Title: Clinical application of circulating tumor DNA (ctDNA) to monitor the molecular tumor burden in patients with late-stage breast cancer

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Contents

1. Background

Breast cancer is one of the most common malignant tumors, and the second major cause of female cancer death. The development of breast cancer treatment has made more and more new drugs emerge and used in clinical practice. According to different patients, the strategy of classified treatment and individual treatment should be given to patients with specific conditions. It can guarantee the clinical benefits of patients to the maximum extent and embody the principle of rational medication.

Circulating tumor DNA (ctDNA) refers to the endogenous DNA of the body which is partially degraded by the part of the circulating blood dissociated from the outside of the cell. Its source is mainly the necrosis, apoptosis and secretion process of tumor cells (Crowley, et al. 2013). CtDNA is common in peripheral plasma of different tumor patients. Many studies have proved that detection of ctDNA mutation level has great potential for early diagnosis, drug guidance, drug efficacy prediction and recurrence monitoring of cancer. In terms of monitoring breast cancer treatment, Dawson et al. (2013) compared the monitoring data of ctDNA, CA 15-3, CTC and imaging in 30 patients with malignant breast cancer during the comprehensive treatment. The results showed that ctDNA has high sensitivity and specificity in monitoring the process of tumor load. Garcia murillas et al. (2015) monitored the plasma ctDNA of patients with early breast cancer receiving neoadjuvant chemotherapy. The results showed that ctDNA could detect small residues and could prompt the recurrence of molecular level 7.9 months before clinical recurrence. Murtaza et al. (2015) conducted three years of ctDNA monitoring in a patient with metastatic breast cancer. The results showed that plasma ctDNA matched the mutation spectrum of biopsy samples, which could reflect the changes of molecular characteristics in tumor development and response to drug treatment. In the absence of multiple tissue biopsies, liquid biopsy can be the second choice for biopsy.

The occurrence and development of tumor are often accompanied by the production of new mutation and accumulation of original mutation. The evolutionary point of view is that the earlier mutation is located near the trunk in the phylogenetic tree, and the later mutation is located in the branch (**Figure. S2A**). It has been shown that metastasis and chemoresistance of breast cancer are accompanied by the changes of subclonal structure of tumor (Yates, et al. 2015). Generally, tissue biopsy is easier to detect regional subclonal groups (**Figure. S2B**), while plasma ctDNA covers DNA fragments from different clones of tumor, and can also reflect the dynamic changes of tumor clone composition at different time nodes (Swanton &govindan, 2016). It has unique advantages in the research of tumor clone evolution and drug resistance cloning development.

2. Research purpose

- to evaluate the feasibility of plasma ctDNA mutation spectrum and clonal spectrum in guiding late-line treatment and in evaluating the prognosis of late-stage metastatic breast cancer patients.
- to establish a model of ctDNA subtyping system to evaluate the molecular load of tumor, to evaluate the curative effect comprehensively and to detect the disease progression in advance.

- to explore the clonal evolution of ctDNA in patients with progressive disease (PD), and to monitor the changes of tumor heterogeneity from the molecular level, so as to provide reference for the analysis of drug sensitivity.

3. Research Route (**Figure S3**)

4. Primary endpoint

- Progression free survival (PFS) was analyzed by Logrank test and Cox regression analysis.
- the dynamic change of the frequency spectrum of ctDNA mutation in plasma
- the dynamic changes of molecular tumor load of plasma ctDNA.
- the dynamic changes of molecular cloning of plasma ctDNA.

6. Sample size

- Until June 2019, ensure that totally more 200 patients were enrolled.
- Until June 2019, ensure the number of trial cases (enrolled in ctDNA-based treatment group) to exceed 100.

7. Inclusion criteria

- Recent progression of TNBC after multiple lines of chemotherapy or of HR+ or HER2+ MBC after multiple lines of endocrine or targeted therapy;
- No available recommendation for the next treatment regimen;
- An Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2; and
- An updated, available pathological HR/HER2 status for metastasis;
- According to RECIST 1.1 standard, there should be at least one measurable target lesion;
- The expected survival time is > 3 months;
- Those aged 18-70 years old;
- Liver and kidney function and blood routine test meet the following conditions: Neutrophil >

2.0g/l, Hb > 9g / L, PLT > 100g / L; ALT and AST < 2.5ULN; TBIL < 1.5ULN; Cr < 1.0ULN

- Signing informed consent;
- Those willing to accept polygenic testing.

8. Exclusion criteria

- Patients with multiple primary tumors;
- Those who are unable to obtain blood samples;
- Those with a history of immunodeficiency or organ transplantation;
- Those with abnormal cardiac function or previous history of myocardial infarction or serious arrhythmia;
- The researchers think it is not suitable to participate in this experiment.

9. Detailed operation procedures

- According to the inclusion criteria, informed consent was signed and patients were enrolled.
- According to the specific sampling scheme (**Figure S4**), the samples were collected and the case report form was recorded. 8 ml of peripheral blood samples were taken from cell free DNA BCT Streck tube.
- The clinical information of the enrolled patients was recorded, the target lesion size was recorded according to RECIST 1.1 standard, and 8 ml of peripheral blood sample was collected before treatment; after two medication cycles, the curative effect was evaluated according to RECIST 1.1 standard, and peripheral blood sample 8 was collected for the second time After two cycles of treatment, 8 ml of peripheral blood samples were collected for the third time according to the efficacy; if it was necessary to continue medication, 8 ml peripheral blood samples were collected for the fourth time when the disease progressed mL.
- DNA extraction, database construction and capture (**Figure S5**). Plasma and peripheral lymphocytes (as control samples) were separated from peripheral blood collected from each node. DNA of plasma and lymphocytes were extracted. After the quality control was qualified, the databases were established respectively. Self designed chip was used for capture and sequencing. The chip region integrates the hot exon regions of tumor drug-related genes, reported proto oncogenes, tumor suppressor genes, and genes with high mutation frequency. For reported clinically related CNV, all exons of the gene are added to the chip region. Only the fusion region was designed into the chip.

- Sample quality control and sequencing: 2100 quality control and qPCR quantitative quality control were carried out on the hybrid samples. After the quality control was qualified, Illumina hiseq 3000 was used for double terminal sequencing of the mixed library.

- Data analysis: the low-quality sequences and splice sequences were excluded from the sequencing data, and the mutations were detected by independent analysis process. The mutation types included single base mutation (SNV), small indel (small indel), copy number variation (CNV) and other important structural variations (SV). Within group and inter group mutation spectrum and clone spectrum were analyzed under each treatment mode.

- Data statistics: Logrank and Cox regression test were used for PFS analysis; double tailed t test was used for difference analysis; Pearson correlation analysis was used for correlation analysis. SAS 9.4 and graphpad prism software will be used for the above statistical analysis. The significance level used is 0.05, and the confidence interval is 95% confidence.

10. Basis of the existing work

(1) High precision detection of ctDNA low frequency mutation with ER-SEQ patent technology.

In view of the fact that liquid ctDNA only has a very small amount in total plasma free DNA, a high-precision low-frequency variation analysis technique (ER-SEQ) was developed. In this method, the positive and negative strands of DNA double strand were independently labeled by random barcode connector (**Figure S6**). After PCR amplification and double terminal sequencing, barcode clustering was used to distinguish PCR introduction error or sequencing error from real mutation, so as to realize the high-precision detection of 1% or even 1 ‰ frequency variation (**Figure S7**).

(2) ctDNA m clone analysis model is used to analyze the clonal structure of tumor.

Tumors are spatiotemporal heterogeneity, that is, tumors are composed of clonal populations with different mutations in different proportions. The analysis of tumor clonal composition is of great theoretical value for the study of tumor origin and evolution. It can also provide clues for the clinical research of tumor metastasis, tumor therapy and tumor drug resistance mechanism. M clone analysis strategy is to analyze the main and branch clones on the basis of ctDNA mutation spectrum (**Figure S8**), and further analyze the dynamic changes of subclonal structure of heterogeneous tumors at different sampling nodes with the help of convenient ctDNA sampling.

(3) Real time monitoring of tumor mutation load based on ctDNA clone structure.

In view of the existence of tumor heterogeneity, the mutation load monitoring method based on single mutation hot spot only involves the fluctuation of partial clone population, which cannot clearly reflect the overall load trend, and has limitations in population coverage and sensitivity. ctDNA contains DNA fragments from different tumor clones. Based on the tumor mutation accumulation model, using the clonal structure analysis strategy of mclone, continuous monitoring of main clones can reflect the overall trend of tumor molecular load (**Figure S9**).

11. Ethical approval and informed consent

(1) Ethical considerations and scheme approval

It is the responsibility of the investigator to provide the ethics committee with the clinical trial protocol, detailed patient information pages and copies of informed consent to obtain independent approval documents for the implementation of the clinical study.

The approval document of the ethics committee must be obtained before clinical study. The approval document of the ethics committee shall be sent to the researcher in writing, and then the researcher shall provide a copy of the approval document to the study sponsor. The approval document of the ethics committee shall be accompanied by a list of all members of the Committee participating in the discussion of the approval document and their respective responsibilities.

In the process of clinical research, any problems related to the safety of clinical research, such as the changes of clinical research protocol or patient information page, and serious adverse events in clinical research, must be reported to the ethics committee in time. The end or early termination of a clinical study must also be reported to the ethics committee.

(2) Informed consent process and informed consent form.

The details of the study, including the purpose, method and process of the study, were provided to the subjects in the language and words that the subjects or their legal representatives / guardians could understand. We make it clear to the subjects that their personal data are confidential, and they have the right to withdraw at any stage of the study without discrimination or retaliation, and their medical treatment and rights will not be affected. After obtaining the signature of the subjects or his legal representative / guardian and indicating the date, the clinical study can be carried out. The informed consent signed and dated by the clinical trial subjects must be kept by the researchers. The content of informed consent is detailed in informed consent.

12.Reference

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