Original Article

DNA damage response as a prognostic indicator in metastatic breast cancer via mutational analysis

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Background: High tumor heterogeneity contributes to breast cancer recurrence and metastasis. However, the lack of indicators to serve as precise and reliable means of predicting breast cancer prognosis has yet to be addressed. This study aims to reveal the prognostic relevance of mutations in metastatic breast cancer (MBC) by large-scale circulating tumor DNA (ctDNA) analysis in China.

Methods: We performed ctDNA panel-captured sequencing of 958 blood samples from MBC patients including 494 hormone receptor (HR)-positive cases, 130 human epidermal growth factor receptor 2-positive cases, and 177 triple-negative breast cancer (TNBC) cases. The somatic mutations and potential targets were assessed. Progression-free survival (PFS) was analyzed using the Kaplan-Meier method.

Results: In 801 of the 958 MBC blood samples, 663 mutated genes and 5,829 nonsynonymous alterations were identified. Mutated genes of the highest frequency were tumor protein p53 (*TP53*, 54%), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*, 41%), estrogen receptor 1 (*ESR1*, 12%), myeloid/lymphoid or mixed-lineage leukemia protein 3 (*MLL3*, 11%), DNA (cytosine-5)-methyltransferase 3A (*DNMT3A*, 10%), erb-b2 receptor tyrosine kinase 2 (*ERBB2*, 10%), GATA binding protein 3 (*GATA3*, 8%), FAT atypical cadherin 1 (*FAT1*, 7%), phosphatase and tensin homolog (*PTEN*, 6%), and mitogen-activated protein kinase kinase kinase 1 (*MAP3K1*, 6%). Enriched mutations and driver genes in MBC varied across stages and in multiple subtypes. Moreover, *TP53*, *ERBB2*, or coexisting *TP53*/*PIK3CA* mutations in MBC were remarkably related with shorter PFS. Mutated DNA damage response (DDR) genes were significantly associated with tumor mutation burden and mutant-allele tumor heterogeneity score, as well as with worse clinical outcome.

Conclusions: Our findings indicate that the mutations of *TP53*, *PIK3CA*, *ERBB2*, and in particular, DDR genes, in MBC might be relevant indicators of unfavorable prognosis in MBC.

Keywords: Metastatic breast cancer (MBC); genomic; circulating tumor DNA; mutation; prognosis

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Introduction

Breast cancer is recognized as the most common female malignant tumor, and is the leading cause of death from metastasis (1). A growing number of studies have reported mutational characteristics of metastatic breast cancer (MBC), indicating genomic evolution and high tumor heterogeneity in this disease (2-7). However, useful prognostic biomarkers that could reliably pinpoint the MBC

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patients most likely to benefit from specific and tailored treatment have yet to be identified. A circulating tumor DNA (ctDNA) analysis of MBC with largest scale in China was undertaken in the current study, to comprehensively interpret the genomic features and reveal the prognostic relevance of somatic mutations in MBC.

Recently ctDNA assays have been used in metastatic and recurrent cancers. Given the intra-tumor heterogeneity of breast cancer, the genomic map of tissue specimens may not represent the whole tumor (8). Additionally, it is difficult to obtain tissue samples of metastatic foci in clinical practice, as MBC commonly involves other metastatic organs such as the liver, lung, bone, or brain. Recently, ctDNA has been proposed as an accurate means to identify mutations, assess tumor burden, and dynamically monitor treatment response with minimally invasive procedures (9-12). In this study, 958 blood samples of MBC were collected, ctDNA was extracted, and target capture sequencing was performed. Survival analysis was subsequently carried out to explore the prognostic relevance of somatic alterations. We present the following article in accordance with the STROBE reporting checklist (available at http://dx.doi.org/10.21037/atm-20-2137).

Methods

Patient selection

The study was approved by the Institutional Review Board and Human Ethics Committee of at National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Science and Peking Union Medical College (No. CH-BC-052). All patients provided written informed consent prior to study enrollment. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

A total of 958 female MBC patients were enrolled at the time of diagnosis (TNM stage: M1) between June, 2015, and August, 2019. Hormone receptor (HR), including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expressions were assessed by two independent pathologists in accordance with standard clinical practice.

(I) DNA Extraction and target capture sequencing:

Blood samples were obtained and the plasma was separated from peripheral blood cells via centrifugation. Cell-free DNA was extracted from the plasma with QIAamp Circulating Nucleic Acid Kits (Qiagen, Hilden, Germany). Genomic DNA was isolated from peripheral blood cells with QIAamp DNA Blood Mini Kits (Qiagen, Hilden, Germany. Capture probes were designed, covering coding sequences and hot exons of 1021 genes. DNA sequencing was performed with the HiSeq 3000 Sequencing System (Illumina, San Diego, CA, USA). All procedures were conducted according to the manufacturers' instructions (13).

(II) Sequencing data analysis:

After removing the terminal adaptor sequences and low-quality data, the reads were mapped to the human reference genome. Variant calling was performed using Genome Analysis ToolKit (https://www.broadinstitute.org/gatk/, GATK) for single nucleotide variants (SNVs), small insertions and deletions (indels) in somatic DNA by filtering peripheral blood sequencing data. All final candidate variants were manually verified using the Integrative Genomics Viewer (IGV) Browser. The method of sequencing has been previously described by Hu *et al*. (14).

(III) Next generation sequencing (NGS) datasets and enrichment analysis:

The NGS dataset comprised 128 non-metastatic breast cancer cases (NMBC, M0 stage), processed with the same panel as the MBC cohort. The public dataset which included MBC information from Memorial Sloan Kettering Cancer Center (MSK) was selected for comparison. These datasets were age-matched to the study group and of a similar HR expression profile. Enrichment analysis was performed via Metascape (15). This web-based tool was used to integrate functional enrichment, interactome analysis, gene annotation, and protein-protein networks (http://metascape.org/gp/index.html#/main/step1).

(IV) Driver mutations analysis:

OncodriveFML (http://www.intogen.org/oncodrivefml) was used to identify driver mutations for pattern analysis of somatic mutations across tumors and elucidate their involvement in carcinogenesis. OncodriveFML integrated different scoring frameworks and predicted the impact of mutations on gene function (16).

(V) Tumor mutation burden (TMB) and mutant-allele tumor heterogeneity (MATH) calculation:

TMB and MATH have been frequently used to assess somatic mutations due to their established association with tumor heterogeneity. TMB was

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Table 1 MBC Patients characteristics

Parameters	Overall (n=801)	HR+ (n=494)	HER2+ (n=130)	TNBC (n=177)	P value
Age (range)	46 [22–76]	45 [24–75]	48 [22–72]	46 [23–76]	0.17
Lung metastasis					
Yes	364 (45%)	216 (44%)	53 (41%)	95 (54%)	0.09
No	437 (55%)	278 (56%)	77 (59%)	82 (46%)	
Liver metastasis					
Yes	310 (39%)	169 (41%)	52 (40%)	51 (29%)	0.03
No	491 (61%)	239 (59%)	78 (60%)	126 (71%)	
Bone metastasis					
Yes	455 (57%)	303 (61%)	68 (52%)	84 (47%)	0.009
No	346 (43%)	191 (39%)	62 (48%)	93 (53%)	
Brain metastasis					
Yes	92 (11%)	40 (8%)	24 (18%)	28 (16%)	0.002
No	709 (89%)	454 (92%)	106 (82%)	149 (84%)	
Number of metastatic organs					
≤2	536 (67%)	327 (66%)	84 (65%)	125 (71%)	0.68
>2	265 (33%)	167 (34%)	46 (35%)	52 (29%)	

MBC: metastatic breast cancer. HR: Hormone receptor. HER2: human epidermal growth factor receptor 2. TNBC: triple negative breast cancer.

calculated using the number of non-synonymous somatic mutations per mega-base in coding regions (14). To analyze the effect of intra-tumor heterogeneity, MATH was the normalized width distribution of mutant-allele fractions among tumor-specific mutated loci (17).

(VI) Statistical analysis:

Differences of categorical and numerical variables were performed using Fisher's and Mann–Whitney U tests, respectively. The relationship between two variables was assessed using Pearson's or Spearman's correlation test. Statistical significance was considered when $P<0.05$. PFS was assessed using a Kaplan-Meier survival plot. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA) or R (18).

Results

Mutational landscape of MBC

Mutations were identified in 801 of 958 blood samples from female MBC patients, comprising 494 HR+ cases (luminal A and B), 130 HER2+ cases, and 177 triple-negative breast cancer (TNBC) cases. All patients were diagnosed at the M1 stage, and the cohort ranged in age from 22–76 years old, with a median age of 46. The involved distant organs included the lung, liver, bone, and brain. Extracted ctDNA was assayed for somatic mutations by target capture NGS with a panel of 1,021 genes. The patient's characteristics of the identified MBC cases are summarized in *Table 1*.

There were 5,829 nonsynonymous mutations identified in 663 genes, including 4,590 missense mutations, 474 nonsense mutations, 370 deletions of small fragment, and 182 insertions of small fragment in coding sequence. The most frequently altered genes were tumor protein p53 (*TP53*, 54%), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*, 41%), estrogen receptor 1 (*ESR1*, 12%), myeloid/lymphoid or mixed-lineage leukemia protein 3 (*MLL3*, 11%), DNA (cytosine-5)-methyltransferase 3A (*DNMT3A*, 10%), erb-b2 receptor tyrosine kinase 2 (*ERBB2*, 10%), GATA binding protein 3 (*GATA3*, 8%), FAT atypical cadherin 1 (*FAT1*, 7%), phosphatase and

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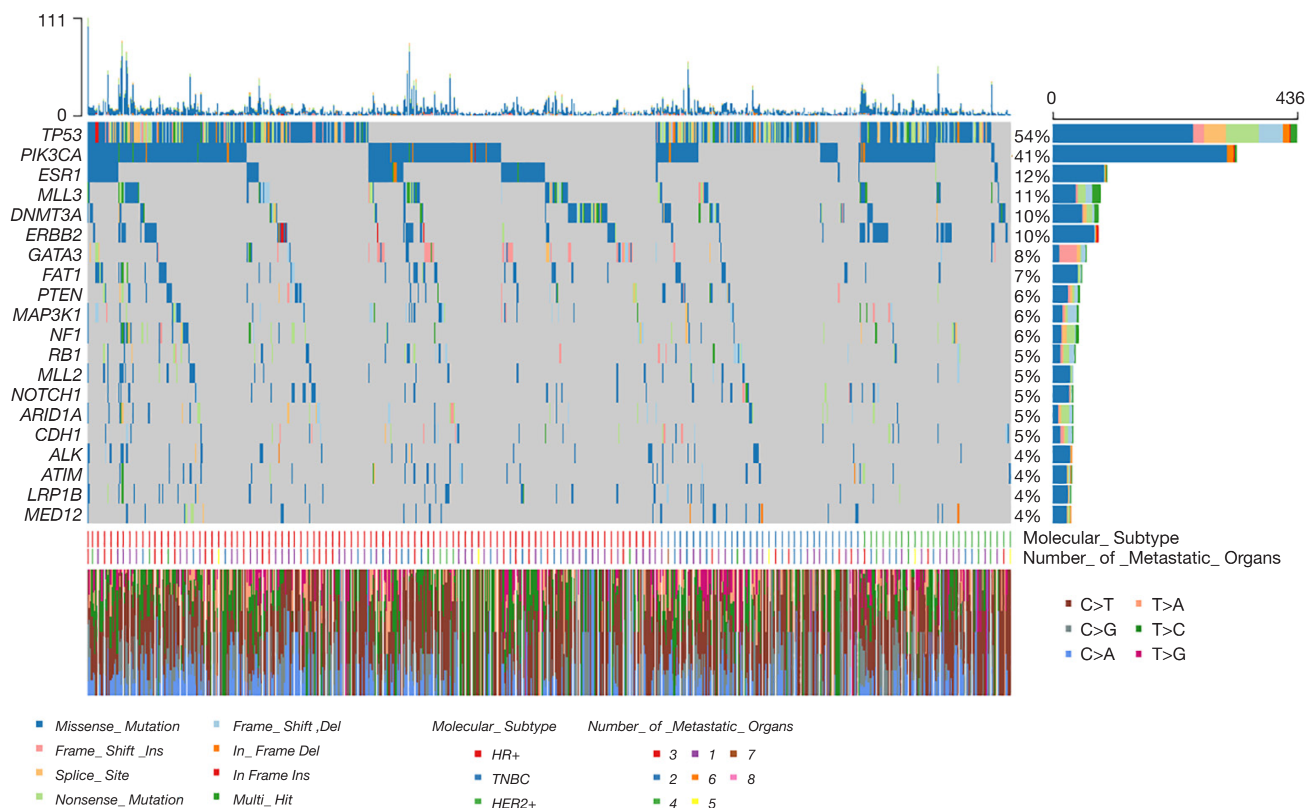

Figure 1 The clinical features and the mutational landscape of MBC samples from ctDNA sequencing. The upper bars indicate the number of mutations in each patient, and the right bars represent the number of mutations in each gene. Summarized data are presented as a stacked bar plot showing fraction of conversions in each sample. MBC, metastatic breast cancer; ctDNA, circulating tumor DNA.

tensin homolog (*PTEN*, 6%), and mitogen-activated protein kinase kinase kinase 1 (*MAP3K1*, 6%) (*Figure 1*). The frequency of mutations observed in this current study was concordant with tissue-based results from the matched MSK dataset (R^2=0.8395) (Figure S1, Table S1). Despite the lower mutation frequency, the variant allele frequency (VAF) of *PTEN* was higher than that of *PIK3CA* (*Figure 2A*). Moreover, the TMB per sample ranged from 1-111, with a median of 5. Mutated *TP53* significantly co-existed with *PIK3CA*, neurofibromatosis type 1 (*NF1*), myeloid/lymphoid or mixed-lineage leukemia protein 2 (*MLL2*), *ERBB2*, and retinoblastoma protein (*RB1*). Meanwhile, *ESR1*, *GATA3*, and *DNMT3A* were revealed to be mutually exclusive genes (*Figure 2B*).

Analysis of our data in the Oncogenic Signaling Pathways in The Cancer Genome Atlas (19) revealed that the affected genes were mostly involved in the RTK-RAS, PI3K/Akt and Notch pathways. It was also noted that the p53 pathway was associated with the highest frequency of affected genes (*Figure 2C*). A total of 16 druggable genes including *PTEN*, *TP53*, *ESR1*, *RB1*, *notch receptor 1* (*NOTCH1*), *ALK receptor tyrosine kinase* (*ALK*), *ERBB2*, *ATM serine/threonine kinase* (*ATM*), *PIK3CA*, *GATA3*, *mediator complex subunit 12* (*MED12*), *cadherin 1* (*CDH1*), *DNMT3A*, *MAP3K1*, *NF1*, and *AT-rich interaction domain 1A* (*ARID1A*) were identified and classified into 20 target categories, based on Drug Gene Interaction database (*Figure 2D*). The mutated genes were associated with a pathway in cancer (hsa05200), disease of signal transduction (R-HSA-5663202), and EGFR tyrosine kinase inhibitor resistance (hsa0521) (Figure S2).

Enriched mutations in MBC

In the current study (n=801), 10 differentially mutated genes of MBC (Table S1) were recognized when compared to matched NMBCs (Table S2) derived from our ctDNA dataset (n=128) (*Figure 3A*). Considering the important

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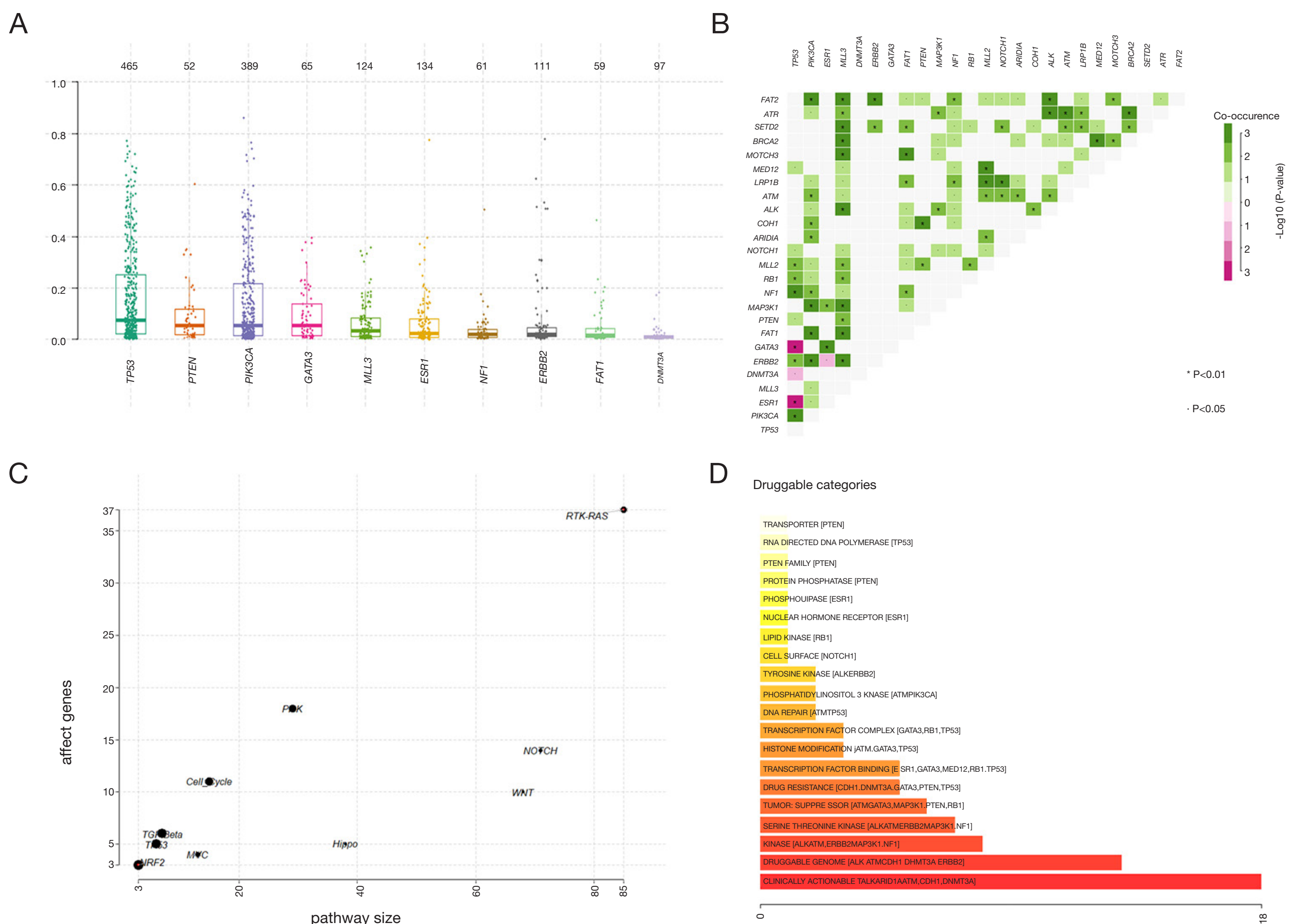

Figure 2 Mutational characteristics of MBC. (A) Top 10 mutated genes according to VAF. (B) Mutual exclusivity and co-occurrence among multiple mutations. Green and magenta dots represented co-occurrence and mutual exclusivity, respectively. The degree of significance is represented by color intensity. (C) Enrichment of known oncogenic signaling pathways. The X-axis represents the total number of genes in the pathway, and the Y-axis represents the number of affected genes in the pathway. (D) A total of 16 druggable genes were identified and classified into 20 targetable categories based on Drug Gene Interaction database. The X-axis represents the number of genes. MBC, metastatic breast cancer; VAF, Variant Allele Frequency.

role of molecular subtypes in the diagnosis and treatment of breast cancer, we compared mutational frequencies of metastatic HR+ (n=494), HER2+ (n=130), and TNBC (n=177) subtypes. *TP53*, *ERBB2*, and *MSH2* were more prevalently altered in metastatic HER2+ cases; *TP53* and *PIK3CA* were differentially mutated in metastatic TNBCs; and *ESR1* and *GATA3* occurred more frequently in HR+ subtype (*Figure 3B*,*C*,*D*).

Through OncodriveFML (q<0.1), 56 driver genes were identified in MBC, with only 3 found in NMBC. Of the top 20 frequently mutated genes in MBC, 14 were interpreted as driver genes. It was found that the driving effect of *PIK3CA* in NMBC was not shown in MBC. Within the molecular subtypes of MBC, 52 driver genes were observed in the HR+ group, 14 in the HER2+ group, and 9 in the TNBC group. *TP53*, *RB1*, and *ARID1A* were shared driver genes, regardless of subtypes. *ESR1*, *GATA3*, *FAT1*, *NF1*, *PTEN*, and *CDH1* were specific driver genes in the HR+ subtype, while *NOTCH1* showed a strong correlation with TNBC cases (*Figure 3E*). Additionally, TMB was found to be significantly higher in MBC than NMBC (*Figure 3F*). An association was observed between the number of metastasis and gene alterations (P=0.0003). Furthermore, a significant difference between mutational burden in lung metastasis

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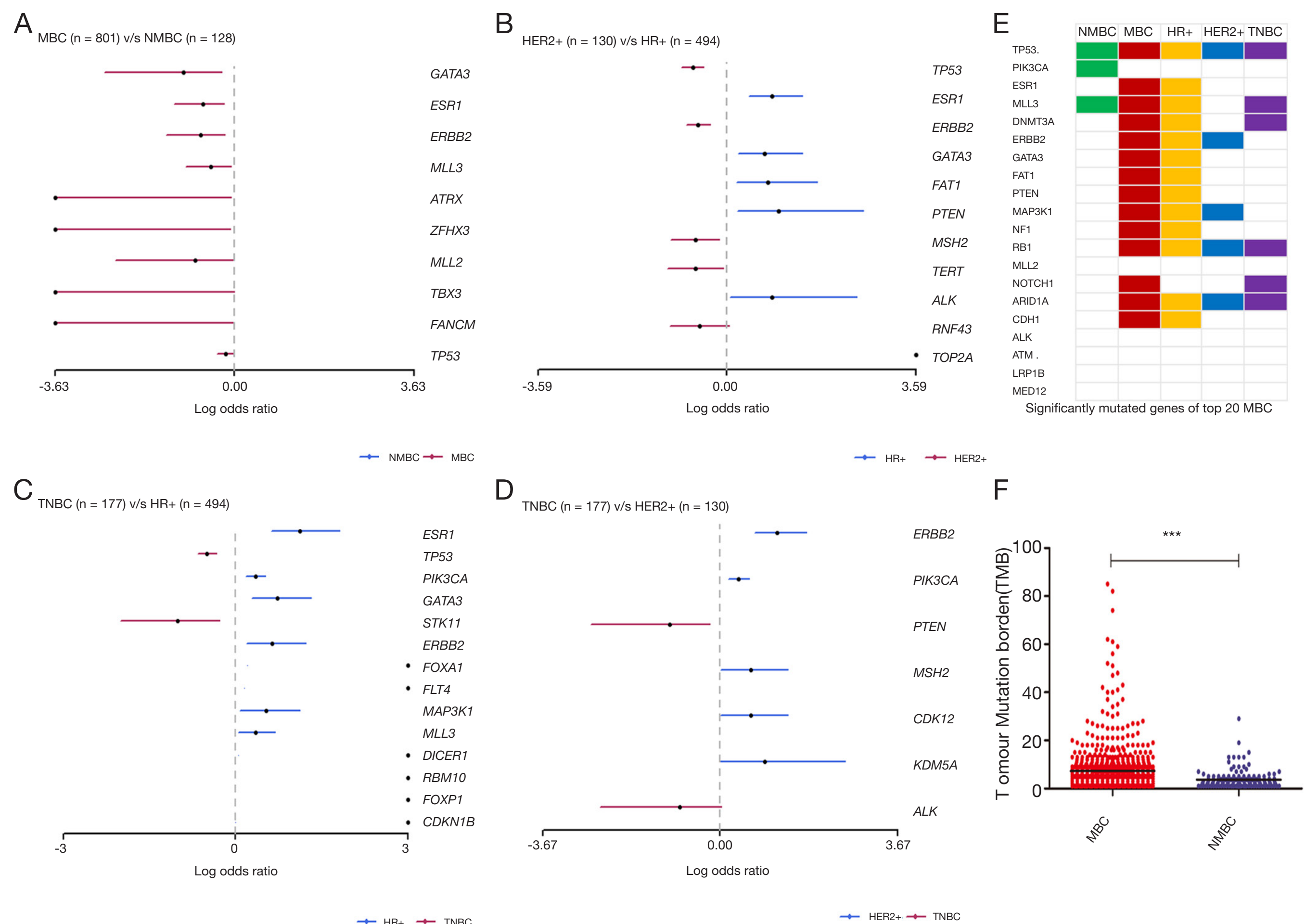

Figure 3 Mutations enriched in MBC. (A) Ten genes were more frequently altered in MBC than NMBC. (B-D) Different mutations observed among the metastatic HR+, HER2+, and TNBC cases. (E) Driver genes varied in MBC and NMBC. (F) The TMB of MBC was significantly higher than that of NMBC. MBC, metastatic breast cancer; NMBC, non-metastatic breast cancer; HR, Hormone receptor. HER2, human epidermal growth factor receptor 2; TNBC, triple negative breast cancer; TMB, tumor mutation burden.

and liver metastasis (P=0.0467) (Figure S3) was noted. Through Metascape, it was shown that different pathways were involved with different metastatic sites in MBC (Table S3).

Prognostic relevance of somatic mutations in MBC

The relationship between highly mutated genes and cancer prognosis was investigated. Follow-up data from 126 MBC cases in this study revealed that *TP53* or *ERBB2* mutations led to shorter PFS (*Figure 4A,B*). Co-existence of *TP53* and *PIK3CA* mutations was significantly associated with worse prognosis (*Figure 4C*). Mutations of DNA damage response (DDR) genes have been considered to play a critical role in maintaining genomic stability (19). Our findings identified a total of 85 mutated DDR genes from 570 MBC cases, while 35 altered DDR genes were found in 71 NMBC cases, highlighting a significant difference between these two cohorts (*Figure 4D*). The distribution of DDR gene mutations varied among different molecular subtypes (*Figure 4E*). MBC cases with DDR mutations showed higher TMB and MATH scores compared to those without DDR mutations (*Figure 4F,G*). More importantly, MBC patients with DDR mutations displayed shorter PFS than those without DDR mutations (*Figure 4H*).

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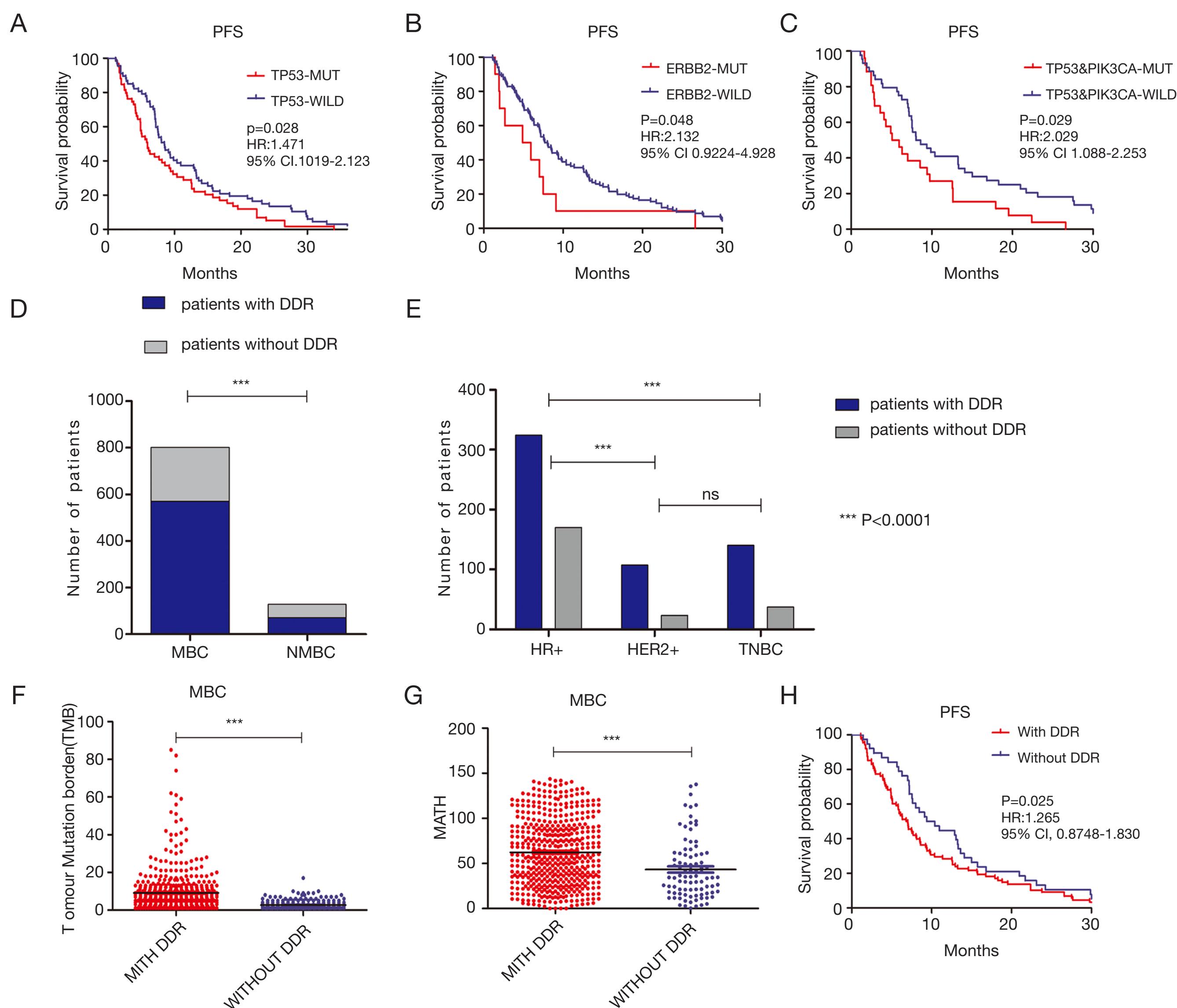

Figure 4 Prognostic relevance of somatic alterations in MBC. (A-C) Mutated genes associated with PFS. (D) The proportion of patients with DDR gene mutations in MBC was higher than that in NMBC. (E) The proportion of patients with DDR gene mutations varied among the different subtypes of MBC. (F) DDR gene mutations were related with higher TMB in MBC. (G) DDR gene mutations were associated with higher MATH score in MBC. (H) DDR gene mutations contributed to significantly shortened PFS in MBC patients. MBC, metastatic breast cancer; PFS, progression-free survival; NMBC, non-metastatic breast cancer; DDR, DNA damage response; TMB, tumor mutation burden; MATH, mutant-allele tumor heterogeneity.

Characteristics of TMB or MATH in MBC

TMB was higher in MBC cases with two or more metastatic organs than those with single distant organ involvement (*Figure 5A*). Mutations in *TP53*, *PIK3CA*, *ESR1*, *MLL3*, *ERBB2*, *GATA3*, *FAT1*, *PTEN*, *MAP3K1*, and *NF1* were closely related with higher TMB (*Figure 5B,C,D,E,F,G,H,I,J,K*). However, no direct association was found between TMB and PFS (*Figure 5L*). MATH score was higher in MBC cases with multiple metastatic organs (*Figure 5M*). *TP53*, *MLL3*, *ERBB2*, and *FAT1* mutations were significantly associated with MATH scores (*Figure 5N,O,P,Q*). No

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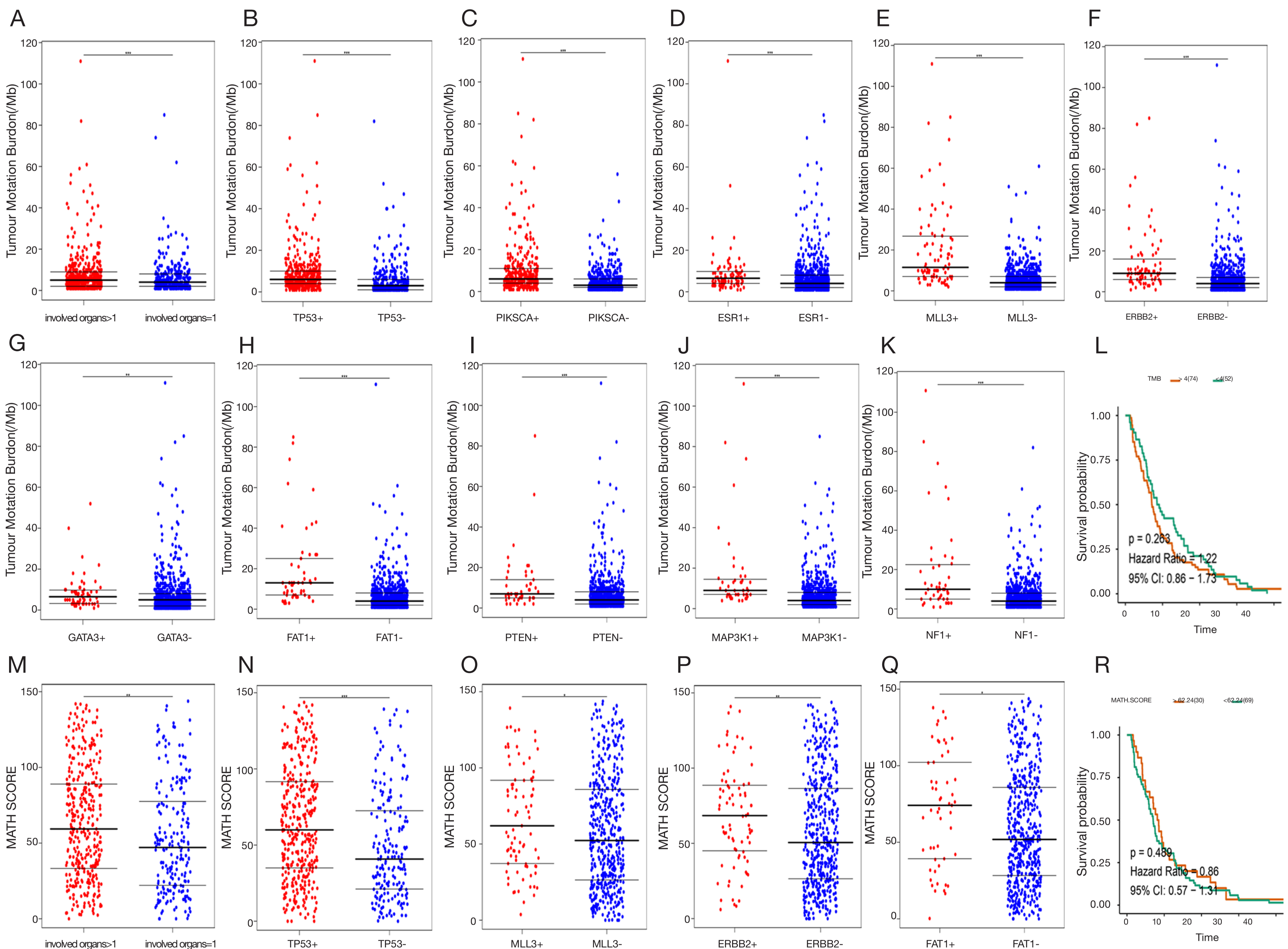

Figure 5 TMB and MATH in MBC. (A-K) TMB characteristics in MBC. (L) TMB was not associated with PFS. (M-Q) The characteristics of MATH score in MBC. (R) MATH score was not associated with PFS. *, P<0.05, **, P<0.01, ***, P<0.0001. TMB, tumor mutation burden; MATH, mutant-allele tumor heterogeneity; MBC, metastatic breast cancer; PFS, progression-free survival.

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relationship was observed between MATH score and PFS in MBC (*Figure 5R*).

Discussion

This study exploited the multiple advantages of using liquid biopsies as a means of conducting large-scale genomic profiling of MBC via ctDNA analysis. *TP53* and *PIK3CA* alterations appeared most prevalent, followed by *PTEN*, which warrants attention. It has been reported that *PTEN* mutations frequently occur in various cancers, particularly in the breast (20) and participate in the PI3K/AKT/mTOR and P53 pathways (21). In the current study, *PTEN* showed significant correlation with *TP53*, displaying a co-existing pattern.

Based on our data, the spectrum of genes identified in MBC during the analysis was differed from NMBC, indicating an evolutionary process and tumor heterogeneity. The differentially mutated genes were reported to be strongly associated with tumor progression and poor prognosis. *RB1* mutation was associated with poor outcomes in the HR+ subtype, resulting in a low response to cyclin-dependent kinase 4 (CDK4) inhibitors (6,22), thereby warranting new target therapies (23). Interestingly, this study showed *RB1* to co-occur with set domain containing 2, histone lysine methyltransferase (*SETD2*) and *MLL2*. *NF1*, which displayed a relationship with low density lipoprotein receptor-related protein 1B (*LPR1B*) and FAT atypical cadherin 2 (*FAT2*), has also been involved in resistance to endocrine therapy (24). *MAP3K1* was reported to be responsible for regulating the transcription of important cancer genes including c-Myc, c-Elk1, c-Jun, and c-Fos (25). Based on our data, *MAP3K1* was strongly associated with the HR+ subtype. *NOTCH1*, one of the key receptors in the Notch signaling pathway, could promote proliferation, invasion and metastasis of cancer cells (26). It was reported that the inhibition of *NOTCH1* may prevent the pathogenesis and metastasis of breast cancer (27). *NOTCH1* mutations were mainly found in the TNBC subtype in this study. Unlike *TP53*, *ERBB2*, or DDR alterations, no prognostic relevance was shown between *RB1*, *NF1*, *MAP3K1*, and *NOTCH1* and MBC according to our data; further research with larger cohorts might be needed.

Compared to normal cells, cancer cells have attenuated DNA repair capacity, driving tumor formation (28). Recent studies have found that DDR alterations are independently associated with response to PD-1/PD-L1 blockade (29). In this study, the proportion of mutated DDR genes in MBC was higher than that in NMBC. The distribution of DDR mutations varied with different subtypes of MBC. DDR mutations were significantly associated with TMB, MATH score, and PFS, suggesting a relationship with heterogeneity and prognosis (30).

It is much argued that ctDNA has a similar mutational profile to gDNA. Given the occurrence of tumor heterogeneity and difficulties in obtaining samples of distant metastasis in clinical practice, ctDNA analysis may therefore provide a convenient way to identify mutations and assess tumor burden in MBC. The frequency of mutations observed in this study was concordant with tissue-based results from the matched MSK dataset (R^2=0.8395). However, the validation of ctDNA application needs further and more comprehensive investigation. The potential target genes identified in the current study were evaluated in relation to the existing Genome-Wide Association Studies (GWAS). Most risk variants detected through GWAS were found in non-coding regions (31,32), highlighting the importance of using large-scale genomic data.

We investigated the genomic characteristics and prognostic indicators, based on a large MBC cohort with ctDNA analysis. The mutational profile of MBC was shown to be different from early stage of breast cancer and varied across multiple subtypes, including driver genes. *TP53*, *ERBB2*, or coexisting *TP53*/*PIK3CA* mutations in MBC contributed to shorter PFS. Furthermore, DDR mutations in MBC were significantly associated with TMB and MATH score, as well as unfavorable prognosis.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-2137). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), approved by the Institutional Review Board and Human Ethics Committee of at National Cancer Center/ National Clinical Research Center for Cancer/ Cancer Hospital, Chinese Academy of Medical Science and Peking Union Medical College (No. CH-BC-052) and informed consent was obtained from all the patients.

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Supplementary

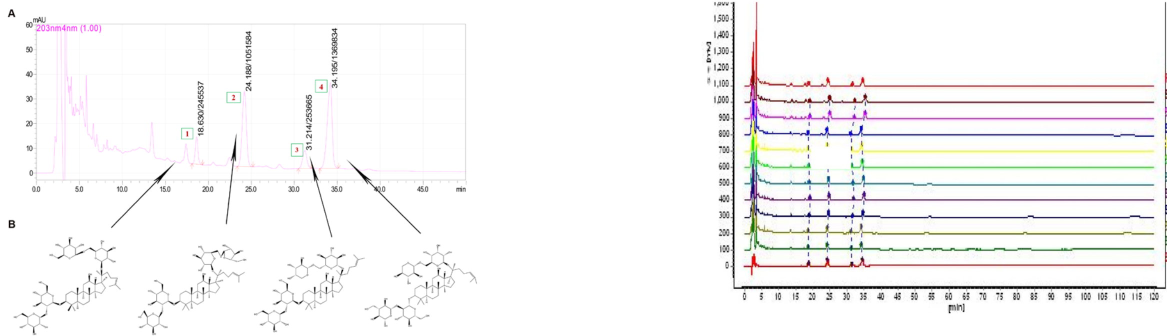

Figure S1 Consistency of the mutational spectrum between this study and MSK tissue-based data. MSK, Memorial Sloan Kettering Cancer Center.

Figure S2 Functional enrichment analysis of mutated genes in MBC. MBC, metastatic breast cancer.

Table S1 Information of MSK group

	Overall(n=374)	HR+(n=317)	HER2+(n=26)	TNBC(n=31)	P-Value*
Age (range)	51(25-82)	51(25-82)	50(30-69)	51(27-74)	0.6915

*Differences between groups were assessed using Kruskal–Wallis test for continuous variables.
MSK: Memorial Sloan Kettering Cancer Center. HR: Hormone receptor. HER2: human epidermal growth factor receptor 2. TNBC: triple negative breast cancer.

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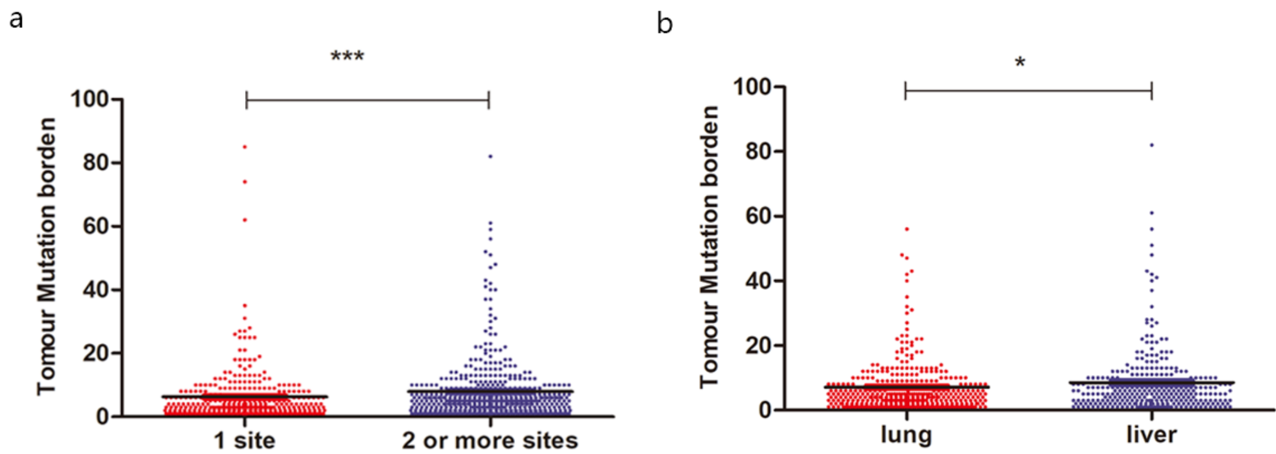

Figure S3 The number and site of metastasis in MBC associated with tumor mutations. (A) The number of metastatic sites was associated with gene alterations (P=0.0003). (B) There was also significant difference between mutational burden in lung metastasis and liver metastasis (P=0.0467). MBC, metastatic breast cancer.

Table S2 Information of NMBC group

	Overall(n=128)	HR+(n=84)	HER2+(n=22)	TNBC(n=22)	P-Value*
Age (range)	45(23-78)	43(23-78)	46(24-72)	44(26-75)	0.7847

*Differences between groups were assessed using Kruskal–Wallis test for continuous variables.
NMBC: non-metastatic breast cancer. HR: Hormone receptor. HER2: human epidermal growth factor receptor 2. TNBC: triple negative breast cancer.

Table S3 Mainly involved pathways according to metastatic sites in MBC

Sites	Mainly involved pathways	Log10(P)
	Pathways in cancer	-37.7
Bone	HDR through Homologous Recombination (HRR)	-36.2
	Homologous DNA Pairing and Strand Exchange	-36.2
	Pathways in cancer	-34.5
Brain	Prostate cancer	-31.3
	Diseases of signal transduction by growth factor receptors and second messengers	-26.8
	Pathways in cancer	-34.5
Liver	HDR through Homologous Recombination (HRR)	-33.3
	Resolution of D-loop Structures through Holliday Junction Intermediates	-33.1
	Homologous DNA Pairing and Strand Exchange	-36.1
Lung	HDR through Homologous Recombination (HRR)	-35.5
	Resolution of D-loop Structures through Holliday Junction Intermediates	-34.9

MBC: metastatic breast cancer.

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