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Genomic characterization of tumor mutational burden-high breast carcinomas



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Tumor mutational burden (TMB) has emerged as a potential surrogate for neoantigen load and an indicator of immune checkpoint (IC)-blockade response; however, its precise significance in breast cancer (BC) is not fully understood. Here, we comprehensively characterized the genomic repertoire of BCs with a TMB ≥ 10 mut/Mb (TMB-high [$n = 527$]) to identify putative predictors of importance. The predominant mutational signature was apolipoprotein B mRNA-editing enzyme catalytic polypeptide (APOBEC) in 64.7% of tumors. TMB-high BCs were enriched in *KMT2C*, *ARID1A*, *PTEN*, *NF1*, and *RB1* alterations, which are associated with APOBEC mutagenesis. Further identified were loss-of-function *ARID1A* and *PTEN* alterations, which are linked to immune cell exclusion. *ESR1* p.E380Q prevailed among all *ESR1* hotspot mutations, supporting APOBEC-mediated effects. Finally, mutations in DNA damage response and repair genes were seen at a higher frequency than in non-TMB-high BCs. These findings provide justification for exploring combined pharmacologic inhibition to improve IC-based efficacy.

Tumor mutational burden (TMB) is a quantifiable numeric indicator of the total number of acquired somatic mutations in a delineated region of the genome (i.e., mutations per megabase [mut/Mb]). Consequently, TMB has emerged as a surrogate biomarker for the predicted neoantigen formation, to identify patients that may benefit from immune checkpoint (IC)-blockade. The latter concept is analogous to microsatellite instability (MSI)-high tumors that are attributed to mismatch repair impairment with ensuing somatic hypermutation¹. Following the authorization of pembrolizumab by the Food and Drug Administration (FDA) on June 16, 2020, in the setting of unresectable or metastatic TMB-high solid tumors², defined as ≥ 10 mut/Mb, TMB determination has emerged as a metric of paramount importance for therapeutic decision making.

Breast cancer (BC) has long been considered an immunologically “cold” tumor, with triple negative breast cancer (TNBC) exhibiting the greatest immune cell enrichment among different subtypes, as demonstrated by tumor-infiltrating lymphocytes (TILs) and gene expression signatures^{3,4}. Nonetheless, only a subset of patients achieves therapeutic benefit from IC-blockade. At present, a practical ascertainment of tumor immune cell activation involves the determination of stromal and intra-tumoral TILs by histopathologic evaluation and application of programmed death-ligand 1 (PD-L1) protein expression by immunohistochemistry. Intuitively, high TMB serves as a marker for eliciting a T-cell immune

response due to an increased neoantigen load, yet IC-blockade response does not necessarily always correlate with TMB. On that account, predictive markers of immune response are urgently needed to better tailor treatment decisions.

Studies have yet to show that a defined TMB threshold can recognize potential patients that may derive benefit from IC-blockade therapy across various tumor types⁵. TNBC is considered the most aggressive and immunogenic BC subtype, with most studies investigating the efficacy of IC-blockade in these tumors. The initial phase Ib clinical trial (KEYNOTE-012) assessed the efficacy of single-agent pembrolizumab in PD-L1⁺ metastatic TNBCs in patients who had predominantly undergone multiple lines of therapy with an overall response rate of 18.5%⁶. Subsequent studies (KEYNOTE-119) explored the effectiveness of single-agent pembrolizumab in the metastatic setting as a second- or third-line treatment option for TNBCs with no change in overall patient survival when compared to chemotherapy⁷. Thereafter, combination regimens with chemotherapy to “prime” the immune system as a result of chemotherapy-induced immunogenic cell death were explored⁸. Importantly, only a subset of PD-L1⁺ metastatic TNBCs appear to be responsive to IC-blockade, which supports the notion of combination therapies⁹. An additional essential parameter to consider is whether the tumoral neoantigen load induces an immune response. Tumors exhibiting a high neoantigen load that also show a

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positive correlation with CD8⁺ T-cell infiltration, such as metastatic endometrial cancer (KEYNOTE-158) and metastatic melanoma¹⁰, have been reported to confer greater response rates with IC-blockade treatment modalities⁵. Conversely, those who lack the latter correlation (e.g., breast and prostate cancer) have shown not to portend a response rate greater than 20%⁵.

In this study, we queried a large dataset of clinically sequenced BCs to characterize the genomic landscape of BCs with a TMB ≥ 10 mut/Mb (i.e., TMB-high BCs), seeking to identify genetic alterations that may impact clinical response to IC-blockade. We report that patients with TMB-high BCs exhibit enrichment in somatic alterations beyond *PIK3CA* and *TP53* that include *KMT2C*, *ARID1A*, *PTEN*, *NFI*, and *RB1*, which have been linked to apolipoprotein B mRNA editing enzyme catalytic polypeptide (APOBEC)-induced mutagenesis and immune cell exclusion from tumor samples. Moreover, DNA damage response and repair (DDR) genes were altered at a higher frequency in TMB-high BCs, suggesting that alternate therapeutic avenues could potentially be exploited. Our study demonstrates that TMB-high BCs harbor a composition of alterations that can be explored beyond IC-blockade and presents insight with promising avenues for improved patient outcomes.

Results

Patient characteristics

To examine the proportion of BCs with a TMB ≥ 10 mut/Mb we leveraged the Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) platform and identified a total of 527 tumors (primary, $n = 121$ [23%]; metastatic, $n = 406$ [77%]) from 462 patients who met the inclusion criteria. The entire MSK-IMPACT BC cohort at the time of this analysis was comprised of 7894 tumors from 6589 patients who underwent targeted genomic profiling. The mean age at time of diagnosis was 54.2 years (range, 28–82) and the TMB spanned from 10.0 to 394.1 mut/Mb (mean: 18.4; median: 14.0). The mean TMB across the entire MSK-IMPACT BC clinical sequencing cohort was 4.6 mut/Mb (median: 3.5). Overall, invasive ductal carcinoma (IDC) was the most common histologic subtype seen in 62.6% of tumors, followed by invasive lobular carcinoma (ILC) at 26%. The baseline patient and clinicopathologic characteristics are collectively summarized in Table 1.

Distribution of genomic alterations in TMB-high BCs

We next sought to explore the genomic landscape of TMB-high BCs and found identifiable differences in the frequency of alterations when compared to the rest of the cohort (i.e., BCs with a TMB < 10 mut/Mb = non-TMB-high BCs). Tumors cataloged as having ≥ 10 mut/Mb comprised 6.7% of the entire MSK-IMPACT BC clinical sequencing cohort (Fig. 1a). Among TMB-high BCs, 75.1% of tumors had a TMB ranging from 10.0 to 19.9 mut/Mb, whereas 24.9% had a TMB ≥ 20 mut/Mb (Fig. 1b). TMB-high BCs showed a statistically significant enrichment in somatic alterations in genes including *PIK3CA* (60% vs. 33%; $p = 1.61 \times 10^{-37}$), *TP53* (57% vs. 41%; $p = 2.36 \times 10^{-11}$), and *CDH1* (34% vs. 13%; $p = 6.48 \times 10^{-43}$), when compared to the non-TMB-high BCs (Fig. 1c). No appreciable differences were seen when stratifying TMB thresholds by biomarker status in TMB-high BCs.

To investigate the distribution of specific genomic alterations in metastatic tumors, we explored curated sites of involvement and determined the alteration frequency of key genes. Among metastatic BC samples, the most frequent site sequenced was the liver ($n = 89$), followed by lymph node ($n = 70$) and bone ($n = 65$) (Fig. 1d). Alterations in *ESR1* were found in 27% of liver metastases, whereas *CDH1* mutations and *ERBB2* amplifications were present in $\geq 50\%$ of bone and brain metastases, respectively. This mutational preference for specific metastatic sites is in keeping with prior findings^{11,12}. Amplification of *CD274*, the gene encoding for PD-L1, was not common and was seen in 0.9% and 0.6% of TMB-high and non-TMB-high BCs, respectively (Fig. 1e).

Lastly, we explored the structural variant distribution across all TMB-high BCs using MSK-IMPACT and in a subset of tumors reflexed to MSK-

Table 1 | Clinical characteristics of patients in the study cohort

Characteristic	Patients, <i>n</i> (%) (<i>n</i> = 462)
Age at diagnosis, mean (range), years	54.2 (28–82)
Total tumors analyzed	527 (100)
<i>Gender</i>	
Female	460 (99.6)
Male	2 (0.4)
<i>Tumor histology</i>	
Invasive ductal carcinoma (IDC)	330 (62.6)
Invasive lobular carcinoma (ILC)	137 (26)
Mixed IDC & ILC (MDL)	18 (3.4)
Invasive BC—not otherwise specified (NOS)	38 (7.2)
Metaplastic carcinoma	2 (0.4)
Other	2 (0.4)
<i>Hormone receptor status of tested sample</i>	
ER ⁺ , HER2 [−]	354 (67.2)
ER ⁺ , HER2 ⁺	47 (8.9)
ER [−] , HER2 ⁺	36 (6.8)
ER [−] , HER2 [−]	87 (16.5)
Unknown	3 (0.6)
<i>TMB (mut/Mb)</i>	
10.00–19.99	396 (75.1)
20.00–49.99	121 (23)
≥ 50.00	10 (1.9)

Invasive BC—not otherwise specified (NOS) represents BCs of unknown histologic subtype.

Fusion ($n = 108$), a targeted RNA-based assay, and identified 42 fusions involving various loci of the genome (Fig. 1f). Among these, the most recurrent in-frame fusions involved *ESR1* (Chr. 6). Further identified were the androgen receptor (AR) splice variant-7 (AR-V7) ($n = 13$) and *ESR1* out-of-frame fusions ($n = 5$) by MSK-Fusion. The latter events resulted in the deletion of the estrogen receptor (ER) alpha (ERα) ligand-binding domain.

TMB-high BCs exhibit a dominant APOBEC signature

Mutational signature analyses were conducted employing the signature multivariate analysis (SigMA) pipeline, a computational tool approach, which is tailored to generate mutational signatures from targeted sequencing panels¹³. Dominant mutational signatures were inferred in tumors with ≥ 8 curated exonic single-nucleotide variants or small indels. We detected a dominant APOBEC signature in 64.7% of TMB-high BCs, followed by a homologous recombination deficiency (HRD) signature in 23.1% (Fig. 2a). Among different BC subtypes, the greatest contribution of the HRD signature was seen in TMB-high TNBCs (Fig. 2b), whereas the highest TMB was seen in BCs with a dominant APOBEC signature (mean: 19.6 mut/Mb; median: 15.8 mut/Mb) (Fig. 2c). Using a non-parametric Wilcoxon Rank-Sum test (Mann–Whitney *U* test), TMB values were significantly higher in tumors with a dominant APOBEC signature when compared to HRD ($p = 1.6 \times 10^{-12}$), clock ($p = 0.002$), and ROS/5FU ($p = 0.005$). The predominant contribution of APOBEC-related mutations is consistent with previous findings implicating genes such as *PIK3CA*, *KMT2C*, *ARID1A*, *NFI*, and *ESR1* in BC oncogenesis^{14–17}.

Immunohistochemistry for mismatch repair proteins was available for select cases with an MSI signature and showed loss of protein expression (Fig. 2d). The mean TMB for TMB-high BCs with a dominant MSI signature was 19.5 mut/Mb (median: 15.3). PD-L1 expression by immunohistochemistry was available for a subset of TMB-high BCs ($n = 38$), with 9 tumors being positive (SP142, $n = 8$; SP263, $n = 1$) and 29 negative (SP142, $n = 24$; SP263, $n = 5$) (Fig. 2e). Collectively, these data underscore the

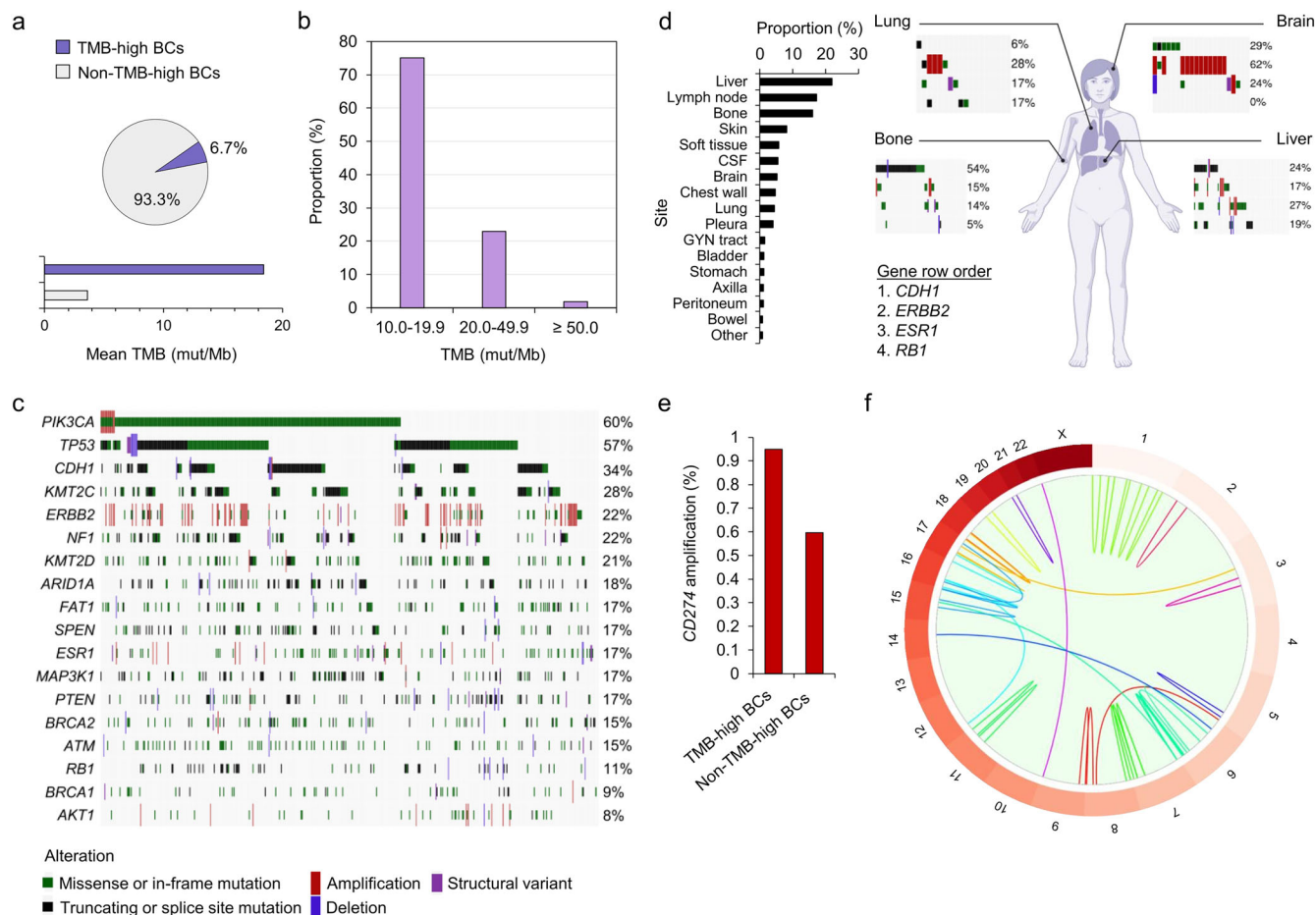


Fig. 1 | Comprehensive molecular characterization of TMB-high BCs.

a Schematic representation of the percentage of TMB-high BCs in the MSK-IMPACT clinical sequencing cohort. Bar chart depicts the mean TMB among the two respective groups. **b** Bar chart showing the TMB distribution across the cohort with the corresponding proportion of tumors. **c** Oncoprint depicting the repertoire of gene alterations in the study cohort by the targeted MSK-IMPACT platform, sorted by decreasing frequency. The top 10 most frequently altered genes are presented, in addition to select genes of clinical importance. Tumors are shown in columns and genes in rows, with the percentage of alterations displayed on the right.

d Distribution of common metastatic sites sequenced within the cohort with corresponding alteration frequency of select genes. Alteration legend is the same as in 1c. The icon of the human figure is created in <https://BioRender.com>. Abbreviations: Cerebrospinal fluid (CSF); Gynecologic tract (GYN tract). **e** Bar chart demonstrating the percentage of *CD274* amplification in TMB-high and non-TMB-high BCs. **f** Circos plot summarizing the distribution of fusions in TMB-high BCs. Inter and intrachromosomal translocations are marked by the colored lines. The numeric digits signify the chromosome number of the corresponding event.

frequency of APOBEC mutagenesis in TMB-high BCs with resultant genomic alterations, in addition to the contribution of tumors with MSI signatures that may benefit from IC-blockade therapy. Moreover, our results corroborate previous findings with a dominance of APOBEC-mediated mutagenesis in tumors with increased TMB^{18,19}.

***ESR1* p.E380Q is the predominant *ESR1* hotspot mutation in TMB-high BCs**

Mutations afflicting the *ESR1* gene were identified in 13% of TMB-high BCs and, surprisingly, 35% of *ESR1*-mutated tumors harbored hotspot missense mutations involving codon 380 (i.e., *ESR1* p.E380Q/K) (Fig. 3a). Overall, the latter variants were seen in 5.8% of non-TMB-high *ESR1*-mutated BCs. Further examining the *ESR1* codon 380 mutations identified, we noted that 89.3% of these variants ($n = 25/28$) demonstrated an APOBEC signature in agreement with a previous report, in contrast to other *ESR1* hotspot mutations, which were primarily non-APOBEC associated (Fig. 3b)¹⁵. Interestingly, a subset of TMB-high BCs harboring *ESR1* p.E380Q mutations co-occurred with likely oncogenic *FOXA1* mutations, including hotspot missense mutations, which are typically reported to be mutually exclusive (Fig. 3c)²⁰. Co-occurring *FOXA1* mutations were subclonal events compared to *ESR1* mutations in all but one tumor. *FOXA1* upregulation has

been reported to activate HIF-2 α transcription in ER⁺/human epidermal growth factor receptor 2 (HER2)[−] metastatic BCs, conferring endocrine resistance²¹. Consistent with previous findings, no *MAP2K1* hotspot mutations were identified in this subset of tumors²⁰. Given that both *ESR1* and *FOXA1* hotspot mutations are well-established mediators of resistance to anti-endocrine therapy, alternate therapeutic avenues need to be explored in TMB-high BCs with concurrent *ESR1* and *FOXA1* hotspot mutations.

TMB-high BCs harbor an increased proportion of alterations in DDR genes

The efficacy of PARP inhibitors in the metastatic setting for germline *BRCA1/2* mutation carriers, based on results from the OlympiAD²² and EMBRACA²³ trials on improved progression-free survival (PFS), has drawn much intrigue in targeting *BRCA* genes. In addition, inferred mutational signature analyses revealed 30% of our dataset to exhibit dominant HRD and MSI signatures that instigated further study. On these grounds, we assessed the distribution of DDR gene alterations across the entire BC cohort and found a higher occurrence of somatic alterations in TMB-high BCs (Fig. 4a). TMB-high BCs were enriched in gene alterations implicated in pathways such as homologous recombination (i.e., *BRCA1*, *RAD50*, and *RAD51*), checkpoint blockade (i.e., *ATM*, *ATR*, and *CHEK1*), and mismatch

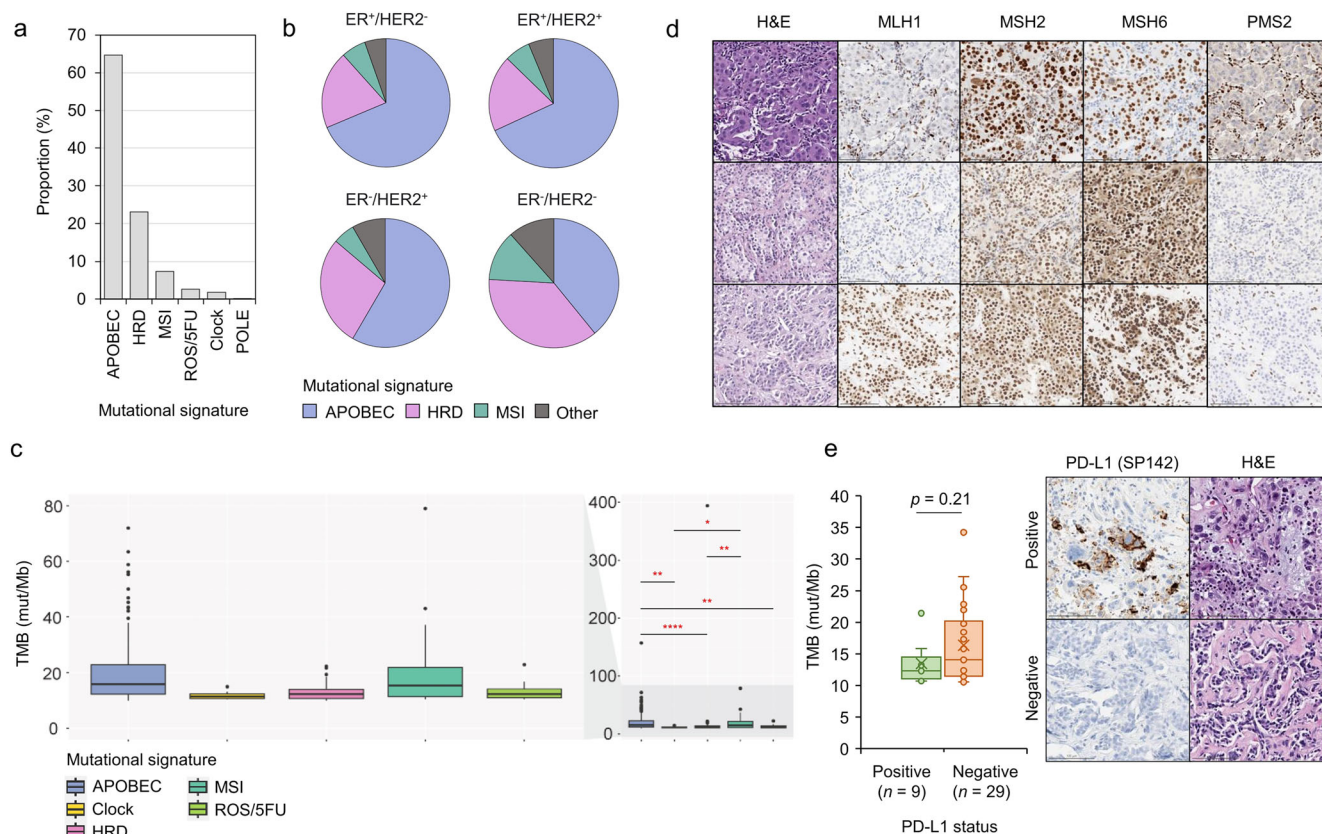


Fig. 2 | Inferred mutational signature analyses assessed by SigMA. a Bar chart demonstrating the distribution of inferred dominant signatures in TMB-high BCs ($n = 515$; signatures were not available for 12 tumors). **b** Pie charts summarizing mutational signatures according to receptor status. Unavailable signatures are incorporated in the other category. **c** Box plots depicting the range of TMB values among different signatures with ≥ 2 tumor samples. Asterisks denote differences of statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Horizontal line in box plots denotes median value. **d** Representative TMB-high BCs with a dominant MSI signature, showing loss of mismatch repair proteins at the protein

level by immunohistochemistry, alongside corresponding H&E-stained photomicrographs. Each row represents one tumor. Rows 1 and 2: Loss of MLH1 and PMS2. Row 3: Loss of MSH2 and MSH6. Scale bars, 100 μm . **e** Box plot (left panel) depicting the range of TMB values in tumors with positive (SP142: $\geq 1\%$; SP263: CPS ≥ 10) and negative PD-L1 expression by immunohistochemistry (unpaired t -test). Mean value is demonstrated, and the quartile calculation is exclusive of the median. Representative PD-L1 positive and negative cases with corresponding H&E-stained photomicrographs (right panel). Scale bars, 100 μm .

repair (i.e., *MLH1*, *MSH2*, *MSH6*, and *PMS2*). Of these genes, we observed likely oncogenic hotspot mutations in *RAD51C* ($n = 6$) (Fig. 4b) and *POLE* ($n = 6$) (Fig. 4c) in multiple tumors when compared to the non-TMB-high BCs at 1 and 3 tumors, respectively. Interestingly, all tumors with the *POLE* p.E18K hotspot mutation had a dominant APOBEC signature; admittedly, these mutations were not present in the exonuclease domain.

Metastatic TMB-high TNBCs are enriched in mutations related to PI3K/AKT signaling and potential immune cell exclusion

Given the modest treatment response in BCs subjected to IC-blockade, we surveyed primary and metastatic TMB-high TNBCs to assess for potential somatic mutations that may be associated with immune cell modulation. We ascertained an enrichment in truncating *ARID1A*, *RB1*, and *MAP3K1* mutations in metastatic TMB-high TNBCs (Fig. 5a). *AKT1* gene amplifications and *AKT1* p.E17K hotspot mutations were present in 13% of metastatic TMB-high TNBCs but were not seen in primary TMB-high TNBCs in our series. One *ESR1* p.G521V missense mutation of unknown significance was identified in a primary TMB-high TNBC in a patient previously treated with anti-endocrine therapy for contralateral breast cancer. Pathogenic *TP53* alterations were present in 77% of metastatic TNBCs, which have been reported to promote immune evasion and abrogation of immune signaling²⁴. Based on SigMA, dominant APOBEC and HRD mutational signatures were seen in 39.1% and 36.8% of TMB-high TNBCs, respectively. Eleven tumors (12.6%) had an MSI signature, of which

one patient had documented Lynch syndrome. These findings imply that the phosphoinositide 3-kinase (PI3K)/AKT pathway and additional intrinsic genomic events may play crucial roles pertaining to treatment response that warrant investigation with functional studies.

Next, primary TMB-high TNBCs were assessed for the percentage of TILs using recommendations by the International TILs Working Group (mean: 17.3%; median: 10%) (Fig. 5b)²⁵. Past work has suggested that early-stage TNBCs with at least 30% TILs have prognostic implications conferring excellent survival²⁶. Among tumors exhibiting $\geq 30\%$ TILs, 4 of 5 tumors lacked mutations in *ARID1A* or *PTEN*, whereas one tumor had a *PTEN* p.T319* nonsense mutation with a dominant MSI signature. Importantly, loss-of-function alterations in these genes have been reported to associate with immune cell exclusion. In addition, 2 TMB-high TNBCs with increased TILs (30% and 60%) had *RB1* splice site mutations. Co-occurring *RB1* and *TP53* alterations have been previously shown to display greater T-cell-inflamed signatures and CD8⁺ lymphocytes in bladder urothelial carcinoma²⁷. Moreover, immunohistochemistry for AR was previously performed in a subset of cases ($n = 45$) and showed positive expression in 53.3% of tumors.

Lastly, we filtered the Fraction and Allele-Specific Copy Number Estimates from Tumor Sequencing (FACETS) results for loss-of-function and splice site mutations to interrogate for copy number alterations and loss of heterozygosity (LOH) in tumors with recurrent (≥ 3) alterations in *ATM*, *BRCA1*, *NF1*, *PTEN*, and *RB1* genes. Most tumors with alterations in these

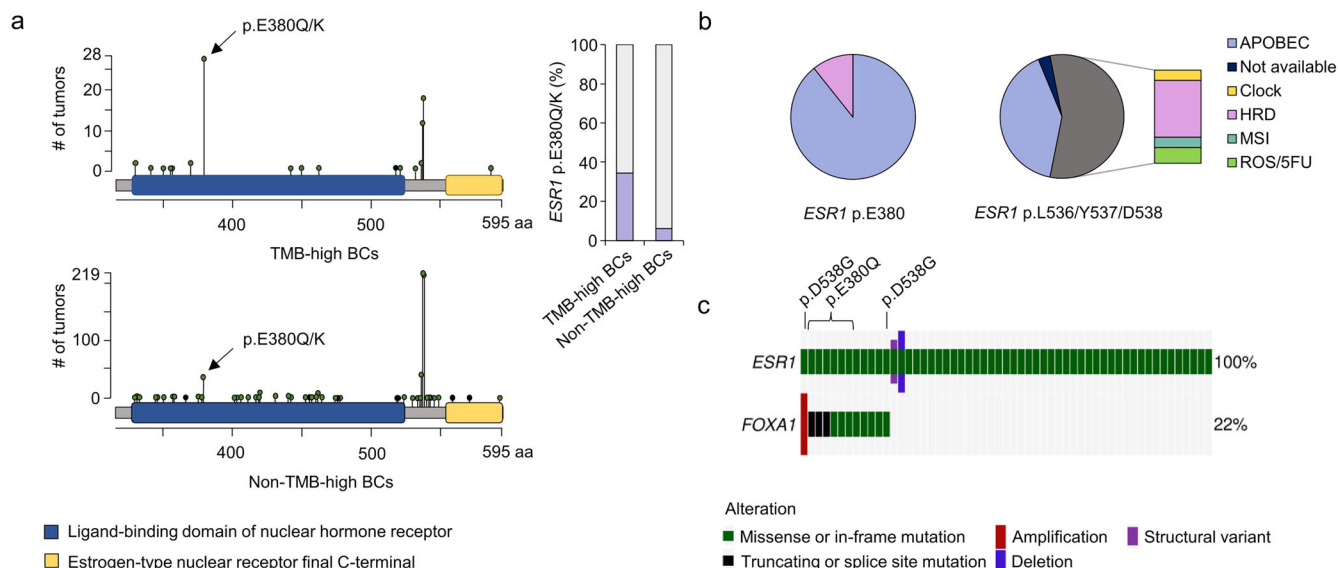


Fig. 3 | Distribution of *ESR1* mutations in TMB-high BCs. a Schematic truncated representation of the ligand-binding domain of ERα demonstrating the prevalence of *ESR1* p.E380Q/K hotspot mutations in TMB-high BCs (top panel) and non-TMB-high BCs (bottom panel). The mutation frequency is represented by the height of each lollipop (y-axis). Green circles: missense or in-frame mutations; Black circles: truncating or splice site mutations. Bar chart (right panel) showing the percentage of

ESR1 p.E380Q/K mutations among all *ESR1*-mutated BCs in each respective cohort. **b** Pie chart and a bar of pie chart summarizing the prevalence of mutational signatures according to the mutated *ESR1* hotspot codon. **c** Oncoprint depicting co-occurring *FOXA1* alterations with *ESR1* gain-of-function hotspot mutations. The annotated *ESR1* hotspot mutations show the corresponding likely oncogenic *FOXA1* mutations, including hotspot missense mutations ($n = 4$).

genes further exhibited either loss, LOH or copy-neutral LOH (CN-LOH) in the other allele (Fig. 5c). There was a significant difference in the overall LOH status of TMB-high TNBCs when compared to the remaining TMB-high BCs (Chi-squared test: $p = 0.0001$). These findings warrant further exploration to determine the prognostic implications and clinical importance of such events.

Discussion

Here, we provide a comprehensive assessment of a large cohort of TMB-high BCs to characterize the genomic alterations that underpin this subset of tumors. We found a preponderance of APOBEC-related gene mutations involving *PIK3CA*, *KMT2C*, *ARID1A*, *NF1*, and *CDH1* in our cohort that have been reportedly linked to endocrine resistance and metastases^{15,28}. Moreover, loss-of-function *ARID1A* and *PTEN* gene mutations reported to be involved in immune cell exclusion were identified at a high frequency in our series. Given the FDA approval of pembrolizumab in the unresectable/metastatic setting for TMB-high solid tumors and the modest treatment responses to IC-blockade in BC, our findings provide insight into treatment plans to better target this group of tumors.

Prior evidence has associated APOBEC mutagenesis with higher TMB and to harbor mutations associated with immune cell exclusion in BC^{18,29,30}. The APOBEC signature in conjunction with *PIK3CA* mutations is largely encountered in metastatic TNBCs, rendering these tumors amenable to direct agents, such as AKT inhibitors (e.g., ipatasertib, capivasertib), that target the PI3K/AKT pathway and may confer improved therapeutic response^{19,31}. *PTEN* is a tumor suppressor that downregulates the PI3K/AKT signaling axis and is implicated in cellular processes such as proliferation, invasion, and survival³². Thus, abrogation of *PTEN* activity is linked to aberrant activation of PI3K/AKT signaling and tumorigenesis. We observed a predilection for driver *PTEN* alterations, mainly loss-of-function truncating and missense mutations, in ER⁺/HER2⁻ TMB-high BCs (15%) and TMB-high TNBCs (28%) when compared to TMB-high HER2⁺ tumors (i.e., ER⁺/HER2⁺: 2%; ER⁻/HER2⁺: 3%). In addition, the predominance of *PIK3CA* p.E542K and p.E545K hotspot mutations, among others, further substantiates APOBEC-mediated effects and a predilection towards the PI3K/AKT pathway that may be amenable to pharmacologic abrogation. In keeping with this finding, recent evidence

showed that APOBEC activity is present in pre-treatment tumor samples and is a mediator of therapeutic resistance, potentially serving as a biomarker for alternative therapies³³.

Deregulation of various oncogenic signaling pathways has been identified to play key roles in immune cell exclusion in different tumor types. This in part, is also attributed to loss or gain-of-function mutations that are intricately linked to certain signaling modules. The archetype mechanism of immune exclusion was principally characterized in metastatic melanoma, whereby activation of the WNT/ β -catenin pathway resulted in a lack of tumor-infiltrating T-cells in mouse models^{34,35}. Multiple lines of evidence have shown that several fundamental BC genes are involved in tumor immune surveillance. The clinical importance of this is further compounded by the paucity of CD8⁺ T-cell infiltration in up to 57.6% of primary BCs^{36,37}. Moreover, higher rates of metastasis with diminished patient survival are witnessed in BCs with a scarcity of CD8⁺ T-cells^{38–40}.

Upregulation of genes related to T-cell activation, co-stimulation, and receptor signaling has been reported in *TP53*-wild type basal-like and ER⁻ tumors with histopathologic immune cell infiltration²⁹. In addition, other frequently altered genes encountered in BC have been linked with a lack of immunotherapeutic response. Diminished clinical response with reduced PFS and overall survival (OS) was observed in response to PD-1/PD-L1 treated metastatic TNBCs harboring loss-of-function mutations in *PTEN*³⁰. A similar phenomenon is seen in *PTEN*-depleted metastatic melanomas with abrogation of T-cell infiltration. Interestingly, the latter study showed increased efficacy of IC-blockade upon treatment with a PI3K β inhibitor in mouse models⁴¹. These findings suggest critical roles for *PTEN* and the interplay between PI3K/AKT signaling and immune evasion.

Studies have found the acquisition of *NF1* mutations in metastatic tumors with induction of resistance to anti-endocrine therapies in the setting of loss-of-function *NF1* mutations in ER⁺ BCs⁴². Accordingly, loss of *NF1* shows sequential activation of the mitogen-activated protein kinase (MAPK) signaling pathway. *ARID1A*, a constituent of the switch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complex, is the most abundantly mutated gene among this multiplex and has been revealed to express genes related to IFN- γ signaling that draw effector T-cell infiltration⁴³. More importantly, the latter study discerned that *ARID1A* mutations correlate with dismal outcomes among IC-blockade therapies

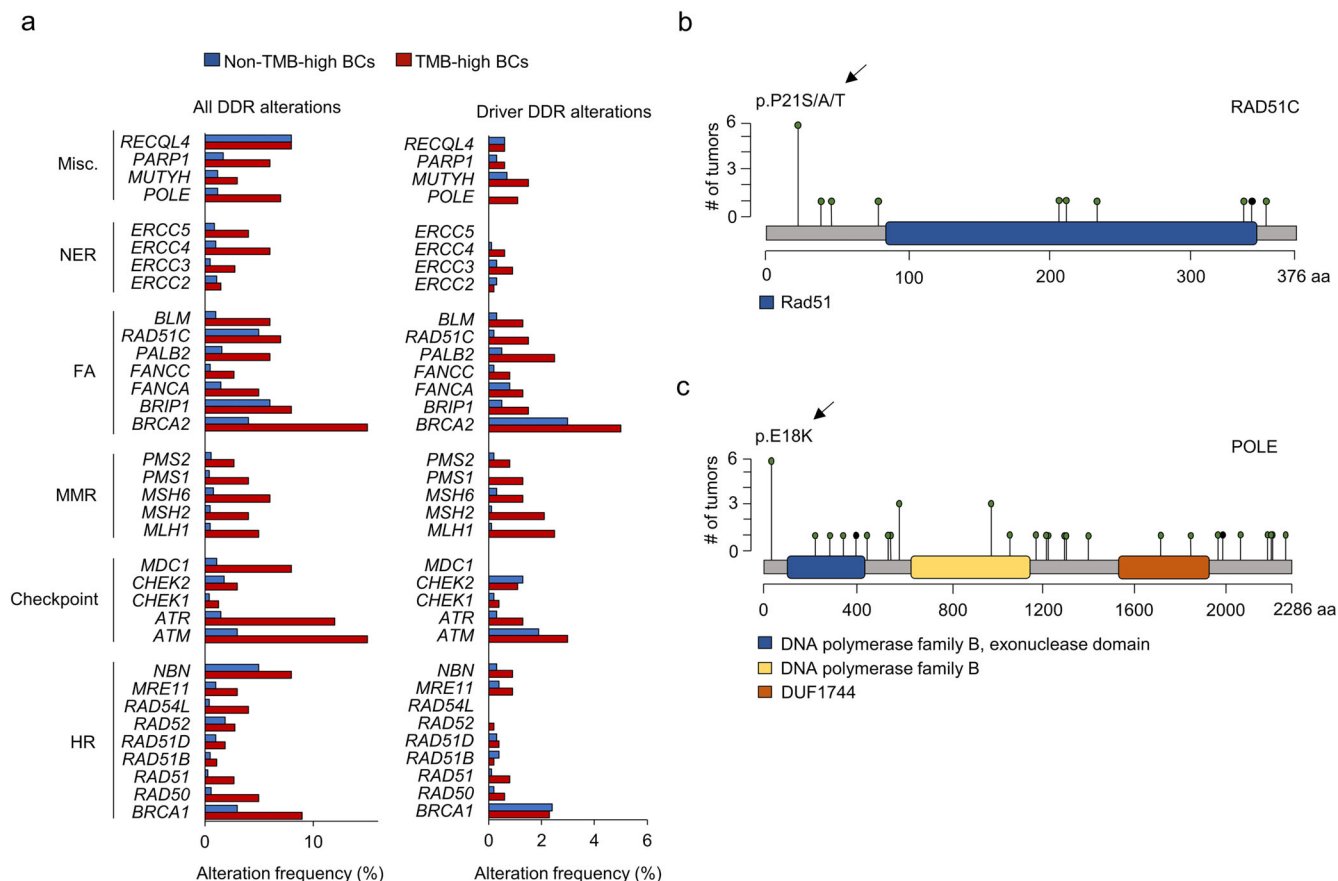


Fig. 4 | TMB-high BCs harbor an increased percentage of alterations pertaining to DDR. **a** Distribution of all (left panel) and driver (right panel) DDR gene alterations in TMB-high BCs (red) and non-TMB-high BCs (blue). Abbreviations: Mismatch repair (MMR); Nucleotide excision repair (NER); Homologous recombination (HR); Fanconi anemia (FA); Miscellaneous (Misc.). Schematic

representation depicting the protein domain(s) with likely oncogenic hotspot mutations in **b** RAD51C and **c** POLE. The mutation frequency is represented by the height of each lollipop (y-axis). Green circles: missense or in-frame mutations; Black circles: truncating or splice site mutations; Arrows: hotspot mutations.

irrespective of TMB. These findings not only shed light on the mechanisms of immune cell evasion in different tumor types but also support the notion of a more multifaceted tumor approach to combat cancer beyond a steadfast TMB value.

Querying for *ESR1* alterations yielded interesting findings. Specifically, we observed an increased proportion of *ESR1* p.E380Q mutations in TMB-high BCs, in contrast to other well-characterized resistance hotspot mutations. This observation has also been documented in a previous report with a TMB-high BC and APOBEC signature⁴⁴. Enrichment in this resistance mutation may potentially infer a high TMB in BCs. Further interrogation of primary TMB-high BCs stratified according to biomarker status revealed a dominant APOBEC signature in > 50% of tumors (i.e., ER⁺/HER2⁻, ER⁺/HER2⁺, and ER⁻/HER2⁺), apart from TMB-high TNBCs. The latter subgroup displayed a more diverse repertoire of mutational signatures, with a dominant APOBEC signature seen in 12% of tumors (Fig. 5a). In addition, mutations concentrated within the ligand-binding domain of ER α , predominantly affecting amino acid residues p.Y537 and p.D538, have been coupled with constitutive receptor activation and BC proliferation^{45,46}. Studies investigating APOBEC-induced mutagenesis resulting in the generation of amino acid changes (i.e., glutamate [E] > glutamine [Q]), have shown this event to occur in *ESR1* p.E380Q and *PIK3CA* p.E453Q hotspot mutations¹⁵. These single-base substitutions are recurrently enriched in metastatic BCs; however, the etiology is undetermined at present.

Our findings are in keeping with other investigators, who report TMB-high tumors in the range of 5% of BCs along with a dominant APOBEC mutational process¹⁹. Yet, the latter study didn't dissect the underlying

genomic alterations that drive these tumors beyond enrichment of *PIK3CA* alterations in tumors with dominant APOBEC activity. The predominance of APOBEC in our series is further substantiated by mutational events in *PIK3CA*, *KMT2C*, *ARID1A*, *NF1*, and *RB1*. We particularly observed a preponderance of recurrent *PIK3CA* hotspot mutations (i.e., p.E542K and p.E545K; oncogenic, gain-of-function) in BCs with APOBEC activity, concordant with previous findings⁴⁷. In a smaller percentage of tumors, we further detected *PIK3CA* p.E453K (oncogenic, gain-of-function) and p.E726K APOBEC-associated events²⁸. Moreover, deficiency of the methyltransferase *KMT2C*, which has been purported to promote APOBEC mutagenesis in different tumor types, was also frequent⁴⁸. Likely oncogenic truncating APOBEC-mediated *NF1* p.Q554* and p.Q1399* events were identified in 5 tumors and were mutually exclusive with *ESR1* hotspot mutations, further corroborating APOBEC dominance¹⁶. In addition, the increase in DDR gene mutations in our study is in keeping with findings from a previous study⁴⁹. Collectively, the contribution of HRD and MSI signatures in TMB-high BCs warrants further investigation in prospective studies.

Prior work has shown that metastatic TMB-high TNBCs treated with IC-blockade had a suboptimal OS when compared to other treatment strategies⁵. Primary phase III clinical trials investigating the efficacy of combined atezolizumab and nab-paclitaxel reported a prolongation in the median PFS (7.5 months) in patients with metastatic PD-L1⁺ TNBC when compared to placebo and nab-paclitaxel (5 months)⁵⁰. This study piloted the expedited FDA approval of combination atezolizumab and paclitaxel for PD-L1⁺ locally advanced/metastatic TNBC based on results from the

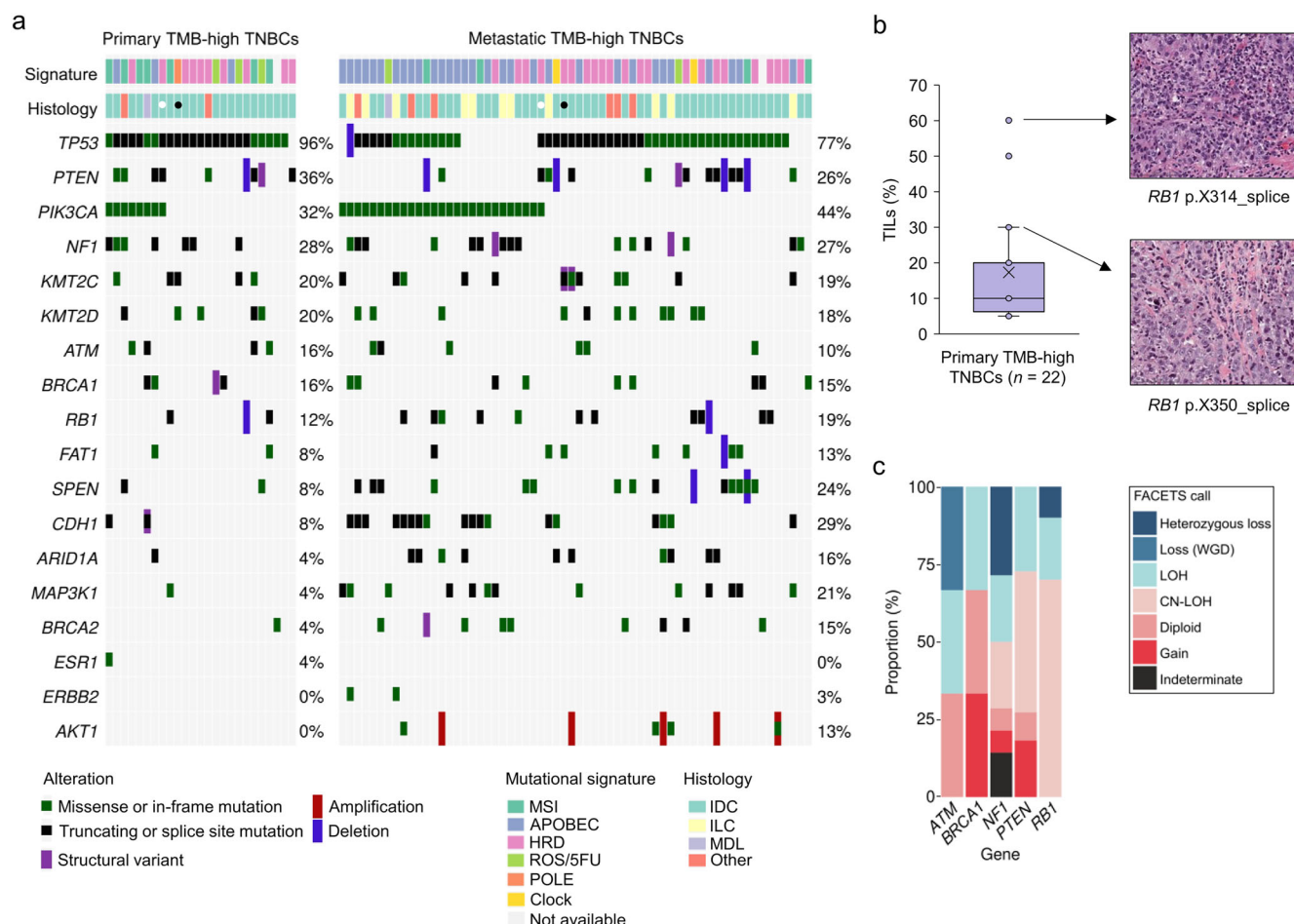


Fig. 5 | Genomic alterations identified in TMB-high TNBCs. **a** Oncoprints displaying select genetic alterations present in primary and metastatic TMB-high TNBCs. Primary and metastatic tumors from the same patient are annotated by the black and white circles. **b** Box plot (left panel) demonstrating the distribution of TILs in primary TMB-high TNBCs ($n = 22$) according to the International TILs Working Group recommendations. Mean value is demonstrated, and quartile calculation is

inclusive of the median. Representative H&E-stained photomicrographs of primary TMB-high TNBCs (right panel) with a prominent lymphocytic infiltrate are shown. Scale bars, 100 μ m. **c** Stacked box plots collectively showing copy number alterations assessed by FACETS in tumors with co-occurring loss-of-function (≥ 3) mutations in specific genes. Abbreviation: Whole genome duplication (WGD).

Impassion130 trial. The succeeding Impassion131 phase III trial⁵¹ failed to show a significant benefit in PFS and OS of statistical significance, ultimately resulting in the withdrawal of the agent. In the KEYNOTE-355 trial, the addition of pembrolizumab to chemotherapy in advanced metastatic TNBCs with a PD-L1 combined positive score (CPS) of ≥ 10 showed improved OS compared to chemotherapy⁵². In our study, retrospective review revealed that 16 and 38 patients received atezolizumab or pembrolizumab, respectively, at some timepoint during their treatment. Most were heavily pretreated patients who received adjuvant therapy and ultimately developed progression of disease with eventual demise (atezolizumab, $n = 14$; pembrolizumab, $n = 21$). While PD-L1⁺ and TMB-high tumors are used as predictive biomarkers for IC-blockade, only a subset of TMB-high BCs were PD-L1⁺ in our series. These findings suggest that alternate mechanisms may contribute to disease progression, given the lack of immunotherapeutic response despite harboring a high TMB, albeit in the metastatic setting.

Our study has some limitations. The retrospective nature of our study may be prone to selection bias, as many patients have been heavily pretreated prior to referral to our institution. In addition, a large proportion of tumors in our series were metastatic in nature, and limited cases had matched primary and metastatic tumors for clonal evolution analysis. Moreover, MSK-IMPACT is a targeted sequencing panel that may preclude the identification of genomic alterations not included in the panel. Yet, our study provides a comprehensive characterization of the largest TMB-high

BC cohort to our knowledge, with novel insights that may help predict response to IC-blockade and other therapies.

In conclusion, the present study comprehensively characterizes the genomic landscape of TMB-high BCs, accompanied by mutational signature analyses that offer deeper insights into this subclass of BCs. The distribution of genomic events in these patients provides further groundwork for better-tailored therapies to improve therapeutic outcomes.

Methods

Case selection

The study was approved by the Memorial Sloan Kettering Cancer Center (MSKCC) Institutional Review Board (IRB) (Protocol #17-287) and has been performed in accordance with the Declaration of Helsinki. The requirement for written informed consent was waived as the study was retrospective, all data were de-identified, and the research contained no more than minimal risk to the participants or their privacy. All BCs that underwent clinical FDA-authorized tumor-normal targeted massively parallel sequencing analysis of up to 505 cancer-related genes by the MSK-IMPACT platform, from September 2014 to August 2022 (data freeze), were evaluated ($n = 7894$). Cases were filtered to identify those with a TMB threshold of ≥ 10 mut/Mb. In total, 527 TMB-high BCs (462 patients: $n = 460$, female; $n = 2$, male) were identified and included in the current study. Clinicopathologic data were collected from the electronic medical records at MSKCC. Patient age at diagnosis ranged from 28 to 82 years.

Receptor information status (ER and HER2) was assessed on the BC samples that underwent genomic profiling.

MSK-IMPACT assay

Details of the MSK-IMPACT assay have been previously published⁵³. Briefly, MSK-IMPACT is a comprehensive molecular profiling assay that involves hybridization capture and deep sequencing of all exons and selected introns of up to 505 oncogenes and tumor-suppressor genes, allowing the detection of point mutations, small insertions or deletions, and rearrangements. In addition to capturing all coding regions of the genes, the assay also captures > 1000 intergenic and intronic single-nucleotide polymorphisms (tiling probes), interspersed homogeneously across the genome, aiding the accurate assessment of genome-wide copy number. In total, the probes target approximately 1.2 megabases of the human genome. The mean tumor sample coverage in the study was 623X (median: 617X). All alterations detected in select genes are shown.

Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue after manual macrodissection to ensure at least 10% tumor content. DNA input ranged from 100 to 200 ng, as measured by fluorometric methods. Matching peripheral blood (EDTA tube) from each patient was also extracted for use as a normal control. Sequence libraries were prepared (Kapa Biosystems, Wilmington, MA) through a series of enzymatic steps, including shearing of double-stranded DNA, end repair, A-base addition, ligation of bar-coded sequence adaptors, and low-cycle PCR amplification. Multiple bar-coded sequence libraries were pooled and captured using our custom-designed biotinylated probes (Roche NimbleGen, Madison, WI). Captured DNA fragments were sequenced on an Illumina HiSeq2500 (Illumina, San Diego, CA) as 100-bp paired-end reads that were then subjected to the bioinformatics analysis pipeline. Sequencing reads were aligned to the human genome (hg19) using Burrows-Wheeler Aligner software version 0.7.5a (<http://arxiv.org/abs/1303.3997>, last accessed October 1, 2013). ABRA⁵⁴ was used for read realignment around indels, and Genome Analysis Toolkit⁵⁵ was used for base quality score recalibration. Duplicate reads were marked and not used in the downstream analyses.

MSK-Fusion assay

The MSK-Fusion assay is a targeted RNA-based panel that utilizes the Archer Anchored Multiplex PCR (AMPTM) technology⁵⁶ and next-generation sequencing to detect gene fusions and oncogenic isoforms in solid tumors^{57–59}. The custom Archer-based MSK-Fusion panel includes up to 85 genes, including the kinase domains of the following genes: *ALK*, *BRAF*, *EGFR*, *ERBB2*, *ERBB4*, *FGFR1*, *FGFR2*, *FGFR3*, *KIT*, *MET*, *NTRK1*, *NTRK2*, *NTRK3*, *RET*, and *ROS1*. The MSK-Fusion panel included *ESR1* as of October 2018. After DNA sequencing, BCs were referred to RNA sequencing during the period studied, including TNBCs with no driver alteration, and ER⁺ metastatic BCs progressing on therapy.

SigMA pipeline

SigMA is a signature analysis tool optimized to detect mutational signatures utilizing information from exomes and whole-genome sequencing¹³. Briefly, SigMA employs a likelihood-based matching approach whereby it associates a tumor mutational spectrum with tumor subtypes according to their signature composition, which is determined by clustering and is used as a reference. The likelihood of the sample to belong to each tumor subtype is calculated, followed by a multivariate analysis, which allows us to obtain a final score for the presence of a signature by combining likelihood and cosine similarity with a non-negative least squares algorithm. Mutational signatures were generated by considering all nonsynonymous mutations in tumors with ≥ 8 mutations. The latter number was determined and adopted by our sensitivity and specificity analysis. A non-parametric Wilcoxon Rank-Sum test (Mann–Whitney *U* test) was used for statistical analysis between different mutational signatures and TMB difference.

FACETS

Allele-specific copy number analysis and assessment of LOH were performed by the FACETS algorithm (<https://github.com/mskcc/facets2n>). Cases were reviewed for proper fitment, and those in which a tumor diploid state was not attainable were excluded from further analysis. TMB-high TNBCs with recurrent loss-of-function gene events (≥ 3) in key genes were further analyzed by FACETS and subsequently compared to the remaining TMB-high BCs that exhibited similar loss-of-function events in the same genes.

Immunohistochemical analysis

Immunohistochemistry for mismatch repair proteins (MLH1: clone ES05 [Leica Biosystems], 1:500 dilution; PMS2: clone A16.4 [556415], 1:100 dilution; MSH2: clone G219-1129 [Cell Marque], 1:750 dilution; MSH6: clone EP49 [Agilent], 1:500 dilution) were performed on select cases. Tumors with positive nuclear expression were classified as normal/retained protein expression. Tumors with loss of nuclear expression for any of the four proteins were classified as abnormal/absent protein expression.

PD-L1 status was assessed by immunohistochemistry using two different assays during the study period on locally advanced or metastatic TNBCs. PD-L1 clone SP142 IHC (ready-to-use) was performed as the companion diagnostic assay for atezolizumab, using Ventana Benchmark Ultra System (Ventana Medical Systems) with antibody detection using the OptiView DAB IHC Detection Kit (Ventana Medical Systems), according to the manufacturer's manual⁶⁰. PD-L1 SP142 positivity is defined as expression on stromal tumor-infiltrating immune cells occupying $\geq 1\%$ of the tumor area as described previously^{61,62}. PD-L1 clone SP263 IHC (ready-to-use) was performed to select patients for pembrolizumab treatment, using Ventana Benchmark Ultra System (Ventana Medical Systems) with antibody detection using the OptiView DAB IHC Detection Kit (Ventana Medical Systems). PD-L1 SP263 has been validated against the companion diagnostic assay PD-L1 22C3 pharmDx and found to be comparable⁶³. PD-L1 SP263 is reported as a CPS, which is the number of PD-L1 staining cells (i.e., tumor cells, lymphocytes, and macrophages) divided by the total number of viable tumor cells, multiplied by 100. PD-L1 positivity is defined as a CPS ≥ 10 .

TMB calculation

TMB was calculated for each individual patient by normalizing the total number of mutations detected in the MSK-IMPACT panel by its total exonic size in megabases.

Assessment of TILs

Primary TMB-high TNBCs were morphologically evaluated for the presence of TILs following recommendations by the International TILs Working Group²⁵. Briefly, two pathologists (T.V. and D.S.R.) quantified the percentage of TILs that occupied the stromal tissue compartment within the borders of the invasive tumor ($n = 22$) in increments of 5%. TILs outside of the tumor borders and at the periphery of ductal carcinoma in situ (DCIS) were excluded. BCs with extensive fragmentation or within lymphoid tissue could not be evaluated and were excluded from assessment of TILs ($n = 3$).

Statistical analyses

Pearson's Chi-squared test with Yates' continuity correction was used for statistical comparisons of genomic alterations between TMB-high and non-TMB-high BCs. For comparing the difference in TMB across different mutational signatures, a non-parametric Wilcoxon Rank-Sum test (Mann–Whitney *U* test) was used. All tests of significance considered a *p*-value < 0.05 as statistically significant. Other analyses are specified within the manuscript.

Data availability

The MSK-IMPACT sequencing dataset is available through the cBioPortal for Cancer Genomics at www.cBioPortal.org.

Code availability

All software used in this manuscript is available online.

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Author contributions

T.V.: conceptualization, data curation, formal analysis, investigation, methodology, writing—original draft and editing; C.V., S.R., A.M.: data curation, formal analysis, investigation, methodology, writing—review and editing; F.P., E.B., C.S., M.E.A., M.L.: data curation, resources, writing—review and editing; H.Y.W.: conceptualization, data curation, investigation, writing—review and editing; D.S.R.: conceptualization, data curation, formal analysis, investigation, writing—review and editing. All authors read and approved the final version of the manuscript.

Competing interests

C.V.: reports equity, intellectual property rights, professional services, and activities (uncompensated) from Paige. AI, Inc. (outside of the submitted work). F.P.: reports membership of the scientific advisory board of MultiplexDx, reports consultancy fees for AstraZeneca, and is a member of their diagnostic advisory board (outside of the submitted work). M.E.A.: reports consulting for Janssen Global Services, Bristol-Myers Squibb, AstraZeneca, Roche, Merck, and Biocartis (outside of the submitted work). M.L.: reports having received honoraria for advisory board participation from Merck, AstraZeneca, Bayer, stock options for advisory board participation from Paige. AI, Inc., and research support from LOXO Oncology, Merus, Elevation Oncology, Helsinn Healthcare SA, ADC Therapeutics, and Rain Oncology (outside of the submitted work). In addition, M.L. is eligible for royalties from a license agreement between MSKCC and Sophia Genetics concerning MSK-IMPACT (outside of the submitted work). H.Y.W. reports personal fees from AstraZeneca and Daiichi Sankyo (outside of the submitted work). D.S.R. reports personal fees from AstraZeneca and Daiichi Sankyo (outside of the submitted work). The rest of the authors declare no potential conflicts of interest.

Additional information

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