Targeted Next-Generation Sequencing of Circulating Tumor DNA Mutations among Metastatic Breast Cancer Patients

Minying Sun (sunmy1220@163.com)
Southern Medical University

Fangqin Lin
Guangzhou Medical University

Lujia Chen
Southern Medical University Nanfang Hospital

Hong Li
Southern Medical University Nanfang Hospital

Weiquan Lin
Guangzhou Center for Disease Control and Prevention

Hongyan Du
Southern Medical University

Xuexi Yang
Southern Medical University

Ming Li
Southern Medical University

Research

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Abstract

Background Liquid biopsy through the detection of circulating tumor DNA (ctDNA) has potential advantages in cancer monitoring and prediction. However, most previous studies in this area were performed with a few hotspot genes, single time point detection, or insufficient sequencing depth.

Methods In this study, we performed targeted next-generation sequencing (NGS) with a customized panel in metastatic breast cancer (MBC) patients. Fifty-four plasma samples were taken before chemotherapy and after the third course of treatment for detection and analysis. Paired lymphocytes were also included to eliminate clonal hematopoiesis (CH)-related alternatives.

Results A total of 1182 nonsynonymous mutations on 419 genes were identified. More ctDNA mutations were detected in patients with tumors> 3cm (P = 0.035) and HER2(−) patients (P = 0.029). For a single gene, the distribution of ctDNA mutations was also correlated with clinical characteristics. Multivariate regression analysis revealed that HER2 status was significantly associated with mutation burden (*OR* 0.02, 95% CI 0–0.62, P = 0.025). The profiles of ctDNA mutations exhibited marked discrepancies between two time points, and baseline ctDNA was more sensitive and specific than that after chemotherapy. Finally, elevated ctDNA mutation level was positively correlated with poor survival (P < 0.001).

Conclusion Mutations in ctDNA could serve as a potential biomarker for the evaluation and prediction, and guide the clinical management of MBC patients with chemotherapy.

Background

Breast cancer is the most common cancer among women in China as well as worldwide[1]. With progress in cancer management, most patients diagnosed in the early stages can be treated satisfactorily by surgical resection and adjuvant therapies. However, metastatic breast cancer (MBC) is still a challenge for clinicians due to the development of drug resistance, and a decreased overall survival rate is closely linked to the incidence of distant metastases[2, 3]. MBC is a heterogeneous and dynamic disease with a range of biological characteristics, genomic alterations, and clinical outcomes[4, 5]. It is important to monitor the clinical progression and therapy responsiveness during the course of personalized treatment[6]. Recent studies have demonstrated that circulating tumor DNA (ctDNA) released from tumor cells into the blood circulation and which contains a great deal of genetic and epigenetic information associated with cancer is a promising biomarker to assess cancer prognosis and the efficacy of treatment[7–10].

Monitoring of ctDNA can be performed more easily, repeatedly, and is non-invasive compared with tissue biopsy[11]. However, there are many scientific and logistical challenges, such as the low level of ctDNA in the “sea” of cell-free DNA (cfDNA) and the small number of validated ctDNA-based driver genes or mutations specific for MBC other than *ER*, *HER2*, *PIK3CA*, and *AKT1*[12]. In addition, most studies to date were performed with detection at only a single time point or with insufficient sequencing depth due to

technical and cost limitations[13]. More data of high quality and comprehensive monitoring are essential to support and promote the application of ctDNA for the evaluation of MBC.

In this prospective study, we designed a personalized target-capture region associated with breast cancer. Plasma samples obtained at two time points were examined by ultrasensitive high-throughput next-generation sequencing (NGS). Meanwhile, parallel sequencing from paired lymphocytes was performed to filter out interference by clonal hematopoiesis (CH) variants. Analyses and comparisons were performed to identify ctDNA mutations related to MBC progression and clinical outcome. The results will provide evidence regarding the clinical utility of ctDNA and potentially explore the molecular mechanism of MBC.

Results

Clinical characteristics of the patients and target-capture sequencing

The main clinical characteristics of the 27 MBC patients included in the study are shown in Table 1. The mean age at diagnosis was 51.30 years (range, 33–68 years). Most patients had infiltrating ductal carcinoma (clinical stage IV) with lymph node, bone, or hepatic metastasis. In total, 14 (51.8%) patients had a maximal tumor diameter> 3cm, and 11 (40.74%) patients had given birth three or more times. The proportions of ER(+), PR(+), and HER2(+)cases were 62.96%,59.26%, and 62.96%, respectively.

Table 1
Clinical characteristics of MBC patients.

Characteristics		N (%)
Diagnostic age (years)	Mean (rang)	51.30 (33–68)
Menarche age (years)	Mean (rang)	14.33 (11–19)
Stage	III	1 (3.70%)
	IV	26 (96.30%)
ER status	ER(+)	17 (62.96%)
PR status	PR(+)	16 (59.26%)
HER2 status	HER2(+)	17 (62.96%)
Menopause	YES	11 (44.44%)
Size of tumor	≥3cm	14 (51.85%)
	≤ 3cm	13 (48.15%)
Parturitions	≥ 3	11 (40.74%)
	≥3	16 (59.26%)
Therapeutic effect*	PR/SD	16 (59.26%)
	PD	11 (40.74%)
*PR: partial remission; SD: stable disease; PD: progressive disease		

After running an iterative algorithm with multiple databases and optimization by the NimbleGen Design portal, we selected a custom panel covering 119.20kb of the genome. The panel included 961 exons of 835 common driver genes distributed over all chromosomes. Details of the target-capture panel are presented in Table S1. DNA was successfully extracted from all 81 samples and qualified for target-capture sequencing. We obtained an average of 810.45 Mb (range 303.08–1424.64Mb) and 403.28Mb (range 183.51–789.27Mb) of high-quality data for the cfDNA and genomic DNA (gDNA) samples, respectively. The average sequencing depths for cfDNA and gDNA were 6799× (range 2543–11952×) and 3383× (range 1540–6621×), respectively.

Identification of ctDNA mutations and related genes

Some mutations originating from CH-related variants in lymphocytes can also be traced in cfDNA, which may interfere with the analysis of ctDNA. After the comparison and elimination of CH variants, we identified 1182 nonsynonymous mutations from all samples, including frameshift indels, stopgains, and single-nucleotide variants (SNVs). They were distributed in 419 genes on all chromosomes (Fig. 1a). All patients had mutations in *FRG1*, *AQP7*, and *DNAJC11*. Mutations were detected most frequently in *FRG1*,

and the highest mutation burden was seen in *MUC16* with 58 mutations. The top 20 genes most frequently mutated in the ctDNA are shown in Fig. 1b. Some typical cancer-related genes such as *TP53*, *PIK3CA*, *MAPK3K1*, *KRAS*, and *PTEN* were also included. The number of ctDNA mutations varied markedly between patients (range 38–171, mean 79.96).

To evaluate the influence of biological features by ctDNA mutations, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted for the mutated genes from three subsets (overall, baseline, and after chemotherapy). The top ten subjects were selected for further comparison and analysis. The analysis revealed that two, seven, and six genes in all three subsets were related to biological process (BP), cellular component (CC), and molecular function (MF), respectively, including regulation of cellular component size, cation channel complex, and calmodulin binding. There were more discrepancies among the three subsets according to BP enrichment than CC and MF (Figure S1).

On KEGG analysis, 96, 97, and 108 pathways were enriched in the baseline, after chemotherapy, and over all subsets, respectively. Among the top ten pathways, six were included in all three subsets (i.e., small cell lung cancer, EGFR tyrosine kinase inhibitor resistance, endometrial cancer, PI3K-Akt signaling pathway, endocrine resistance, and cholinergic synapse; Fig. 2). Most pathways were typical and indeed meaningful for cancer development, progression, or therapeutic response. The results revealed no significant discrepancies in overall enriched pathways among the three subsets.

Distribution of gene mutations and clinical characteristics

The patients were divided into different groups according to their clinical characteristics for further analysis. The results show that the ctDNA mutations were significantly associated with tumor size and HER2 status. Patients with tumors> 3cm carried more mutations than those with tumors≤ 3cm (92.29 vs. 66.69, respectively, $P = 0.035$), while HER2(+) patients carried fewer mutations than HER2(−) patients (68.65 vs. 99.20, respectively, $P = 0.029$). For single genes, the mutation profiles of ctDNA also exhibited discrepancies. We selected the top 20 genes for further analysis. The results show that mutations in *AQP7* and *PTEN* were significantly increased in patients with a poor therapeutic effect showing progressive disease (PD) ($P < 0.05$). *PIK3CA* mutations occurred more frequently in patients with tumors> 3cm ($P = 0.039$). The same was also observed for *DNAJC11*, *MAP3K1*, and *PGAP1* in patients with late menarche (≥ 14 years) and *KRAS* in older patients (age at diagnosis> 51 years, $P < 0.05$) (Table S2).

A multivariate regression analysis was conducted to examine the relations between mutation burden and clinical parameters (Table 2). The results show that ctDNA mutations were significantly associated with HER2 status. Patients with HER2(+) carried fewer mutations than HER2(−) patients (*OR* 0.02, 95% CI 0–0.62, $P = 0.025$). No significant associations were found for other characteristics.

Table 2. Multivariate regression analysis of the relationship between ctDNA mutations

and clinical parameters.

Factors	Coefficient (SE)	Adjusted *OR*	95% *CI*	*P* value
Diagnostic age	0.12	0.92	0.73–1.16	0.475
Menarche age	0.41	1.57	0.70–3.55	0.274
ER status, ER(+) vs ER(-)	2.29	2.51	0.03-222.34	0.687
PR status, PR(+) vs PR(-)	2.07	0.07	0-4.30	0.210
HER2 status, HER2(+) vs HER2(-)	1.70	0.02	0-0.62	0.025
Menopause, YES vs NO	2.02	1.48	0.03–77.85	0.847
Size of tumor, ≥3cm vs ≤ 3cm	1.55	10.28	0.50-213.38	0.132
Parturitions NO. ≥3 vs ≤3	0.50	1.02	0.38–2.72	0.965
Therapeutic effect, PR/SD vs PD	1.33	4.26	0.31–58.24	0.277

Dynamics of ctDNA mutations during chemotherapy

A total of 768 ctDNA mutations were detected in 302 genes at baseline, which decreased to 633 in 291 genes after three courses of treatment. The mean number of mutations for each patient also decreased after three courses of treatment (50.93 vs. 46.59, respectively). To examine the dynamic changes, we compared the distribution of ctDNA mutations in genes before and after chemotherapy (Fig. 3). The mutations reduced sharply after chemotherapy for *MUC16*, *NCOR1*, *TTN*, *PIK3R1*, and *TP53*. In contrast, more mutations were detected after chemotherapy for *MYO6*, *FLNC*, *FMN2*, *CDH1*, and *RHO*.

An analysis based on clinical information showed different mutation patterns in plasma DNA between baseline and after chemotherapy (Table S2). For baseline ctDNA, mutations in *PTEN* increased significantly in patients with PD compared to patients with a partial response/stable disease (PR/SD) ($P = 0.028$). More mutations were detected in gene *PIK3CA* in patients with tumors> 3cm ($P = 0.0039$), in *MAP3K1* and *PGAP1* in patients with late menarche (≥ 14 years, $P = 0.006$ and 0.008, respectively), and in *KRAS* in older patients (age > 51 years, $P = 0.018$). These associations were consistent in all samples, but not in samples obtained after treatment. In addition, fewer ctDNA mutations were found in *AQP7* in PR(+) patients ($P = 0.023$), and in *KRAS* and *PIK3CA* in HER2(+) patients ($P = 0.041$ and 0.030, respectively). In ctDNA after chemotherapy, the number of mutations decreased significantly in *TP53* in both HER2 (+) and PR(−) patients ($P = 0.011$ and 0.024, respectively). Mutations in *LUC7L2* were also strongly associated with HER2(−) status ($P = 0.020$).

ctDNA mutations and clinical outcomes

A Kaplan–Meier analysis was performed to explore risk factors correlated with PFS, defined as the duration from sampling to first disease progression. The mean PFS was 537 days (range 324 to 823 days). All patients were stratified with the mean number of ctDNA mutations as the threshold. The results indicate that HER2 status, therapeutic effect, and number of ctDNA mutations exhibited significant

associations with PFS (Fig. 4). HER2(+) patients had significantly longer PFS compared with HER2(−) patients (adjusted HR 0.26, $P = 0.038$), and PD was related to shorter PFS than PR/SD (adjusted HR 6.54, $P = 0.038$). Patients carrying more ctDNA mutations overall and in baseline plasma both had poor PFS rates ($P < 0.001$). No associations were found for other clinical characteristics and mutations in ctDNA after chemotherapy.

Discussion

In clinical practice, the diagnosis and evaluation for breast cancer are based on tissue biopsy with immunohistochemical and cytogenetic tests, but it is invasive and difficult to perform multiple sampling[14, 15]. Carcinoembryonic antigen (CEA) and cancer antigen 15 − 3 (CA15-3) are also used extensively as predictive markers. However, it has been demonstrated that only 7.2% and 12.3% of patients exhibited significantly elevated serum CEA and CA15-3 levels, respectively, among Chinese women[16]. Therefore, there is a need for more convenient and specific genetic markers of breast cancer. As ctDNA is double-stranded nucleic acid shed by tumor cells into the circulating blood, it should contain all of the genetic information present in tumor tissue, and could capture both spatial and temporal heterogeneity of tumors[17, 18]. The application of ctDNA-based real-time liquid biopsy represents a noninvasive and highly sensitive biomarker for cancer diagnosis, early prediction, and therapeutic response assessment[7, 19–22]. Although there have been many studies regarding ctDNA and breast cancer, some challenges and limitations remain for its clinical implementation, including the identification of specific driver mutations for the multiple demands of breast cancer management, the high costs of detecting the low proportion of ctDNA in the “sea” of normal DNA, and the development of standardized methods for data processing[12, 23, 24].The development of NGS and bioinformatics has provided new opportunities for the establishment and validation of specific panels of mutation biomarkers for routine clinical use in breast cancer management[25, 26].

The prevalence of mutations in ctDNA was strikingly similar to matched tumor DNA, and they were more often detected in patients with advanced or metastatic disease[27]. However, most studies to date focused only on a limited repertoire of genes. They also varied in the quality of samples and sequencing data[28]. In this study, we performed target-capture NGS, which is more sensitive and specific than traditional sequencing, to detect and evaluate the ctDNA in MBC patients. In our cohort, 1182 nonsynonymous mutations in 419 genes were identified. More attention should be paid to some frequently mutated genes, including *FRG1*, *AQP7*, *DNAJC11*, and *MUC16*, in future studies. Mutations were detected in genes closely associated with cancer development, progression, or therapeutic response.

Mutations in ctDNA could reflect intratumoral heterogeneity and disease processes[29, 30]. The mutation burden was therefore associated with clinical parameters, representing the phenotype of cancer development[27, 31]. We hypothesized that patients with “risk” clinical factors for breast cancer should harbor more mutations[32, 33]. As predicted, our statistical analysis showed that an elevated ctDNA mutation burden was positively correlated with large primary tumor size, HER2(−) status, and poor survival outcome. These were all detrimental to cancer management. In addition, this study suggests that

ctDNA mutations could be used as potential biomarkers for the evaluation and prediction of MBC to guide clinical management. Mutations in baseline ctDNA were more sensitive and specific than those after chemotherapy.

The remarkable advantages of this study are the use of a custom-designed panel with broad coverage, standardized sequencing with high depth, sampling at two time points, and parallel sequencing to eliminate the interference of CH variants. However, this study also has some limitations. First, the sample size was small due to the lack of willingness among MBC patients to supply adequate amounts of blood. Some patients did not agree to participate in genomic testing even though it was provided without cost. Another limitation was the lack of paired tumor tissue samples, which could have been used to verify the mutations in ctDNA. These limitations restricted further analysis. Despite these limitations, our study contributes to the identification of MBC-related mutations and strengthens the evidence for the clinical applicability of ctDNA detection. Subsequent studies with larger numbers of participants and more complete information are already in progress in our laboratory.

Conclusions

Mutations in ctDNA were successfully detected in MBC patients by targeted NGS. The mutation burden was significantly associated with clinical factors such as age at diagnosis, tumor size, hormone receptor status, therapeutic effect, and survival. The results suggest that ctDNA could be used to predict the progression and treatment outcomes of MBC.

Methods

Study subjects and blood collection

Patients with newly pathologically diagnosed MBC receiving consecutive gemcitabine and capecitabine treatment at Nanfang Hospital between February 2017 and October 2019 were enrolled in the study. The exclusion criteria were a previous cancer diagnosis within the last 5 years and an inability to provide adequate blood samples for NGS or undergo medical follow-up. A total of 27 Han Chinese women were included in the study population. Follow-up was performed to estimate the association between ctDNA profile and prognosis. Clinical data were obtained from the medical records and follow-up results. Therapeutic effects and prognosis were evaluated according to Response Evaluation Criteria in Solid Tumors guidelines.

Peripheral blood samples were collected in 10-mL Streck tubes at two time points: before the initiation of chemotherapy and the end of the third course of treatment (63 ± 6days). Samples were shipped at 4–8°C to the laboratory and processed within 3 h. Plasma and lymphocytes were isolated by centrifugation (1600 × *g*, 4°C for 10min,and then 16000 × *g*, 4°C for 10min) immediately and stored at − 80°C.

DNA extraction and assessment

The cfDNA and gDNA were extracted from plasma and lymphocytes, respectively, using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA, USA) and QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. The DNA concentration was quantified using a Qubit 2.0 Fluorometer with Qubit dsDNA High Sensitivity (HS) Assay Kit (Fisher Scientific, Newark, DE, USA). An Agilent 2100 Bioanalyzer and DNA HS Kit (Agilent Technologies, Santa Clara, CA, USA) were used to assess the fragment length distribution. Only samples containing > 20ng of cfDNA and > 500ng of gDNA were processed for further analysis. All 81 DNA samples (54 cfDNA and 27 gDNA) were prepared and stored at − 20°C before library construction.

Gene panel design and target-capture sequencing

In this study, a personalized target panel was designed according to NCBI Build 37.1/GRCh37. Genomic exons that are frequently mutated in breast cancer or related to chemotherapy, immunotherapy, and targeted drugs were identified by searching the COSMIC database, The Cancer Genome Atlas, and published studies. An iterative algorithm was applied to maximize the number of mutations per patient while minimizing the region size. Target panel selection involved tradeoffs between sequencing cost, sensitivity, and coverage. Target-capture hybrid probes were custom designed through the NimbleGen Design portal (v1.2.R1, Roche, Basel, Switzerland).

DNA libraries were prepared with a KAPA HyperPrep Kit (Kapa Biosystems, Woburn, MA, USA), including end repair and A-tailing, adapter ligation, post-ligation cleanup, library amplification, and post-amplification cleanup. The DNA polymerase displayed strong 3'→5' exonuclease activity and a low error rate. Agencourt AMPure XP beads (Beckman Coulter, Fullerton, CA, USA) were employed for "with-bead" enzymatic and cleanup steps. Aliquots of 20–30 ng of plasma DNA were used directly for library construction. For gDNA, 500–1000ng DNA was sheared with a Covaris S2 instrument (Boston, MA, USA) set for 200-bp fragments and then used for library construction.

Groups of four libraries were incorporated in a single capture pool. Hybridization and target capture were performed with custom probes from the SeqCap EZ Choice Library (Roche). The captured DNA products were amplified and purified with NimbleGen SeqCap Kits (Roche). After quality control of the DNA concentration and fragment distribution, we applied targeted NGS using 2 × 75bp or 2 × 150bp paired-end reads on the MiSeq Sequencing system (Illumina, San Diego, CA, USA) in accordance with the manufacturer's recommendations.

Data processing and statistical analysis

Software fastQC (version 0.11.9) was used for sequencing data quality control. Low-quality data, including N bases≥ 50%, proportion of bases with a Phred quality score≤ 15 above 80%, or read length≤ 30bp, were excluded. The terminal adaptor sequences were removed with cutadapt (version 3.0). The reads were then mapped to the hg19 reference human genome using Burrows-Wheeler Aligner (BWA, version 0.7.17). Genome Analysis Toolkit (GATK, version 4.1.6), Picard (version 2.22.3), and SAMtools (version 1.10) were used to call small insertions/deletions (indels) and mutations according to the COSMIC and dbSNP databases. Annovar (2020Apr01) was used for annotation with multiple databases.

Variations were filtered out if they had low depth< 2000×in cfDNA or< 1000×in gDNA, they were supported by less than five high-quality sequencing reads for cfDNA and two high-quality reads for gDNA, or if they were synonymous variants, including SNVs and indels. Variants detected in the plasma DNA that were wild type in the gDNA were considered ctDNA mutations and included for statistical analysis.

GO and KEGG enrichment analyses of the mutated genes were performed using topGO_2.40.0 (clusterProfiler_3.16.1). The associations between ctDNA mutations and clinical characteristics were analyzed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). The endpoint of clinical observation for the survival analysis was the last follow-up (Oct.31, 2019) or death. A progression-free survival (PFS) analysis was estimated by the Kaplan–Meier method using survival_3.2-7(survminer_0.4.9). Survival curves with a hazard ratio (HR) and 95% confidence interval (CI) were plotted with ggplot2_3.3.3 (circlize_0.4.12). The tests were two-sided, and $P < 0.05$ was taken to indicate statistical significance.

Abbreviations

MBC: Metastatic breast cancer; ctDNA: Circulating tumor DNA; cfDNA: Cell-free DNA; gDNA: Genomic DNA; NGS: Next-generation sequencing; PR: Partial remission; SD: Stable disease; PD: Progressive disease; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; SNVs: single-nucleotide variants.

Declarations

Ethics approval and consent to participate

This observational study was approved by the ethical committee of Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong Province, China.

Consent for publication

Not applicable

Availability of data and materials

The data of this study are available from the corresponding authors upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SM, YX and LM designed the study. CL enrolled the patients and performed follow-up. SM and LH performed the NGS and collected all the data. LF and LW analysed and interpreted the data. SM drafted the manuscript. DH revised the manuscript. YX and LM oversaw the work. All authors have read and approved the final manuscript.

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Authors' information

[1] Institute of Antibody Engineering, School of Laboratory Medicine and Biotechnology, Southern Medical University, Guangzhou 510515, China. [2] GMU-GIBH Joint School of Life Science, Guangzhou Medical University, Guangzhou, 511436, China. [3] Nanfang Hospital, Southern Medical University, Guangzhou, 510515, China. [4] Department of Primary Public Health, Guangzhou Center for Disease Control and Prevention, Guangzhou 510440, China. [5] Institute of Public Health, Guangzhou Medical University & Guangzhou Center for Disease Control and Prevention, Guangzhou 510440, China.

References

1. Fahad Ullah M. Breast Cancer: Current Perspectives on the Disease Status. Adv Exp Med Biol 2019; 1152: 51-64.
2. Peart O. Metastatic Breast Cancer. Radiol Technol 2017; 88: 519M-539M.
3. Welch HG, Gorski DH, Albertsen PC. Trends in Metastatic Breast and Prostate Cancer--Lessons in Cancer Dynamics. N Engl J Med 2015; 373: 1685-1687.
4. Liang Y, Zhang H, Song X, Yang Q. Metastatic heterogeneity of breast cancer: Molecular mechanism and potential therapeutic targets. Semin Cancer Biol 2020; 60: 14-27.
5. Tang W, Guo X, Niu L et al. Identification of key molecular targets that correlate with breast cancer through bioinformatic methods. J Gene Med 2020; 22: e3141.
6. Winters S, Martin C, Murphy D, Shokar NK. Breast Cancer Epidemiology, Prevention, and Screening. Prog Mol Biol Transl Sci 2017; 151: 1-32.
7. Wang R, Li X, Zhang H et al. Cell-free circulating tumor DNA analysis for breast cancer and its clinical utilization as a biomarker. Oncotarget 2017; 8: 75742-75755.
8. Cheng F, Su L, Qian C. Circulating tumor DNA: a promising biomarker in the liquid biopsy of cancer. Oncotarget 2016; 7: 48832-48841.

9. Campos-Carrillo A, Weitzel JN, Sahoo P et al. Circulating tumor DNA as an early cancer detection tool. Pharmacol Ther 2020; 207: 107458.
10. Oliveira KCS, Ramos IB, Silva JMC et al. Current Perspectives on Circulating Tumor DNA, Precision Medicine, and Personalized Clinical Management of Cancer. Mol Cancer Res 2020; 18: 517-528.
11. Rohanizadegan M. Analysis of circulating tumor DNA in breast cancer as a diagnostic and prognostic biomarker. Cancer Genet 2018; 228-229: 159-168.
12. Arnedos M, Vicier C, Loi S et al. Precision medicine for metastatic breast cancer–limitations and solutions. Nat Rev Clin Oncol 2015; 12: 693-704.
13. Buono G, Gerratana L, Bulfoni M et al. Circulating tumor DNA analysis in breast cancer: Is it ready for prime-time? Cancer Treat Rev 2019; 73: 73-83.
14. Budny A, Staroslawska E, Budny B et al. [Epidemiology and diagnosis of breast cancer]. Pol Merkur Lekarski 2019; 46: 195-204.
15. Reiland-Smith J. Diagnosis and surgical treatment of breast cancer. S D Med 2010; Spec No: 31-37.
16. Garcia-Murillas I, Schiavon G, Weigelt B et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci Transl Med 2015; 7: 302ra133.
17. Pessoa LS, Heringer M, Ferrer VP. ctDNA as a cancer biomarker: A broad overview. Crit Rev Oncol Hematol 2020; 155: 103109.
18. Alimirzaie S, Bagherzadeh M, Akbari MR. Liquid biopsy in breast cancer: A comprehensive review. Clin Genet 2019; 95: 643-660.
19. Zhang X, Ju S, Wang X, Cong H. Advances in liquid biopsy using circulating tumor cells and circulating cell-free tumor DNA for detection and monitoring of breast cancer. Clin Exp Med 2019; 19: 271-279.
20. Butler TM, Spellman PT, Gray J. Circulating-tumor DNA as an early detection and diagnostic tool. Curr Opin Genet Dev 2017; 42: 14-21.
21. Zhang X, Zhao W, Wei W et al. Parallel Analyses of Somatic Mutations in Plasma Circulating Tumor DNA (ctDNA) and Matched Tumor Tissues in Early-Stage Breast Cancer. Clin Cancer Res 2019; 25: 6546-6553.
22. Ribeiro IP, de Melo JB, Carreira IM. Head and neck cancer: searching for genomic and epigenetic biomarkers in body fluids - the state of art. Mol Cytogenet 2019; 12: 33.
23. Eigeliene N, Saarenheimo J, Jekunen A. Potential of Liquid Biopsies for Breast Cancer Screening, Diagnosis, and Response to Treatment. Oncology 2019; 96: 115-124.
24. Nicolini A, Ferrari P, Duffy MJ. Prognostic and predictive biomarkers in breast cancer: Past, present and future. Semin Cancer Biol 2018; 52: 56-73.
25. Clatot F. Review ctDNA and Breast Cancer. Recent Results Cancer Res 2020; 215: 231-252.
26. Tzanikou E, Lianidou E. The potential of ctDNA analysis in breast cancer. Crit Rev Clin Lab Sci 2020; 57: 54-72.

27. Zhou Y, Xu Y, Gong Y et al. Clinical factors associated with circulating tumor DNA (ctDNA) in primary breast cancer. Mol Oncol 2019; 13: 1033-1046.
28. Gorgannezhad L, Umer M, Islam MN et al. Circulating tumor DNA and liquid biopsy: opportunities, challenges, and recent advances in detection technologies. Lab Chip 2018; 18: 1174-1196.
29. Ma F, Guan Y, Yi Z et al. Assessing tumor heterogeneity using ctDNA to predict and monitor therapeutic response in metastatic breast cancer. Int J Cancer 2020; 146: 1359-1368.
30. Romero D. Breast cancer: Tracking ctDNA to evaluate relapse risk. Nat Rev Clin Oncol 2015; 12: 624.
31. Zhang Y, Yao Y, Xu Y et al. Pan-cancer circulating tumor DNA detection in over 10,000 Chinese patients. Nat Commun 2021; 12: 11.
32. Fernandez-Garcia D, Hills A, Page K et al. Plasma cell-free DNA (cfDNA) as a predictive and prognostic marker in patients with metastatic breast cancer. Breast Cancer Res 2019; 21: 149.
33. Cullinane C, Fleming C, O'Leary DP et al. Association of Circulating Tumor DNA With Disease-Free Survival in Breast Cancer: A Systematic Review and Meta-analysis. JAMA Netw Open 2020; 3: e2026921.

Figures

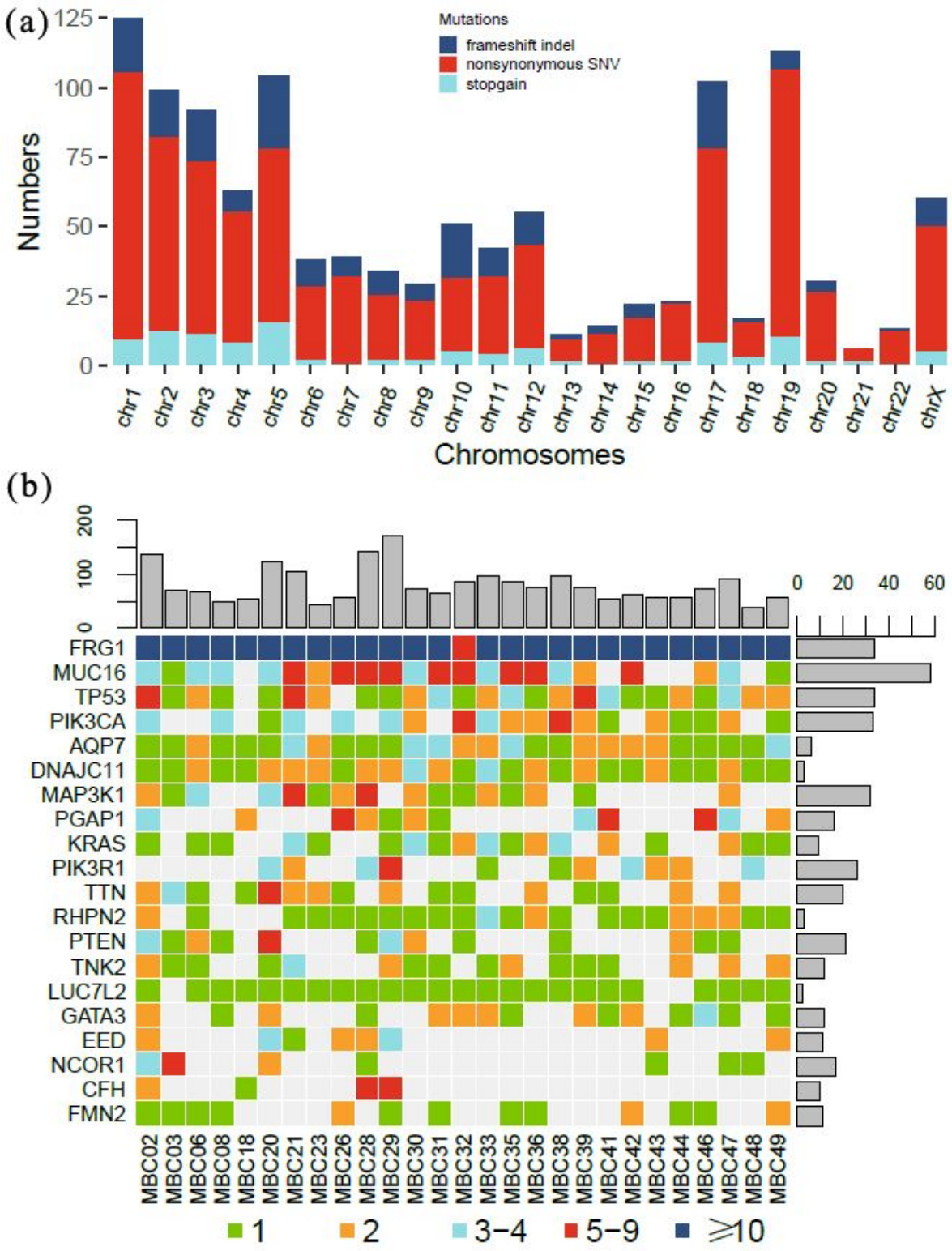

Figure 1

The profiles of ctDNA mutations identified. (a) Distribution of ctDNA mutations on all chromosomes. The height of each column represents the number of mutations. (b) Mutation burden of ctDNA in the top 20 genes for 27 MBC patients. The number of mutations in each gene and patients are listed to the right and top respectively.

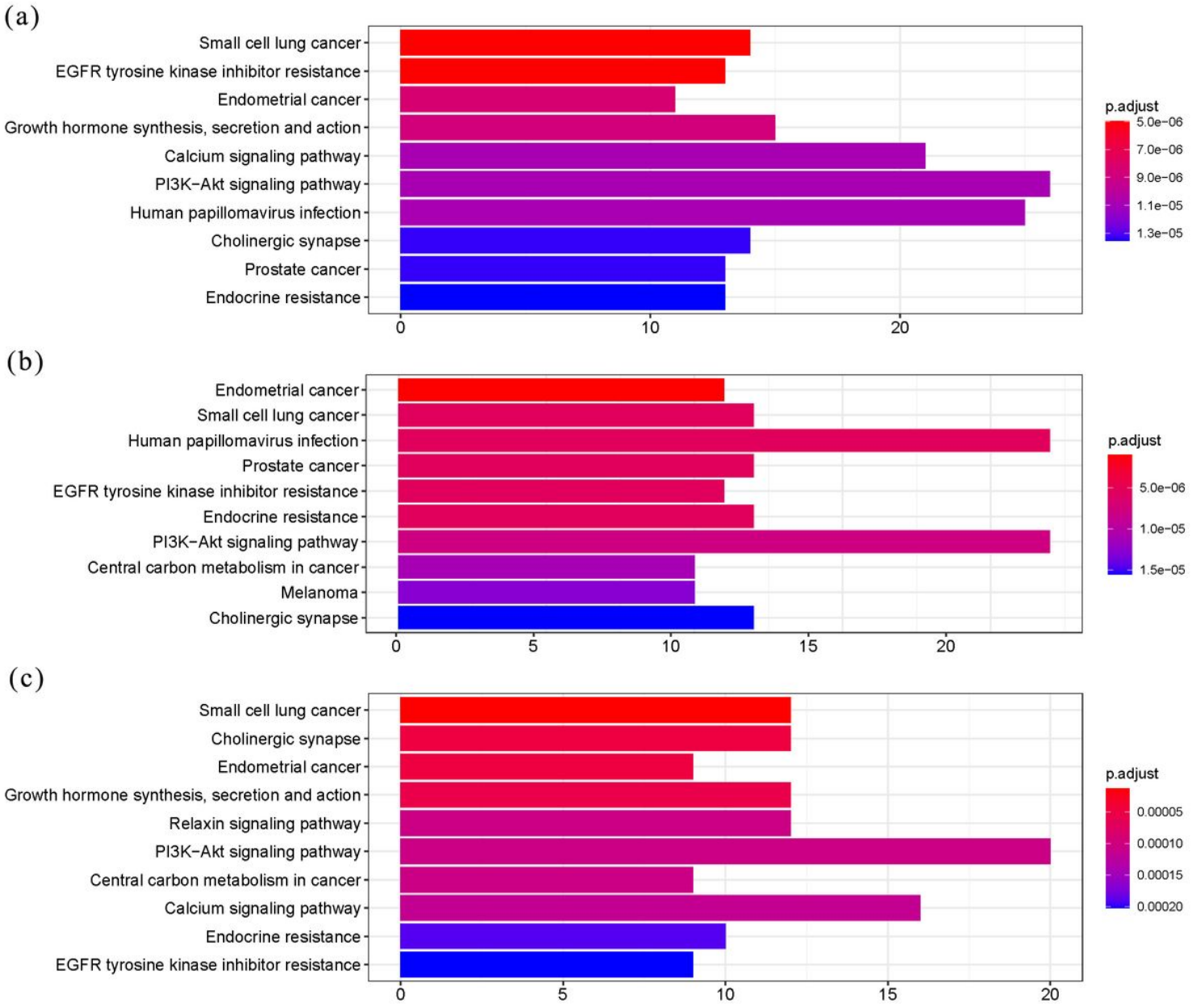

Figure 2

KEGG analyses for the mutated genes from three subsets. (a) Enriched pathways for overall mutated genes. (b) Enriched pathways for baseline mutated genes. (c) Enriched pathways for mutated genes after chemotherapy. The top 10 pathways were shown. The length of each column indicates the number of enriched genes, and the colour of bars rep-resents statistical significance.

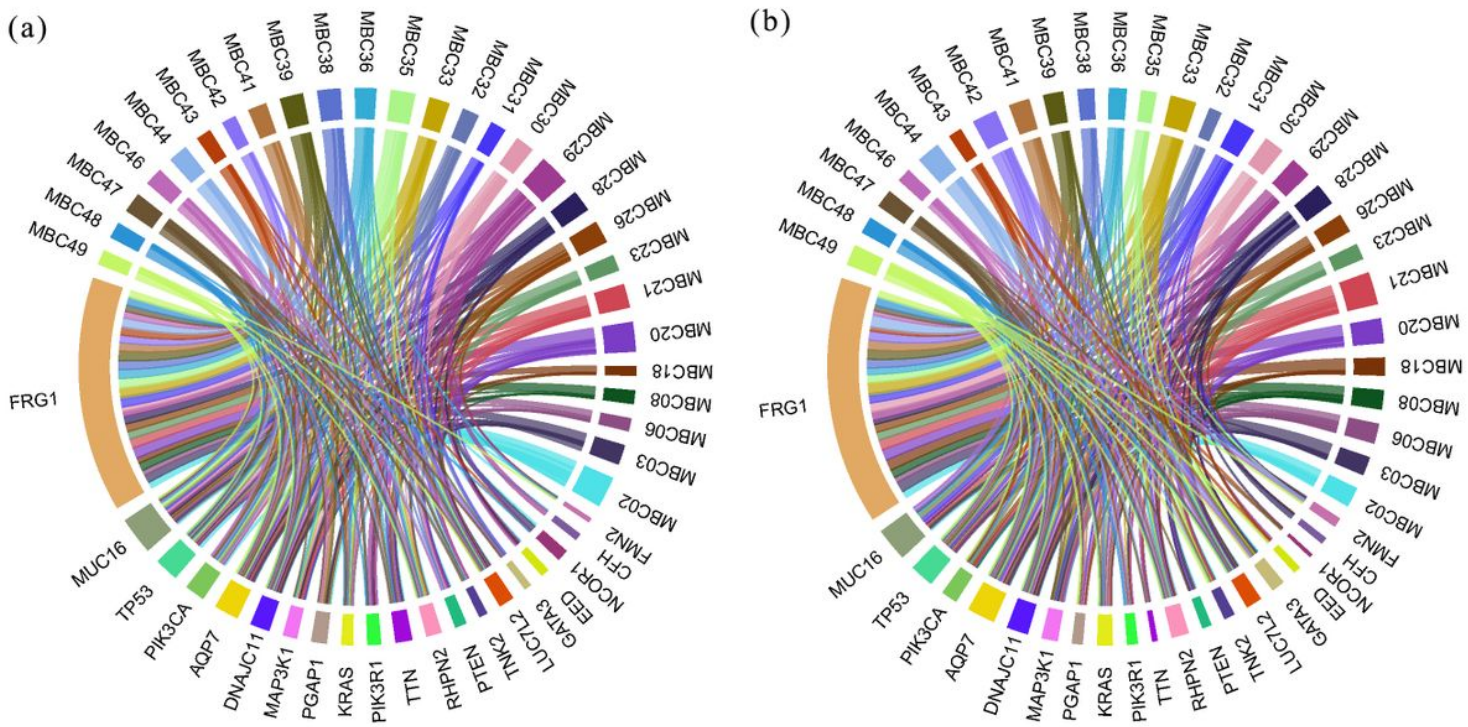

Figure 3

The distribution of ctDNA mutations before and after chemotherapy for all patients. The top 20 genes were shown. (a) ctDNA mutations at baseline. (b) ctDNA mutations after chemotherapy. The width of each part represents the number of mutations detected in corresponding patients or genes.

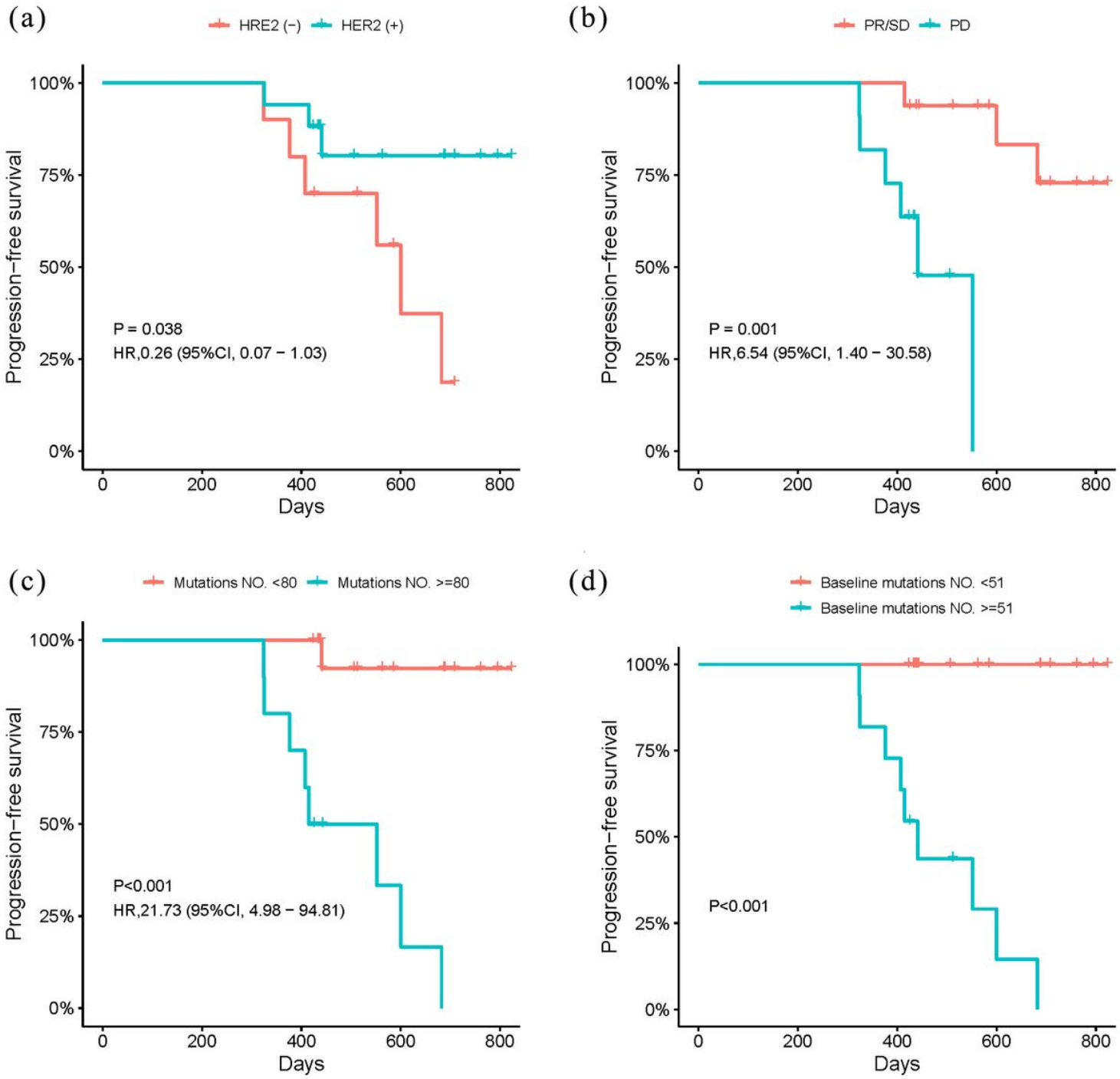

Figure 4

Kaplan–Meier estimate of PFS. (a) Kaplan-Meier curves for HER2 status. (b) Kaplan-Meier curves for therapeutic effect. (c) Kaplan-Meier curves for mutations in overall ctDNA. (d) Kaplan-Meier curves for mutations in base-line ctDNA.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterialsSunmy2021.5.doc