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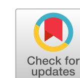
Research Paper

Identifying Circulating Tumor DNA Mutation Profiles in Metastatic Breast Cancer Patients with Multiline Resistance



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ABSTRACT

Purpose: In cancer patients, tumor gene mutations contribute to drug resistance and treatment failure. In patients with metastatic breast cancer (MBC), these mutations increase after multiline treatment, thereby decreasing treatment efficiency. The aim of this study was to evaluate gene mutation patterns in MBC patients to predict drug resistance and disease progression.
Method: A total of 68 MBC patients who had received multiline treatment were recruited. Circulating tumor DNA (ctDNA) mutations were evaluated and compared among hormone receptor (HR)/human epidermal growth factor receptor 2 (HER2) subgroups.
Results: The baseline gene mutation pattern (at the time of recruitment) varied among HR/HER2 subtypes. *BRCA1* and *MED12* were frequently mutated in triple negative breast cancer (TNBC) patients, *PIK3CA* and *FAT1* mutations were frequent in HR+ patients, and *PIK3CA* and *ERBB2* mutations were frequent in HER2+ patients. Gene mutation patterns also varied in patients who progressed within either 3 months or 3–6 months of chemotherapy treatment. For example, in HR+ patients who progressed within 3 months of treatment, the frequency of *TERT* mutations significantly increased. Other related mutations included *FAT1* and *NOTCH4*. In HR+ patients who progressed within 3–6 months, *PIK3CA*, *TP53*, *MLL3*, *ERBB2*, *NOTCH2*, and *ERS1* were the candidate mutations. This suggests that different mechanisms underlie disease progression at different times after treatment initiation. In the COX model, the ctDNA *TP53* + *PIK3CA* gene mutation pattern successfully predicted progression within 6 months.
Conclusion: ctDNA gene mutation profiles differed among HR/HER2 subtypes of MBC patients. By identifying mutations associated with treatment resistance, we hope to improve therapy selection for MBC patients who received multiline treatment.

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1. Introduction

Breast cancer is the most prevalent cancer among females, with a relatively high survival rate. Indeed, the Surveillance Epidemiology and End Results (SEER) dataset (SEER 18, 2006–2012) in the United States indicates that the 5-year survival rate is 89.7%. This survival rate has actually improved in recent decades, in part due to the prevalence of population-based mammography screening and the systematic use of adjuvant therapies [1]. As a result, long-term tumor-bearing survival is now common among advanced breast cancer patients [2]. Importantly, many long-term surviving metastatic breast cancer (MBC) patients receive multi-line chemotherapy. Unfortunately, such treatment reduces the sensitivity of MBC tumor cells to most commonly used

Abbreviations: ctDNA, circulating tumor DNA; MBC, metastatic breast cancer; HR, hormone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple negative breast cancer; SEER, Surveillance Epidemiology and End Results; PFS, progression-free survival; ER, estrogen receptor; PR, progesterone receptor; IHC, immunohistochemistry; cfDNA, cell-free DNA; gDNA, genomic DNA; SNV, Single nucleotide variants; Indels, insertions and deletions; TMB, Tumor mutation burden; WES, whole exome sequencing; ROC, receiver operating characteristic; AUC, area under the curve; HR, hazard ratio; CI, confidence interval; GX, gemcitabine+capecitabine; AI, aromatase inhibitor.

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drugs. This complicates the process of selecting effective drugs for progressed MBC patients. One way to streamline this drug selection process is by identifying potential drug-sensitive gene mutations in circulating tumor DNA (ctDNA).

ctDNA can be derived from liquid biopsies (minimally-invasive blood from cancer patients). While traditional imaging tools or serum biomarkers (e.g., CEA and CA153) are not timely nor sensitive enough to reflect small changes in tumor mutations, monitoring ctDNA provides valuable and sensitive blood-based biomarkers in advanced cancers. Indeed, ctDNA levels correspond to the tumor burden [3]; thus, ctDNA screening can help monitor the tumor response to treatment [4–7]. ctDNA mutations can also indicate tumor sensitivity to specific chemotherapy drugs [8–12]. For example, *PIK3CA* and *BRCA1* are common mutations in MBC [13,14]. While *PIK3CA* mutations suggest sensitivity to the mTOR inhibitor Everolimus [15], *BRCA1* mutations suggest sensitivity to the PARP inhibitor Olaparib [16]. Thus, screening for ctDNA mutations provides a minimally-invasive tool for doctors to identify effective drug-based therapies in MBC patients.

Conversely, monitoring ctDNA can also aid early detection of genetic events underlying drug resistance and inform potential combination therapy approaches [17]. For example, increased *PIK3CA* mutations following treatment initiation suggest tumor progression and poor progression-free survival (PFS) [18]. In estrogen receptor (ER)-positive patients, *ESR1* mutations following endocrine therapy (except Fulvestrant) indicate treatment resistance [19–23], while in HER2+ patients, mutations in *TP53* and *PIK3CA* are associated with anti-HER2 therapy resistance [24]. According to NCCN guidelines, breast cancer patients are recommended to be diagnosed and treated based on their hormone receptor (HR)/human epidermal growth factor receptor 2 (HER2) status. In general, triple negative (HR-/HER2-), HR-positive and HER2-positive are three main subtypes. However, previous studies did not fully and systematically characterize ctDNA gene mutation patterns in MBC patients related to HR/HER2 status and treatment. Such information is critical for both efficient surveillance of tumor gene mutations and accurate treatment. In this study, we recruited 68 MBC patients to systematically investigate their ctDNA mutation profiles relative to HR/HER2 status. Potential ctDNA markers for monitoring tumor progression, drug resistance, and treatment response were analyzed.

2. Methods

2.1. Patient Cohort and Clinical Data Collection

This study was approved by the Ethics Committee at the Affiliated Cancer Hospital of Xiangya School of Medicine at Central South University. A total of 68 metastatic breast cancer patients were enrolled in this study who were treated from January 2016 to November 2017 at the Department of Breast Oncology in the Affiliated Cancer Hospital of Xiangya School of Medicine. Informed consent was obtained from each patient prior to study onset. According to the American Joint Committee on Cancer staging system, patients diagnosed with stage III/IV primary breast malignant tumors (site: C50.0–C50.9; histology type: invasive ductal carcinomas or lobular carcinomas) were recruited. Other inclusion criteria included: 1) pathologically confirmed triple negative MBC patients, HER2+ positive recurrent or MBC patients, and ER+/progesterone receptor (PR)+recurrent or MBC patients; 2) according to RECIST 1.1 standards, patients had at least one measurable lesion; 3) aged between 18 and 70 years; 4) liver, renal, and blood tests showed a neutrophil count > 2.0 g/l, Hb > 9 g/l, platelet count > 100 g/l, AST and ALT >0.5 ULN, TBIL <1.5 ULN, and Cr < 1.0 ULN. The exclusion criteria included: 1) multiple primary cancers; 2) patients with immunodeficiency or organ transplantation history; 3) patients with heart disease or heart abnormalities such as cardiac infarction and severe cardiac arrhythmia (Fig. S1). Basic demographic and clinical information including age, pathology, laterality, stage, metastatic sites, HR/HER status, imaging records, and treatment history were collected [25].

2.2. Receptor Status Evaluation

ER and PR status were evaluated by immunohistochemistry (IHC) based on the American Society of Clinical Oncology guidelines [26,27]. The steps for IHC evaluation were as follows: 1) calculate the percent of ER/PR positively stained invasive tumor cells; 2) record the intensity of staining; 3) interpret that a minimum of 1% of invasive tumor cells staining positive for ER/PR in a specimen is HR+, while specimens exhibiting <1% of tumor cells staining for ER or PR of any intensity is HR-. For HER2 status, HER2+ was defined as a tumor area >10% with contiguous and homogeneous tumor cells indicated as HER2+ via gene amplification or IHC (HER2 copy number or HER2:CEP17 quantified by fluorescent in situ hybridization based on counting at least 20 cells within the area). The latest record of HR/HER2 status from recurrence biopsies was applied in this study.

2.3. Blood Sample Collection and DNA Extraction

Peripheral blood samples were collected 7 days before treatment, at 2–3 cycles of treatment when the first evaluation was performed, and at disease progression (image evaluation based on RECIST 1.1 standards). Peripheral blood samples were collected in Streck tubes (Streck, Omaha, NE, USA) and centrifuged within 72 h to separate the plasma from peripheral blood cells. Cell-free DNA (cfDNA) was extracted from plasma usinga QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). Genomic DNA (gDNA) was extracted from peripheral blood cells using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Both DNA extractions were performed according to the manufacturer's instructions. gDNA was sequenced as the normal control sample. Volumes of blood collected and of circulating-free DNA extracted (concentrations and total amounts) for all patients are listed in Table S1.

2.4. Target Capture and Next-Generation Sequencing

Both cfDNA and gDNA libraries were constructed with the KAPA DNA Library Preparation Kit (Kapa Biosystems, Wilmington, MA, USA) using the manufacturer's protocol. Capture probes were designed to cover coding sequences and hot exons of 1021 genes that are frequently mutated in solid tumors. A detailed description of the capture experiments has been reported previously [28]. Libraries were hybridized to custom-designed biotinylated oligonucleotide probes (Integrated DNA Technologies, Iowa, IA, USA). DNA sequencing was performed using the HiSeq 3000 Sequencing System (Illumina, San Diego, CA) with 2 × 101-bp paired-end reads. In Table S2, all genes included in our panel are listed. Clonal hematopoietic mutations were filtered as previously described [29], including those in *DNMT3A*, *IDH1*, and *IDH2* and specific alterations within *ATM*, *GNAS*, and *JAK2*.

2.5. Sequencing Data Analysis

From raw data, terminal adaptor sequences and low-quality reads were removed. The BWA (version 0.7.12-r1039) tool aligned clean reads to the reference human genome (hg19), and Picard (version 1.98) marked PCR duplicates. Realignment and recalibration was performed using GATK (version 3.4–46-gbc02625). Single nucleotide variants (SNV) were called using MuTect (version 1.1.4) and NChot, a software developed in-house to review hotspot variants [28]. Small insertions and deletions (Indels) were called using GATK. Somatic copy number alterations were identified with CONTRA (v2.0.8). Significant copy number variation was expressed as the ratio of adjusted depth between ctDNA and control gDNA. The final candidate variants were all manually verified in the Integrative Genomics Viewer. Sequencing stats of all samples are shown in Table S1. This sequencing method was previously proven credible with simulated cfDNA [28], so we did not validate the mutations found in ctDNA by sequencing tumor biopsies.

2.6. Tumor Mutation Burden (TMB) Calculation

Previously published whole exome sequencing (WES) data demonstrated that mutation burden is a prognostic factor [30]. Usually, TMB is calculated from very large datasets (e.g. whole exome), but Chalmers et al. showed that gene panels can accurately assess TMB compared with WES [31]. In our analysis, we also found that the TMB of our pan-cancer panel significantly correlated with that of WES data from The Cancer Genome Atlas and paired sequencing data from Geneplus (data not shown).

TMB analysis interrogated SNVs and small indels with the variant allele frequency ≥ 3%. TMB-high patients were identified with ≥11 mutations/MB (upper quartile of all data). All others were identified as TMB-low patients.

2.7. Image Evaluation and Definition of Drug Resistance

Image evaluation was performed every 2–3 treatment cycles according to RECIST 1.1 standards. Intargeted therapy-based treatment trials of MBC patients, PFS closely correlates with overall survival [32,33]. In this study, PFS was applied to evaluate the drug treatment response. Drug resistance was defined as disease progression within three months of treatment (at the first evaluation, PFS $<$ 3 months).

2.8. Statistical Analyses

Numerical variables were summarized as the mean (standard deviation) and median (interquartile range). Categorical variables were reported as counts (percentage). An analysis of variance was used to compare continuous variables with symmetrical distributions across subgroups. Chi-square tests or Fisher's exact tests ($n < 5$) were used to compare categorical variables among HR/HER2 subtypes. Cox regression analysis was used to evaluate the univariate and multivariate risk of candidate gene mutations in progression. Kaplan-Meier (KM) curves were used to plot survival distributions against progression, and the log-rank test was used to assess differences in PFS among subgroups. To identify putative ctDNA mutation profiles in HR/HER2 subtypes, a logistic regression model was performed. A receiver operating characteristic (ROC) curve was calculated to determine prediction efficiency. All tests

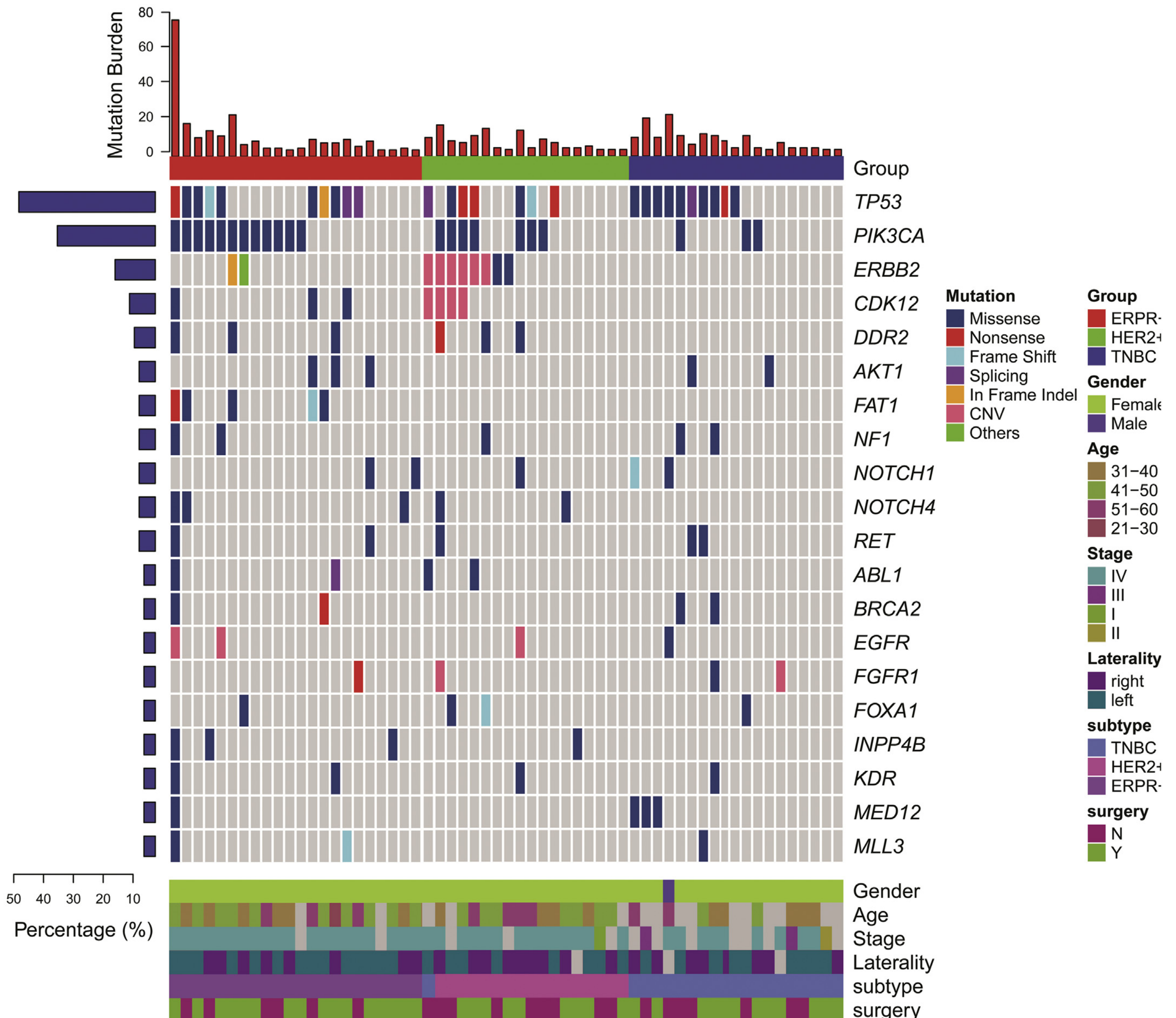


Fig. 1. Circulating tumor DNA (ctDNA) gene mutation profiles (top) and patient demographic/clinical data (bottom).

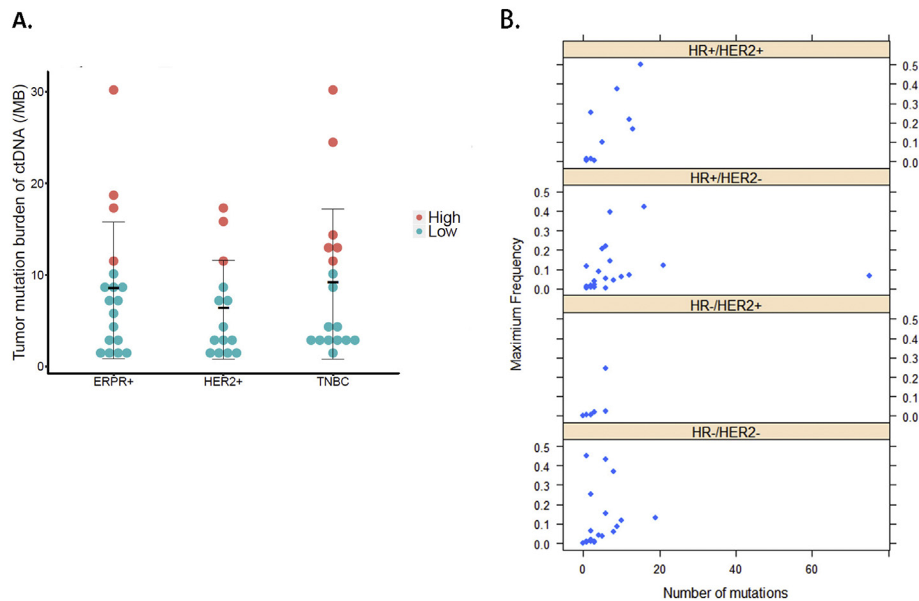


Fig. 2. Tumor mutation burden (TMB) for different metastatic breast cancer subtypes. A. TMB comparison among the different HR/HER2 subtypes: ER/PR+, HER2+, and triple negative breast cancer (TNBC). B. Number of mutated circulating tumor DNA (ctDNA) genes and their maximum frequency in each patient among the four HR/HER2 subtypes.

of hypotheses were two-tailed and conducted at a significance level of 0.05. Statistical analyses were conducted using SAS 9.4.

3. Results

3.1. Demographic and Clinical Features of Patients

In this study, 68 MBC patients were included and the demographic and clinical features of these patients were analyzed. As shown in Table S3, the average diagnostic age was 44.1 yrs. All patients except one were female. The majority of patients had invasive carcinoma, and all patients were in stage III/IV. Of the 68 included patients, 23 were metastatic triple negative breast cancer (TNBC), 25 were ER/PR+ (HR+/HER2-), and 20 were HER2+ (6 HR-/HER2+ and 14 HR+/HER2+). There were no differences in histology, laterality, or surgical treatment between the three subtypes. Most patients (91.18%) received primary tumor surgery, and all received at least 2nd line chemotherapy. All HR + and HR+/HER2+ patients had received endocrine therapy, while all HER2+ patients had received anti-HER2 treatment, except one who could not afford the drug. As described in the designed schematic (Fig. S1), recruited patients received ctDNA testing analysis.

3.2. Mutation Profiling of MBC Patients Using ctDNA Sequencing

To determine the tumor gene mutation profiles for each HR/HER2 subtype, patients' blood samples were collected and ctDNA was extracted for sequencing. We captured 1021 hot-mutated genes for next-generation sequencing, and mutations were detected by aligning to a reference human genome (hg19). Among the 68 patients, 62 (87%) were mutation-positive before treatment. Specifically, 22 (100%) TNBC, 18 (85.71%) HER2+, and 22(88%) ER/PR+ patients were ctDNA mutation-positive at baseline (Fig. 1). As shown in Fig. 1 and Table S4, the top four most commonly mutated somatic genes were *TP53*, *PIK3CA*, *ERBB2*, and *CDK12*, with mutation frequencies of 38.24% (26 pts), 33.82% (23 pts), 14.71% (10 pts), and 10.29% (7 pts), respectively. For missense mutations, the hotspot locus in *TP53* was p. C238F/Y and in *PIK3CA* was p.H1047L/R (Fig. S2). In addition, 7 (31.82%) TNBC, 1(4%) HR+, and 1 (4.76%) HER2+ sample harbored *BRCA1* mutations (Table S4). Among these 9 *BRCA1* mutations, five were germline mutations and four were somatic (Table S5).

3.3. ctDNA Gene Mutation Profiles Differ among TNBC, HER2+, and ER/PR+ Patients

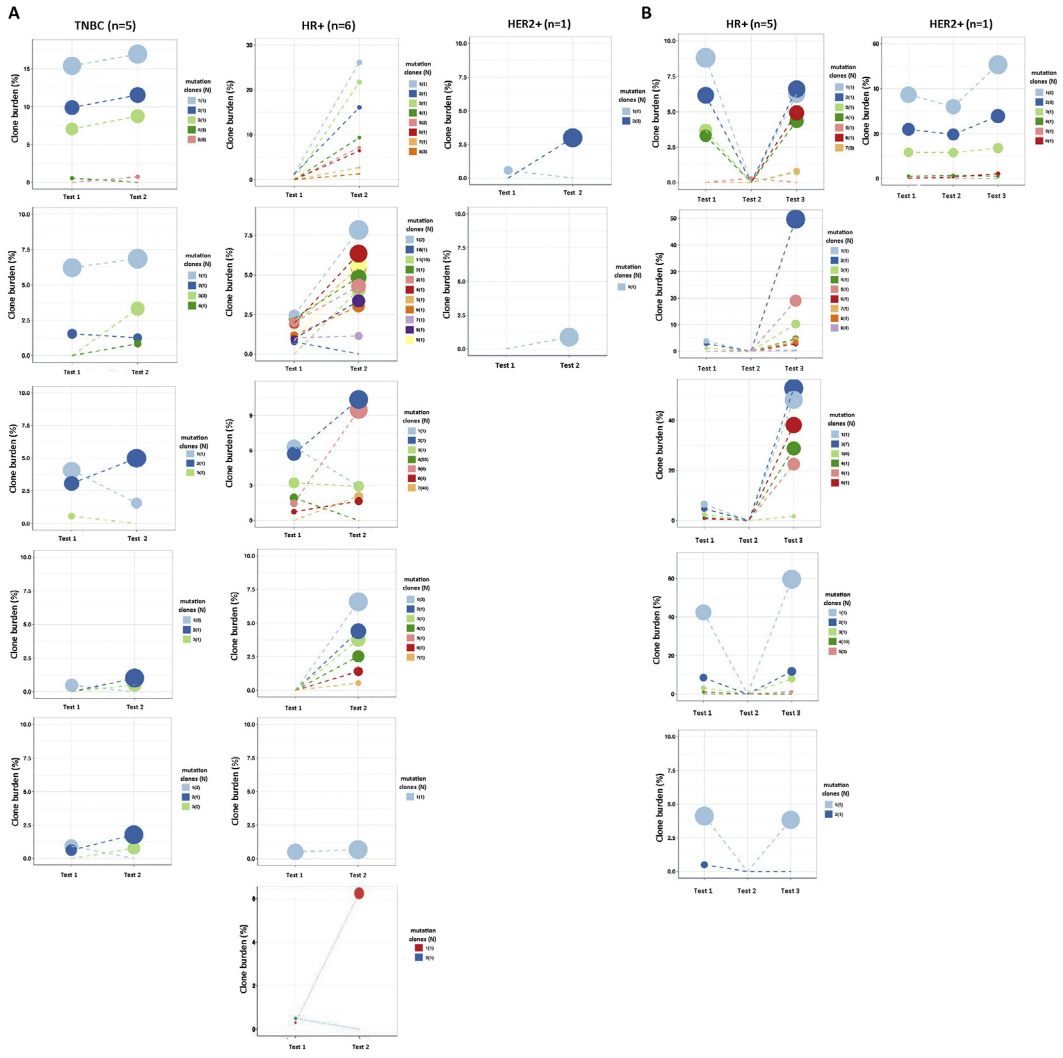
Analyzing the ctDNA gene mutation profiles of each subtype revealed that these profiles differed among HR/HER subgroups. Among all HR/HER2 subtypes, *TP53* mutations were frequent (Table S4). Specifically, *TP53* mutations occurred in 10 (45.45%) TNBC patients, 9 (36.00%) HER2+ patients, and 7 (22.22%) HR+ patients. However, in the TNBC group, *PIK3CA* and *ERBB2*mutations were rare, while *BRCA1* germline mutations were significantly more frequent than in the HER2+ or HR+ groups ($p = 0.009$). Alternatively, *PIK3CA* mutations were significantly more frequent in the HER2+ and HR+ patients than in TNBC ($p = 0.020$). Likewise, *ERBB2* mutations were most frequent in the HER2+ group ($p < 0.0001$).

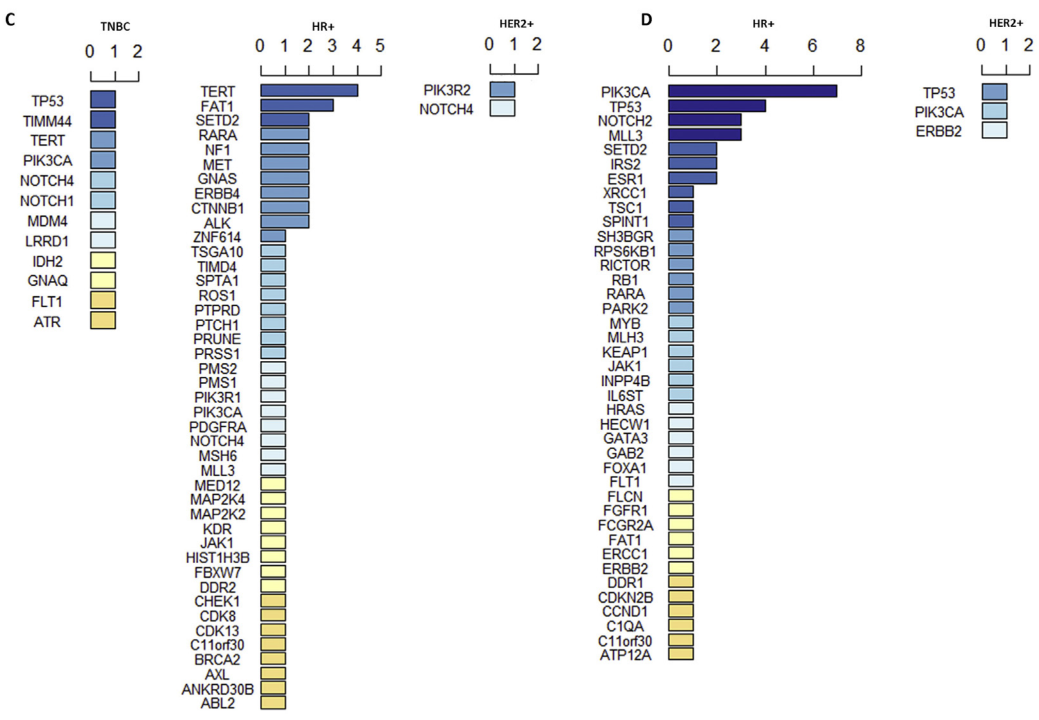
Furthermore, in TNBC patients, *TP53, BRCA1*, and *MED12* were all frequently mutated, while in HR+ patients, *TP53*, *PIK3CA*, and *FAT1* mutations were the most common (Fig. 1, Table S4). Finally, *ERBB2*, *PIK3CA*, and *TP53* were the most frequently mutated genes in HER2+ patients. Upon dividing HER2+ patients into HER2+/HR+ and HER2+/HR- subgroups, we found that *ERBB2* mutations were concentrated in HER2+/HR+ patients, specifically (Table S5C).

3.4. Tumor Mutation Burden (TMB) Did Not Significantly Differ among HR/HER2 Subgroups

We next evaluated how TMB varied among the different HR/HER2 subgroups. With our current sample size and algorithm, TMB did not significantly differ among the HR+, HER2+ and TNBC groups. However, the TNBC and HR+ groups had a moderately higher TMB than the HER2 + group (Fig. 2A). Further dividing the HER2+ group into HR-/HER2+ and HR+/HER2+ subgroups revealed that HR-/HER2+ patients had fewer ctDNA mutations and lower mutation frequencies compared to the other three patient groups (TNBC, ER/PR+, and HR+/HER2+)

Fig. 3. Ranking circulating tumor DNA (ctDNA) gene mutations increased in triple negative breast cancer (TNBC), HR+, and HER2+ patients with progression. A. ctDNA gene mutations increased in patients who had progression within 3 months of treatment. B. ctDNA gene mutations increased in patients who had progression within 3–6 months. C. Ranking of ctDNA mutations increased in patients who had progression within 3 months. D. Ranking of ctDNA gene mutations increased in patients who had progression within 3–6 months.


A
TNBC (n=5)
HR+ (n=6)
HER2+ (n=1)
B
HR+ (n=5)
HER2+ (n=1)
Clone burden (%)
mutation clones (N)
Test 1
Test 2
Test 3


C
TNBC
0 1 2
TP53
TIMM44
TERT
PIK3CA
NOTCH4
NOTCH1
MDM4
LRRD1
IDH2
GNAQ
FLT1
ATR
HR+
0 1 2 3 4 5
TERT
FAT1
SETD2
RARA
NF1
MET
GNAS
ERBB4
CTNNB1
ALK
ZNF614
TSGA10
TIMD4
SPTA1
ROS1
PTPRD
PTCH1
PRUNE
PRSS1
PMS2
PMS1
PIK3R1
PIK3CA
PDGFRA
NOTCH4
MSH6
MLL3
MED12
MAP2K4
MAP2K2
KDR
JAK1
HIST1H3B
FBXW7
DDR2
CHEK1
CDK8
CDK13
C11orf30
BRCA2
AXL
ANKRD30B
ABL2
HER2+
0 1 2
PIK3R2
NOTCH4
D
HR+
0 2 4 6 8
PIK3CA
TP53
NOTCH2
MLL3
SETD2
IRS2
ESR1
XRCC1
TSC1
SPINT1
SH3BGR
RPS6KB1
RICTOR
RB1
RARA
PARK2
MYB
MLH3
KEAP1
JAK1
INPP4B
IL6ST
HRAS
HECW1
GATA3
GAB2
FOXA1
FLT1
FLCN
FGFR1
FCGR2A
FAT1
ERCC1
ERBB2
DDR1
CDKN2B
CCND1
C1QA
C11orf30
ATP12A
HER2+
0 1 2
TP53
PIK3CA
ERBB2

(Fig. 2B). However, more samples are needed to fully illustrate this difference.

3.5. Increased ctDNA Mutation Frequency in Patients with Drug Resistance

To further address the influence of tumor mutations on treatment response, mutation changes over time were investigated. Among the 68 patients, 41 received 2nd or 3rd ctDNA evaluation (Table S6). At the 2nd evaluation (at about 3 months of drug treatment), 5 TNBC, 6 HR+, and 2 HER2+ patients had increased ctDNA gene mutation frequencies. After 3 months of treatment, 5 HR+ and 1 HER2+ patient had increased ctDNA gene mutation frequencies. Another 22 patients exhibited decreased ctDNA gene mutation frequencies within 180 days and were characterized as drug sensitive (Table S6A).

We then compared the results of ctDNA gene mutation frequencies to IHC analysis of disease progression. In general, the results of these two outcomes correlated (kappa = 0.8010). According to Recist 1.1 standards, of the 41 patients that received 2nd or 3rd ctDNA evaluation, 15 patients had IHC-observable disease progression. However, four patients had increasing ctDNA mutation frequencies but did not show progression by image analysis, indicating that ctDNA testing can detect changes earlier than imaging (Table S6B). Fig. S3 shows the images for these four patients at the time of ctDNA testing. These metastatic tumors were detected by computed tomography (CT). In Table S6C, the mutated genes and variant allele frequencies for these four patients are specified.

3.6. ctDNA Mutations Related to Chemotherapy Resistance

We next evaluated the effect of ctDNA mutations on chemotherapy resistance. We used both image analysis and ctDNA testing to evaluate disease progression following treatment initiation. Of the 41 patients who received 2nd or 3rd ctDNA evaluation, all TNBC patients received chemotherapy, except one who received PD-1 antibody, while all HR + patients received chemotherapy. To identify candidate gene mutations associated with chemo-resistance, we ranked the ctDNA mutations that increased in resistant patients with PFS < 3 months (Fig. 3A) and with PFS >3 months (Fig. 3B). In patients with PFS < 3 months, *TERT* and *FAT1* mutation clones were frequently detected in HR+ patients (Fig. 3C). Similarly, in both the TNBC and HR+ subgroups, *Notch4* mutations were commonly detected (Fig. 3C). Other chemo-resistance related mutations included *SETD2*, *RARA*, and *MLL3*. For chemo-resistance after 3 months (PFS > 3 months), *PIK3CA*, *TP53*, *NOTCH2*, *MLL3*, and *SETD2* mutations were all frequently detected (Fig. 3D). Thus, these ctDNA mutations were likely related to chemo-resistance and disease progression.

3.7. ctDNA Mutations Related to Anti-HER2 Treatment Resistance

We also evaluated the effect of ctDNA mutations on anti-HER2 treatment. All evaluated HER2+ patients received anti-HER2 treatment, except one with GX (gemcitabine+capetabine) chemotherapy. For HER2 + patients who received anti-HER2 treatment, two had anti-HER2 treatment resistance within 3 months (Fig. 3A). Within this time, *PIK3R2* and *NOTCH4* mutations were elevated (Fig. 3C). For patients who had anti-HER2 treatment resistance after 3 months of treatment, *TP53*, *PIK3CA*, and *ERBB2* mutations increased (Fig. 3B and D).

3.8. Effects of ctDNA Mutations on PFS

Thus, of the most commonly mutated genes in MBC patients (*TP53*, *PIK3CA*, *ERBB2*, and *BRCA1/2*), some were related to treatment resistance. We next wanted to quantify the efficiency of these mutations in predicting disease progression. To do this, ROC curves were drawn, and sensitivity/specificity were calculated for different mutation patterns. The area under the curve (AUC) of the*TP53* + *PIK3CA* mutation pattern, the *TP53* + *PIK3CA* + *ERBB2* pattern, and the top five mutation combination (*TP53* + *PIK3CA* + *ERBB2* + *BRCA1* + *CDK12*) pattern were 0.7890, 0.8402 and 0.8502, respectively (Fig. 4). Of these, the *TP53* + *PIK3CA* mutation pattern had the lowest AUC value, with optimal sensitivity and specificity among the three models (Table S7).

To evaluate the effect of ctDNA mutations on PFS, lifetest and univariate Cox proportional hazards analyses were performed. Interestingly, survival analysis with KM curves revealed that baseline *TP53* and *PIK3CA* mutations were risk factors for PFS (Fig. 5A&B, Log-rank $p =$ 0.0059 for *TP53* and $p = 0.0278$ for *PIK3CA*). When the *TP53* and *PIK3CA* mutations were combined, the*TP53-/PIK3CA-* group (no *TP53* nor *PIK3CA* mutations) showed marginally better PFS compared to the *TP53+/PIK3CA+*, *TP53-/PIK3CA+*, and *TP+/PIK3CA-* groups (*TP53* or *PIK3CA* mutations; $p = 0.0116$, Fig. 5C). Thus, *TP53* and *PIK3CA* ctDNA mutations likely limit survival and promote disease progression.

Baseline *ERBB2* mutations were also a risk factor for PFS (Fig. 5D, $p =$ 0.0450). Univariate Cox regression analysis further confirmed the effect of *TP53* (hazard ratio [HR] (95% confidence interval [CI]) = 2.576(1.283, 5.175), $p = 0.008$), *PIK3CA* (HR (95% CI) = 2.167 (1.073, 4.379), $p =$ 0.03), and *ERBB2* mutations (HR (95% CI) = 2.162 (1.000, 4.676), $p =$ 0.05, Table S8) on PFS. Due to our relatively small sample size, baseline *BRCA1* and *CDK12* mutations did not significantly affect PFS (Fig. 5E–F). To eliminate potential confounding variables, a multivariate Cox regression analysis was performed. The results of this model suggested that *TP53* and *ERBB2* mutations were significant risk factors for disease progression (Table S8).

4. Discussion

In this study, we identified many ctDNA mutations in MBC patients that were associated with resistance to chemotherapy or anti-HER2 treatment. This is important because according to National Comprehensive Cancer Network guidelines, the main treatment strategies for

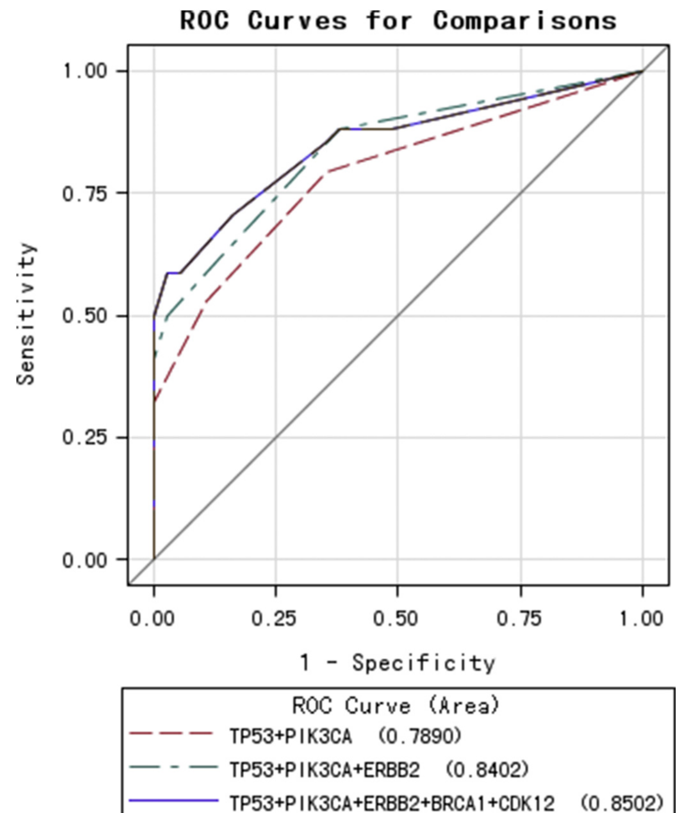


Fig. 4. Receiver operating characteristics (ROC) curves for four joint models of *TP53*, *PIK3CA*, *ERBB2*, *BRCA1*, and *CDK12* mutations in predicting progression-free survival.

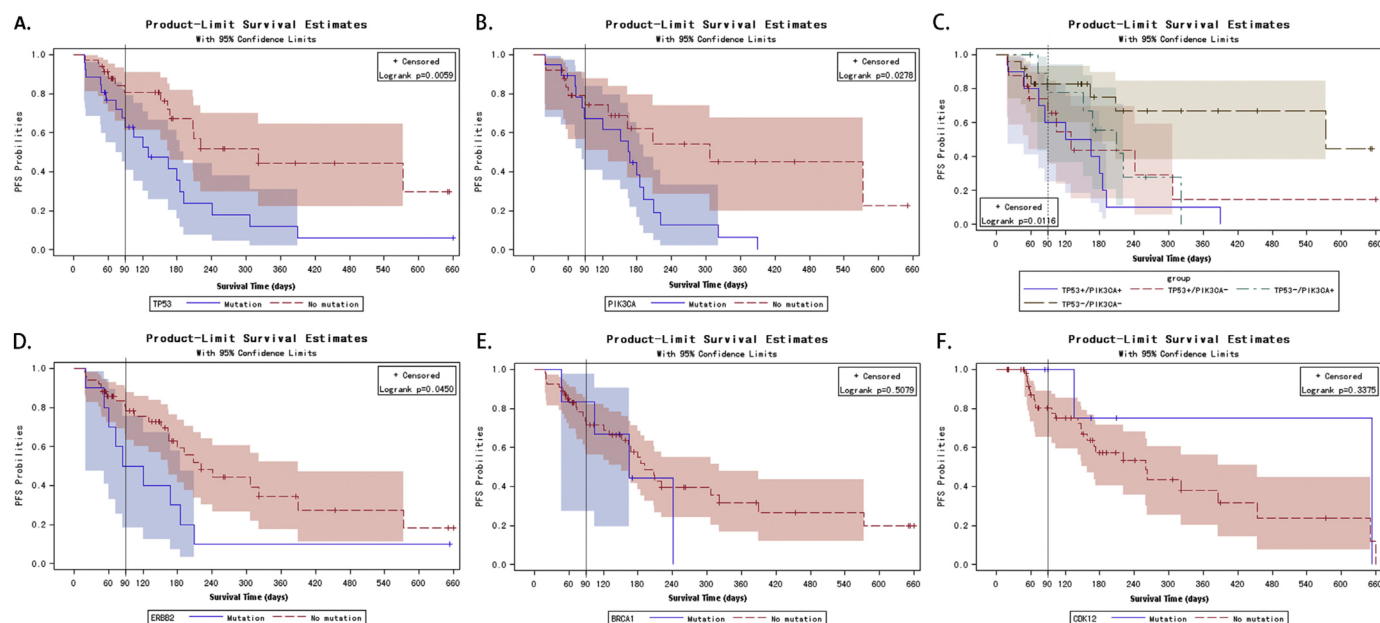


Fig. 5. Kaplan-Meier curves for progression-free survival probabilities stratified by circulating tumor DNA (ctDNA) mutations, *TP53* (A), *PIK3CA* (B),*TP53*/*PIK3CA* (C), *ERBB2* (D),*BCRA1* (E), and *CDK12* (F).

inoperable metastatic or recurrent breast cancer are chemotherapy combined with endocrine and/or targeted therapy. Treatment strategies depend upon a patient's HR/HER2 status, which is delineated by biopsy results. Specifically, ER+ and/or PR+ patients are appropriate candidates for endocrine therapy, including nonsteroidal aromatase inhibitors (anastrozole and letrozole), steroidal aromatase inhibitors (exemestane), serum ER modulators (tamoxifen and toremifene), and ER down-regulators (fulvestrant). Aromatase inhibitors (AI) are superior to serum ER modulators [34]; however, many AI-treated patients relapse due to *ESR1* mutations [35] and acquired CYP19A1 (encoding aromatase) amplification [36]. Alternatively, for HER2 + MBC patients, anti-HER2 target therapy is the first choice.

In this study, among the patients who received ctDNA evaluation at 3 months after treatment, all HR+ (HER2-) patients received chemotherapy alone, except one who received chemotherapy (Capetabine + Vinorelbine) + Letrozole. All HER2+ patients received chemotherapy plus anti-HER2 treatment, except one who received chemotherapy (GX) only. Only limited patients received endocrine therapy; therefore, in this study, we could not evaluate genes that conferred resistance to endocrine therapy. Instead, we evaluated genes associated chemotherapy resistance in HR+ (HER2-) patients, and anti-HER2 treatment resistance in HER2+ patients. Of the HR+ patients, those who had PFS < 3 months exhibited increased mutation frequencies in *TERT*, *FAT1*, *RARA*, and *ERBB4*, those who had progression with PFS > 3 months had increased mutations in *PIK3CA*, *TP53*, *NOTCH2*, and *MLL3* (Fig. 3). This suggests a distinct pattern or mechanism for drug resistance between HR+ patients with PFS < 3 months and PFS > 3 months.

In this study, two HER2+ patients had anti-HER2 treatment resistance with PFS < 3 months. Following anti-HER2 treatment, ctDNA mutation frequencies increased for *PIK3R2* and *NOTCH4* (Fig. 3C). ForHER2 + patients who had anti-HER2 treatment resistance with PFS > 3 months, *TP53*, *PIK3CA*, and *ERBB2* mutations increased (Fig. 3D). Previously, reactivation of HER2 (*ERBB2*) mutations have been related to acquired resistance to Lapatinib-containing HER2-targeted therapy [37]. *DNMT3A* mutations or activation is also associated with resistance to docetaxel in breast cancer [38].

In this study, all progressed TNBC patients had PFS <3 months. In these patients, *TP53*, *TERT*, *PIK3CA*, *NOTCH1/4* were commonly mutated. Previously, *TERT* SNPs rs10069690 and rs2242652 have been associated with ER and *BRCA1*-mutated breast cancer without altering telomere length [39]. *TERT*-CLPTM1L locus mutations are also significantly associated with TNBC, particularly in younger women [40]. In this study, *TERT* was not a baseline ctDNA mutation candidate (Fig. 1). However, its increase was common in patients with chemo-resistance, especially for HR+ and TNBC patients. Thus, the mechanisms underlying the effects of *TERT* mutations on treatment resistance require further investigation.

Ultimately, we clarified the baseline ctDNA mutation patterns for MBC patients based on HR/HER2 status. We also identified treatment resistance related ctDNA mutations. However, our study was limited by a relatively small sample size. About 20 patients in each subgroup likely created noise in the baseline mutation frequency calculation. In addition, patients in the same subgroup received different treatment regimens. For example, some HER2+ patients were treated with Herceptin while others were treated with Herceptin + Lapatinib. Future studies should include a larger sample size to compare ctDNA mutation profiles within subgroups based on different treatment regimens. Future studies should also investigate the mechanisms underlying the effects of these ctDNA mutations on treatment resistance.

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Author Contributions

Dr. Quchang Ouyang had full access to all data in the study and takes responsibility for the integrity and accuracy of the data analysis.

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