presence of $ESR1^{MUT}$ (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2024.103731).

ESR1 genomic alterations

A total of 136 $ESR1^{MUT}$ were observed among 131 cases. Of them, 97.79% (133 of 136) consisted of SNV, while indels accounted for the remaining 3 (2.21%) cases.

The most common $ESR1^{MUT}$ consisted of the missense point mutation p.D538G (41.91%, 57 of 136), followed by p.Y537S (22.06%, 30 of 136), and p.E380Q (10.29%, 14 of 136) (Figure 1B). Considering $ESR1^{MUT}$ mutations according to the functional protein domain, 85.29% (116 of 136) of $ESR1^{MUT}$ consisted of class I alterations located around helix 11-12 and within the LBD, followed by mutations affecting helix 5 (p. E380Q) (10.29%, 14 of 136), and class II mutations located in the *ESR1* dimerizing domain (4.41%, 6 of 136), including in this latter group p.M421V, p.F461V, p.V422del, p.V418E, p.G442R, and p.S463P mutations.

Five patients (3.8%) displayed multiple $ESR1^{MUT}$, which included p.D538G and p.Y537C; p.E380Q and p.Y537C; p.Y537N and p.V422del; p.D538G and p.G442R; p.Y537S and p.Y537N.

Genomic co-alterations with $ESR1^{MUT}$

In both $ESR1^{MUT}$ and $ESR1^{WT}$ tumors, *PIK3CA* represented the most commonly altered gene (41% and 38%, respectively; Figure 1C, Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2024.103731). Compared with $ESR1^{WT}$ tumors, $ESR1^{MUT}$ tumors showed a lower rate of genomic alterations in *TP53* {15% versus 36%, odds ratio (OR) 0.33 [95% confidence interval (CI) 0.19-0.56], $q < 0.001$} and *ERBB2* [1% versus 7%, OR 0.10 (95% CI 0.01-0.62), $q = 0.07$] (Figure 2A). Considering signaling pathways, $ESR1^{MUT}$ showed a lower occurrence of *MAPK* [OR 0.34 (95% CI 0.14-0.73), $q = 0.02$] and *TP53* pathway [OR 0.49 (95% CI 0.31-0.74), $q = 0.003$] alterations (Figure 2A and B, Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2024.103731).

In the co-occurrence and mutual exclusivity analysis, $ESR1^{MUT}$ showed mutual exclusivity with genomic variants in *TP53* ($q < 0.001$), *CDH1* ($q < 0.001$), and *ERBB2* ($q < 0.001$) (Figure 2C; Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2024.103731). Conversely, $ESR1^{MUT}$ did not demonstrate any significant co-occurrence with other genetic alterations (Supplementary Table S4, available at https://doi.org/10.1016/j.esmoop.2024.103731).

Finally, considering the availability of different genomically-driven therapies in ER+/HER2− advanced BC, we further evaluated the different distribution of genomic biomarkers for which at least one therapy is clinically approved among $ESR1^{MUT}$ and $ESR1^{WT}$ tumors. While we did not observe any case showcasing microsatellite instability, genomic fusion involving *NTRK1/2/3* or *RET* and class I *BRAF* alterations, between $ESR1^{MUT}$ and $ESR1^{WT}$ BC we did not observe any statistically different distribution of gene alterations in *PIK3CA* (38.6% versus 44.1%, $P = 0.29$), *PTEN* (5.3% versus 8.7%, $P = 0.26$), *AKT1* (7.6% versus 5.3%, $P = 0.50$), and in the proportion of TMB-high tumors (11.4% versus 10.5%, $P = 0.77$) (Figure 2D).

Genomic signatures

Compared with $ESR1^{WT}$ tumors, tumors showing $ESR1^{MUT}$ exhibited a higher median TMB [4.89 (IQR 3.46-6.85) versus

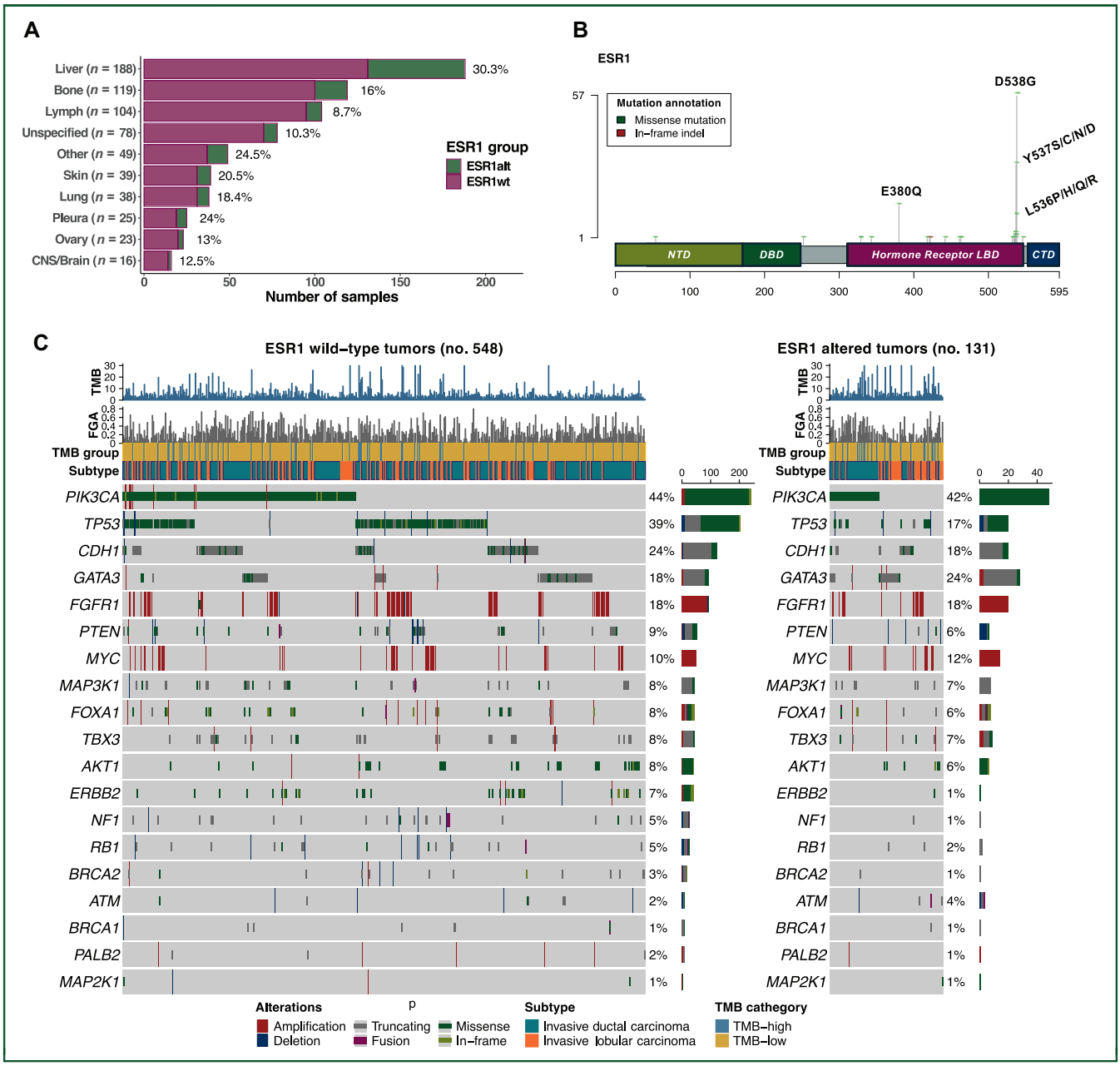

Figure 1. Proportion of *ESR1*^{MUT} according to metastatic site, type of *ESR1* mutations, and driver genes among *ESR1*-mutated and *ESR1* wild-type tumors. (A) Sampled sites with proportion of *ESR1*^{MUT} detected by each site (shown in red). (B) Spectrum of *ESR1*^{MUT} detected among 679 ER+ mBC. (C) Oncoprints of driver genes in breast cancer among *ESR1*^{WT} and *ESR1*^{MUT} tumors.
CTD, C-terminal domain; DBD, DNA-binding domain; ER, estrogen receptor; FGA, fraction of genome altered; LBD, ligand binding domain; mBC, metastatic breast cancer; MUT, mutant; NTD, N-terminal domain; TMB, tumor mutational burden; WT, wild-type.

3.92 (IQR 2.59-6.05), respectively; $P < 0.001$] and FGA [0.28 (IQR 0.15-0.43) versus 0.22 (IQR 0.10-0.38), respectively; $P = 0.047$] (Figure 3A and B). Considering the class of *ESR1*^{MUT}, tumors carrying class H5 mutations showed higher median TMB [8.24 (IQR 5.06-15.3)] compared with class I/H11-12 *ESR1*^{MUT} [4.32 (IQR 3.46-6.66), $P = 0.01$] and class II *ESR1*^{MUT} [4.66 (IQR 4.31-5.14), $P = 0.39$] (Figure 3C). Of note, 5 of 14 p.E380Q *ESR1*^{MUT} (class H5) showed a TMB above 10 mutations per megabase (mut/Mb). Conversely, no difference in FGA was observed according to the class of *ESR1*^{MUT} [0.28 (IQR 0.15-0.43) Class I/H11-12; 0.24 (0.13-0.41) H5; 0.24 (0.11-0.41) Class II; $P = 0.88$] (Figure 3D).

DISCUSSION

In the present work, we outlined the clinical and genomic landscape of ET-resistant ER+/HER2− mBC showing *ESR1*-*MUT*, and aimed to define clinical variables to better select diagnostic strategies and genomic correlates that could potentially inform and tailor therapeutic decision-making.

Distinctive genomic mechanisms may account for ET resistance in ER+/HER2− mBC.

Consistent with previous reports,[7,33] in our cohort tumors showcasing *ESR1*^{MUT} represented about 20% of the cases. In the multivariable analysis, *ESR1*^{MUT} tumors demonstrated enrichment in liver metastasis, as previously shown[34,35] and

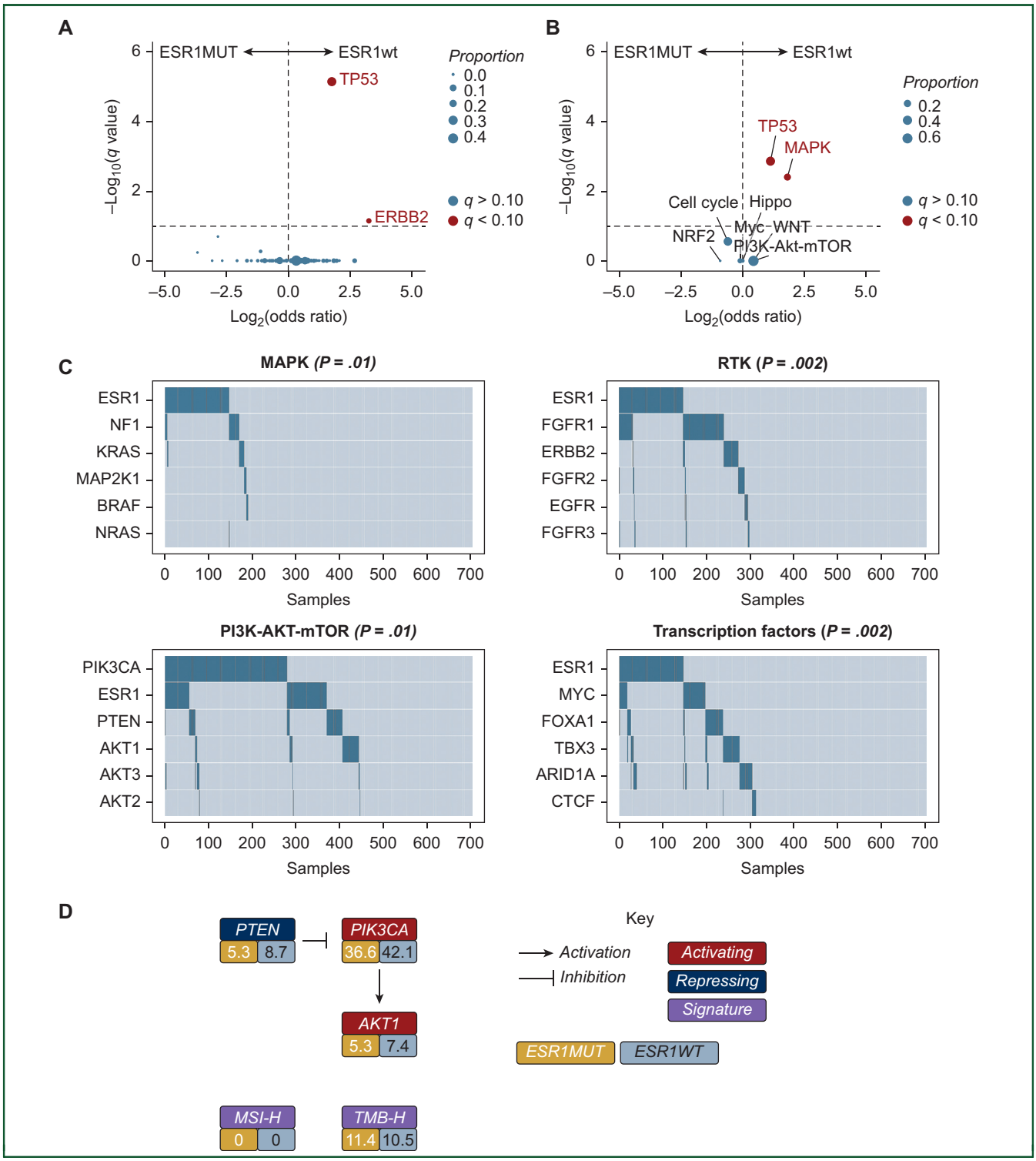

Figure 2. Enrichment gene alteration analysis among *ESR1*-mutated and *ESR1* wild-type tumors. (A) Different distribution of gene-level genomic alterations among $ESR1^{MUT}$ and $ESR1^{WT}$ tumors. (B) Different distribution of pathway-level genetic alterations among $ESR1^{MUT}$ and $ESR1^{WT}$ tumors. (C) Groupwise comparisons of mutual exclusivity of selected list of genes from included in the *MAPK*, *RTK*, *PI3K-AKT-mTOR*, and transcription factors pathways. (D) Proportion of detected genetic biomarkers with approved biomarker-matched targeted therapy available among $ESR1^{MUT}$ (in yellow) and $ESR1^{WT}$ (in gray) tumors. *NTRK1/2/3* fusions, *RET* fusions and MSI-H not displayed as no case was observed.
MUT, mutant; WT, wild-type.

occurred with lower frequency in younger patients and among ILC. The genomic landscape of ER+/HER2− BC differs according to age,[36] with tumors arising in younger women demonstrating lower endocrine dependence and reduced expression of *ESR1* and ER-related genes.[37-39] Therefore, it is possible different mechanisms of ET resistance may emerge in tumors affecting younger women, which may not be directly related to the ET pathway, but rather occur in non-overlapping signaling pathways. Similarly, ILCs have been described to carry recurrent genomic

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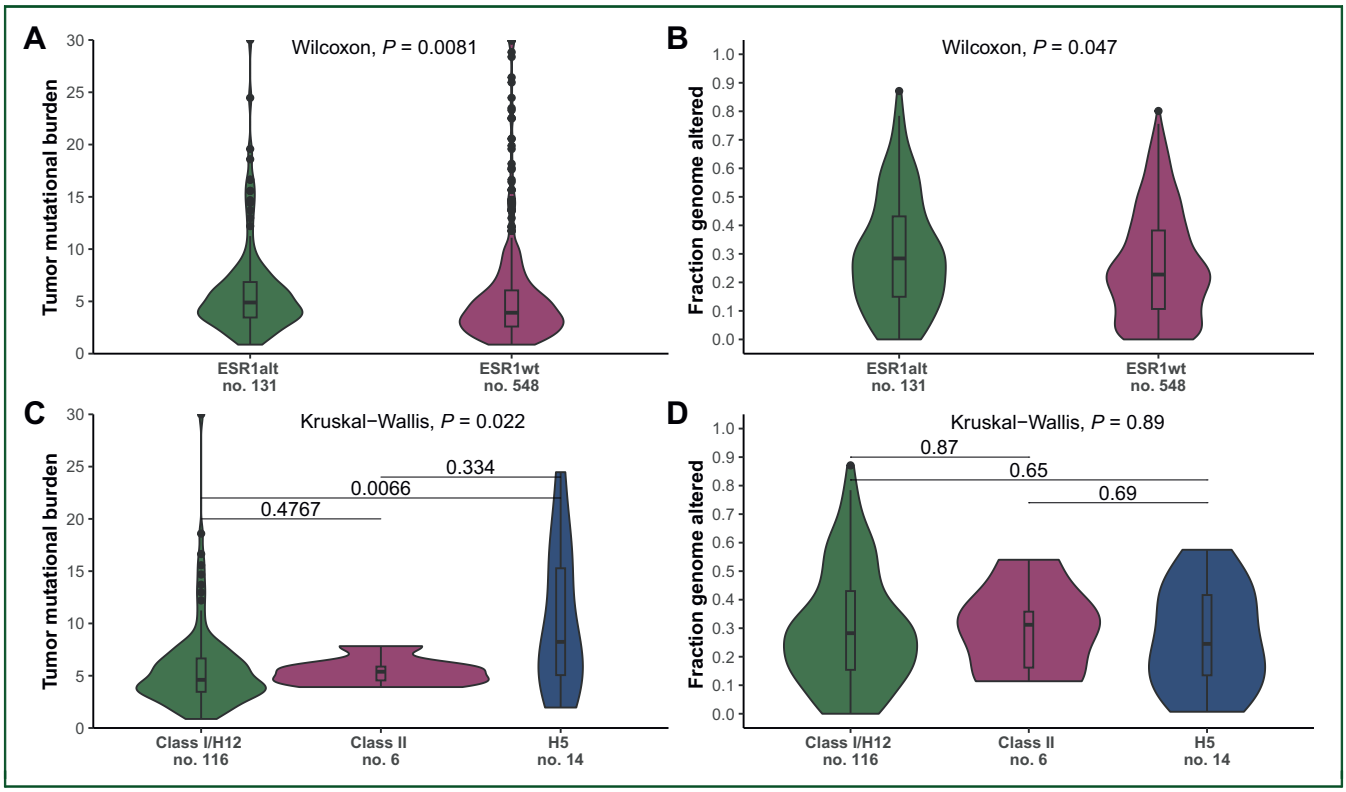

Figure 3. Genomic signatures among *ESR1*-mutated and *ESR1* wild-type tumors, and across classes of *ESR1* mutations. (A) Comparison of tumor mutational burden and (B) fraction genome altered among $ESR1^{MUT}$ and $ESR1^{WT}$ tumors. Comparison of tumor mutational burden (C) and fraction genome altered (D) across different class of *ESR1* mutations.
MUT, mutant; WT, wild-type.

alterations in oncogenes including *MYC*, *PIK3CA*, *FGFR1*, *AKT1*, *ERBB2*, and *ERBB3*.[40-42] In our analysis, we observed ILC to carry a lower frequency of $ESR1^{MUT}$ compared with IDC (11.9% versus 20.1%, respectively; $P = 0.052$). Moreover, in contrast to previous studies showing recurrent *ESR1* copy number gains occurring in ILC,[41] in our analysis, we observed only one case of ILC exhibiting *ESR1* amplification, not suggesting *ESR1* amplifications to be enriched among ILC. Accordingly, our result supports the notion that ILC may leverage with higher frequency on non-estrogen-related genomic pathways to acquire ET, with our hypothesis further reinforced by the mutual exclusivity we observed between $ESR1^{MUT}$ and genetic alterations occurring in the *CDH1* gene ($q < 0.001$).

Alterations involving oncogenes included within the *MAPK* pathway carry a critical role in determining ET resistance. Copy number losses and truncating mutations in *NF1*, along with hotspot mutations in *KRAS*, *BRAF*, and *MAP2K1* (encoding *MEK1*) promote secondary resistance to ET and hence are found to be enriched among pre-treated ER+ mBC.[43,44] Importantly, in our analysis, of 51 oncogenic alterations in the *MAPK* pathway among 46 cases, 42 (91.3%) of them occurred in $ESR1^{WT}$ tumors, the association of which with the $ESR1^{WT}$ status was further confirmed by the significant inferior rate of *MAPK* alterations at the pathway level among $ESR1^{MUT}$ tumors ($q = 0.02$). Accordingly, our results support the concept that mutations in *ESR1* and *MAPK* represent two non-overlapping mechanisms of ET resistance, with agents targeting the *MAPK* pathway potentially representing a ground of research for overcoming ET resistance particularly among $ESR1^{WT}$ tumors.

Besides *MAPK* oncogenes, alterations in tyrosine kinase receptors (*TKR*) play a pivotal role in determining estrogen-independent tumor growth in ER+/HER2− BC. While *ERBB2* copy number gains and hotspot mutations have been reported to be shared by primary tumors and thus contribute to primary ET resistance,[40,43,45,46] alterations involving *EGFR* and *FGFR* are positively selected by acquired mechanisms of resistance to ET.[40,43,47-50] Similarly to *MAPK* alterations, we observed a superior rate of TKR alterations occurring among $ESR1^{WT}$ tumors (27.3% versus 19.0%, respectively, $P = 0.051$). Of note, $ESR1^{WT}$ tumors exhibited 38 of 39 (97.4%) and 10 of 11 (90.9%) *ERBB2* and *EGFR* alterations, respectively. Conversely, we did not observe a different distribution of *FGFR1* alterations among $ESR1^{MUT}$ and $ESR1^{WT}$ tumors (15.2% and 16.9%, respectively), which may be related to the reported co-occurrence of $FGFR1^{MUT}$ and $ESR1^{MUT}$ in tumors showing resistance to cyclin-dependent kinase (CDK) 4 and 6 inhibitors (CDK4/6i) plus ET.[50] Notably, most *ERBB2* alterations (89.1%) consisted of SNV and indels, supporting the notion that *ERBB2* alterations other than amplifications represent a relevant and potentially actionable mechanism of ET resistance, which should be further investigated along with agents targeting other *TRK*, such as *EGFR*, particularly for tumors not showing $ESR1^{MUT}$

and for tumors showcasing pre-treatment TRK genomic alterations.

Considering the expanding genomically-driven therapeutic option for pre-treated ER+/HER2− BC, and particularly for agents targeting the *PI3K-AKT-mTOR* cascade, we investigated whether *ESR1*^{MUT} could account for a different prevalence of alterations in this signaling pathway. While previous studies reported a higher prevalence of *PIK3CA*^{MUT} among *ESR1*^{MUT} BC,[9] in our cohort, *PIK3CA* represented the most commonly altered gene in both *ESR1*^{MUT} and *ESR1*^{WT} tumors, with no association between the *ESR1* status and *PI3K-AKT-mTOR* alterations in our pathway-level analysis. As such, our results demonstrate that concomitant alterations in the *PI3K-AKT-mTOR* pathway occur with a relevant frequency also among *ESR1*^{MUT} tumors, which may be particularly true for tumors showing resistance to CDK4/6i, as previously shown,[50] which emphasizes the necessity to gather additional biomarkers to further personalize second-line treatment options for *ESR1*^{MUT} tumors showing *PIK3CA*/*AKT1*/*PTEN* alterations.

In addition to targeting *ESR1*^{MUT} and the PI3K-AKT-mTOR pathway, several trials assessed the continuation of CDK4/6i beyond progression, with conflicting clinical results that underscore the importance of biomarker selection. Different mechanisms of resistance may account for CDK4/6i resistance, including alterations in *RB1*, *AURKA*, *CCNE1*, *ERBB2*, and *FGFR1*.[51-53] Of note, in *ESR1*^{MUT} compared with *ESR1*^{WT} tumors, we observed a relevant lower occurrence of alterations in *RB1* (2 of 131, versus 23 of 548, $P = 0.19$) and *AURKA* (2 of 131 versus 26 of 548, $P = 0.13$), suggesting a different mechanism of resistance to CDK4/6i may emerge according to the *ESR1* status. To support our observation, in the phase II PACE study, continuing palbociclib while switching to ET showed superior outcomes among *ESR1*^{MUT} ($n = 78$),[54] whose finding was yet not replicated in the phase II MAINTAIN trial, in which ribociclib in combination with fulvestrant following progression to ET plus CDK4-6i (mostly receiving palbociclib) showed inferior benefits among *ESR1*^{MUT} tumors ($n = 36$).[55] It must be noted that in the MAINTAIN study, 50% of *ESR1*^{MUT} tumors showed co-occurrent *FGFR1* alterations,[55] which along with the small population size, does not discredit our hypothesis for which resistance mechanisms to CDK4/6i may occur with a lower frequency among *ESR1*^{MUT} tumors, warranting further investigation as to whether *ESR1*^{MUT} may act as a surrogate biomarker for which CDK4/6i continuation may portend clinical benefits.

Consistent with previous reports,[56,57] we observed a mutual exclusivity between *ESR1*^{MUT} and *TP53* genomic alterations, the relationship of which remained significant at the pathway-level analysis. A reciprocal antagonism between *ER* and *TP53* signaling has been reported, with *TP53* aberrations negatively affecting the downstream *ER* activation, and conversely by *ER* blocking the transactivation of *TP53* by means of activation of TP53 co-repressors.[35,50,57,58] Consequently, our results further confirm the concept that ER+ mBC can leverage distinctively on either *ESR1*^{MUT} or *TP53* pathway under the selective pressure of ET. Moreover, tumors carrying co-occurrent *TP53*^{MUT}-*ESR1*^{MUT} have been described to be enriched in liver metastasis and to carry an immune-enriched imprinting with a higher prevalence of CD8+ T cells and programmed death ligand 1 (PD-L1) expression.[35] In our study cohort, of 19 patients affected by tumors showing co-occurrent *TP53*^{MUT}-*ESR1*^{MUT}, 15 of them (78.9%) showed liver involvement. Furthermore, tumors showing *TP53*^{MUT}-*ESR1*^{MUT} exhibited higher TMB compared with both *TP53*^{WT}-*ESR1*^{MUT} and *TP53*^{MUT}-*ESR1*^{WT} tumors (5.87 versus 4.89 mut/Mb, $P = 0.28$; and 5.87 versus 4.89 mut/Mb, $P = 0.39$, respectively), as well as higher FGA (0.36 versus 0.24, $P = 0.002$; and 0.36 versus 0.30, $P = 0.02$, respectively). As such, our findings expand the limited evidence suggesting tumors carrying *TP53*^{MUT}-*ESR1*^{MUT} exhibit higher genomic complexity, potentially rendering them susceptible to immunotherapy, and to display aggressive clinical behavior, warranting further characterization of their genomic landscape and investigation for tailored treatment regimens.

Lastly, we investigated whether different *ESR1*^{MUT} account for different genomic signatures. In line with previous reports, we observed most *ESR1*^{MUT} (85.29%) occur in the loop bridging helix 11 (H11) and H12, which are known to promote conformational changes in *ESR1* to drive ligand-independent downstream activation. Conversely, a second hotspot alteration involves the aminoacidic position 380, affecting H5, which represented 10.29% of our observed cases and lies topographically near the dimer interface in which class II *ESR1*^{MUT} occur, which is believed to promote *ESR1*-ligand independent heterodimerization, as these latter. While we observed superior TMB and FGA for *ESR1*^{MUT} compared with *ESR1*^{WT} tumors, we observed a relevant higher TMB particularly for H5 mutations (8.24 mut/Mb) compared with class I *ESR1*^{MUT} (4.32 mut/Mb, $P = 0.01$) and class II *ESR1*^{MUT} (4.66 mut/Mb, $P = 0.39$), with 35.7% (5 of 14) showing a TMB above 10 mut/Mb. To our knowledge, no previous reported data revealed a higher TMB specifically in the context of E380Q *ESR1*^{MUT}, which in light of the encouraging progression-free survival observed in the PACE trial with avelumab added to fulvestrant and continuation of palbociclib in ET-resistant ER+ mBC,[54] should be further investigated to possibly identify subgroups of ER+ MBC which may potentially benefit from immunotherapy. Of note, E380Q *ESR1*^{MUT} has been described as a context-mutation frequently occurring in mBC carrying an APOBEC3 dominant signature, which in turn has been shown to associate with higher TMB and to account for most ER+/HER2− BC showing ≥10 mut/Mb,[59] Accordingly, our data suggest the tumor detection of E380Q *ESR1*^{MUT} could act as a surrogate biomarker of HR+/HER2− BC with potential high neoantigen load, for which the use of immunotherapy should be further explored.

It must be noted our work presents limitations. First, our analysis refers to a retrospective, single-institution case series, for which findings should gather external replication and validation. Second, regardless we selected post-

treatment, metastatic ER+ BC, we did not have access to specific treatment data and to the line of therapy on which *ESR1*^{MUT} was detected, and accordingly to whether the genomic landscape could differ according to the previous exposure to distinct ET regimens; nevertheless, it is reasonable most of the patients were exposed to AI and CDK4/6i, according to international guidelines, and corroborated by the relevant frequency of *ESR1*^{MUT} we observed. Third, for the same reason, we could not perform correlations between genomic biomarkers and clinical outcomes to ET as well as analyze different genomic mechanisms possibly governing different resistance to AI and fulvestrant.

Despite these limitations, our study provides a comprehensive analysis of the genomic landscape differentiating *ESR1*^{MUT} from *ESR*^{WT} tumors, revealing distinct mechanisms of ET resistance and further potential and distinct therapeutic targets among the two groups of ER+/HER2− mBC.

Conclusion

Our genomic analysis revealed divergent acquired mechanisms driving clinical resistance to ET, with non-overlapping signaling cascades possibly separating resistance trajectories according to the *ESR1* status. While optimal second-line targeted treatments should be guided in a biomarker-driven fashion, further research is needed to elucidate the best treatment options for tumors carrying multiple actionable biomarkers. Moreover, novel therapeutic options, such as agents targeting *MAPK* and *TRK*, should be further explored to revert ET resistance, and particularly among *ESR1*^{WT} tumors. Lastly, our findings showcased specific mutations in *ESR1* associate with predictive biomarkers of immunotherapy efficacy, warranting further research to address their potential actionability by using immuno-oncology agents.

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STATEMENT OF ETHICAL APPROVAL

Data used for the reported analyses were retrieved from a publicly available database (cBioPortal), with cases anonymized and assigned to a unique code for de-identification, for which no ethical approval nor specific informed consent for the present study was required.

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