Whole Genome Sequencing in single CTC improves clinical outcome in Her-2 negative breast cancer patients

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Research article

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Abstract

Background Tumor tissues are usually highly heterogeneous and difficult to characterize which could mislead treatment strategy. Circulating tumor cells (CTCs) represent the most active and invasive tumor cells. This study explored the feasibility of individualized treatment of breast cancer patients based on genome sequencing of single cell CTC.

Methods Twenty-four CTCs were identified in three patients with breast cancer. For each patient, one polyploid CTC was captured and on which the whole genome sequencing (WGS) was performed. Based on the histopathological Her-2 status in tumor tissue and the HER2 gene status in WGS results of CTC, we adjusted treatment strategies, and monitored disease progression.

Results Patient ID1 and ID2 are Her-2 positive in both primary tumor and HER2 abnormal in the DNA of CTC. In patient ID3, histological examination of primary tumor and liver metastases revealed Her-2 negative, but the WGS analysis of CTC showed that the HER2 gene was amplified and mutated. After adjusting treatment according to the results of CTC sequencing, the liver metastases and pleural effusion were significantly reduced, CTC number and ctDNA burden were decreased. In addition, some potential therapeutic targets and mutations in drug-resistant genes were found.

Conclusion The results of CTC sequencing effectively guided treatment of a patient with HER2 gene amplification/mutation in CTC but with Her-2 negative on tumor tissue. CTC sequencing is useful in resolving the heterogeneity of tumors and providing precision medicine for patients.

Background

Breast cancer is the most common malignant tumors of women in the world and remains the second leading cause of tumor mortality in women. The latest global and Chinese cancer statistics show that the incidence of breast cancer in women is still ranked the first, and the morbidity trend is increasing[1–4]. The median survival time of advanced breast cancer from diagnosis is 2–3 years. Only about 5–10% of patients can survive for more than 5 years[5].

Treatment of breast cancer mainly includes surgery, radiotherapy, chemotherapy, endocrine therapy, immunization therapy and targeted therapy. According to the tumor status of the patients, various treatments are combined to formulate an effective comprehensive treatment program[6]. Targeted therapy for patients who are Her-2 positive by immunohistochemistry (IHC) is an internationally accepted method. Currently available HER2-targeting drugs have vastly improved outcomes in patients with HER2/Her-2 positive status [7]. Accurate and sensitive detection of this targeted factor plays a critical role in screening patients for targeted treatment. However, the IHC/FISH technology, which is mainly used to assess the Her-2 status on tissue, cannot reveal the real gene status in tumors of the patient due to the potentially inconsistent HER2 gene status between primary and metastatic tumors.

Liquid biopsy is a newly developed non-invasive technique for molecular pathological detection of tumors[8]. Circulating tumor cells (CTCs), a liquid biopsy specimen, are highly active tumor cells that detach from solid tumors and enter the peripheral blood. It has been confirmed that CTCs derived from highly invasive tumors and are associated with tumor metastasis[9]. Lots of previous studies have shown that CTCs are significantly correlated with the prognosis and survival of multiple solid tumors, such as metastatic breast cancer[10], prostate cancer[11], and colorectal cancer[12]. Emerging studies also showed that CTC can be a potential biomarker for patient stratification and targeted therapy selection[13]. It is difficult to obtain multiple tumor tissue samples in advanced and metastasis cancer. In this situation, CTCs could be an ideal source of cancer cells for genomic analysis. Tumor tissue sample analysis is usually unable to detect all the genetic variants present in the heterogeneous tumors such as breast cancer[14]. Evidence has pointed out that molecular profiling of CTCs provides a better representation of tumor diversity than a single biopsy[15].

Multiple gene variations in single CTC can be investigated through whole genome sequencing (WGS) based on the next generation sequencing (NGS) technology [16, 17]. With the development of CTC isolation and NGS technology, our understanding of intra-tumor heterogeneity has significantly improved, and the individualized treatment and precise treatment of tumors are further advanced.

In this study, CTC single cell sequencing was performed and the WGS data of CTCs was compared with tumor pathologic results of 3 patients with breast cancer. We evaluated the tumor heterogeneity, optimized the treatment decision of these patients and achieved significant clinical results.

Methods

Patients and sample collection

Patients enrolled in this study were diagnosed at Shanghai Pudong Hospital. Our study was approved by the Ethics Committee Shanghai Pudong Hospital (No. W2001). Written informed consent were obtained to allow the sample collection and data analysis for research purposes. For CTC analysis, 7.5 ml peripheral blood was drawn from patients into ACD anti-coagulant tube. For ctDNA analysis, another 10 ml peripheral blood was collected into an EDTA anti-coagulant tube. Clinical characterizations of these patients were shown in supplementary table1.

Investigation of CTCs

Circulating tumor cell (CTC) detection was carried out by CTC detection kit (Majorbio, China). Briefly, this method combines subtraction enrichment, immunofluorescence staining and chromosome in situ hybridization to achieve the separation and identification of CTC [18]. Firstly, plasma was removed by centrifugation, and red blood cells were removed by density gradient centrifugation. White blood cells were specifically removed by antibody-coated magnetic beads, and remaining cells including CTCs were coated on a slide for subsequent cell identification. Secondly, cells on the slide were stained with multi-tumor immunofluorescence markers CD45 and Her-2. The centromere of chromosome 8 (CEP8) was

detected by fluorescence in situ hybridization. Finally, CTCs were identified according to the criteria of CD45-, DAPI+, and CEP8 ≥ 3. The expression of Her-2 on the surface of CTC was also recorded.

Whole genome amplification and sequencing of single CTC

For each patient, a single CTC was collected into a tube by microdissection performed on a PALM MicroBeam instrument (Zeiss) for subsequent CTC single cell whole genome amplification (WGA). Single-cell WGA was performed using the single-cell whole genome amplification kit (Yikon Genomics, China) which is based on the MALBAC method [19]. The amplified DNA product was assessed by a Qubit® dsDNA HS Assay kit in a Qubit 3.0 Fluorometer (Life Technologies, USA), evaluated the molecular weight on 1% agarose gel electrophoresis, and checked the genomic integrity of the amplified DNA product by quantitative PCR (qPCR) with eight randomly selected loci was performed. DNA samples with a total mass more than 2 μg, and DNA fragment range 300 bp to 2000 bp were considered to meet the further sequencing criteria.

Library preparation was performed according to the SureSelectXT Illumina Paired-End Sequencing Library protocol. Library quality was assessed by 2100 Bioanalyzer and qPCR with TBS380 picogreen (Invitrogen,USA). Next Seq CN500 High-throughput Sequencer (Illumina) was used for whole-genome sequencing of the captured single cells.

ctDNA analysis of the metastatic patient

In this study, plasma DNA of patient ID3 with metastasis breast cancer was extracted for ctDNA analysis. A total of 67 cancer-related genes including 57 drugs related genes, 2 endocrine therapy related genes, and 9 chemotherapy related genes. Point mutations, small fragment insertions and deletions, copy number variants, and fusions of these genes were detected. Details of the gene panel are shown in Supplementary Table 2.

Results

CTC enumeration of breast cancer patients

A total of 24 CTCs with chromosome aneuploidy were enriched and identified in all three patients (Table 1,Fig. 1). The number of CTCs for patient ID3 was 7 cells/7.5 ml blood, whereas that for patients ID1 and ID2was 10 and 7 cells/7.5 ml blood respectively. All these cells were negative for Her-2 expression in the membrane surface (Table 1). A single CTC for every patient was retrieved by micro dissection for the WGS analysis. It was pentaploid cell that collected from patient ID1 and patient ID3, and tetraploid cell from patient ID2. Paired white blood cells were used as control.

Table 1

CTC number assessed for each patient based on chromosome aneuploidy

Patient ID	Number of singles for CEP8 in CTCs			CTC with Her2 expression	Total
	Triploid	Tetraploid	Pentaploid and above		
1	7	1	2	0	10
2	6	1	0	0	7
3 [bT]	6	0	1	0	7
3[aT]	0	0	0	0	0

[bT]: before treatment; [aT]: after treatment

Somatic mutation and CNV in CTCs

We identified 2,934, 497, and 2,901 mutations in the CTCs of the three patients respectively. About 20% of these mutations were recorded in the COSMIC database (Table 2). Among the drug target genes included in the 57-panel, 2 mutations (HER2 R310Q and TP53 S261C) were identified in patient ID1,none in ID2, and 4 mutations (EGFR R160I, G228D, HER2 A467T and TP53 R248W) were identified in patient ID3. Interestingly, the 57-panel only detected TP53 R248W in patient ID3.

HER2 Amplification was observed in all the 3 patients (Table 2). Besides, CNV gains of other 2 drug-related genes (FGFR1 and FGFR2) were revealed in patient ID3.

Table 2
DNA status in CTCs and ctDNA of patients with breast cancer

Patient ID	DNA of CTCs			ctDNA in patient 3	
	1	2	3	before TM	after TM
Mutation					
Total recorded	2934	497	2901		
Cosmic recorded	583	95	538		
Breast cancer-related	30	8	39		
Chromosome 17	174	35	201		
Drug target genes	2	0	4		
EGFR (ERBB1)	0	0	R160L, G228D	-	-
HER2 (ERBB2)	R310Q	0	A467T	-	-
TP53	S261C	0	R248W	R248W	-
CNV					
ERBB2	3	4	8	-	-
FGFR1	2	2	5	3	-
FGFR2	2	2	7	-	-
TM: target medicine					

Correlation between HER2 status and patients' treatment

Patient ID1 with Her-2 expression in the tumor by IHC (Fig. 2A) showed both amplification and mutation in the HER2 gene according to the WGS analysis of CTC (Table 2). The first diagnosis of nonspecific invasive breast cancer was made in Oct 2017. After a mastectomy, she received chemotherapy combined with molecular targeted therapy TCH (Paclitaxel-Carboplatin-Trastuzumab) every 21 days for 6 cycles. Trastuzumab continued for a year. Endocrine therapy is ongoing. Disease free survival (DFS) reached 18 months after followed up to May 2019. (Fig. 3).

Patient ID2 with Her-2 expression in the tumor by IHC (Fig. 2A) showed both amplification and mutation in HER2 gene in the WGS analysis of CTC. (Table 2) Nonspecific invasive breast cancer was diagnosed in Nov 2017. After a mastectomy, she was treated with anthracycline plus cyclophosphamide (AC) every 21 days for 4 cycles followed docetaxel combined with trastuzumab (TH) for 4 cycles. Trastuzumab

continued for a year. Endocrine therapy is ongoing. Disease free survival (DFS) reached 18 months after followed up to May 2019. (Fig. 3).

Patient ID3 was identified as Her-2 negative in tumor by both IHC and FISH (Fig. 2). However, both amplification and mutation in HER2 gene according to the WGS analysis of CTC was observed in this patient (Table 2). Diagnosis of breast infiltrating ductal cancer was made in Nov 2015. Neoadjuvant chemotherapy with docetaxel, epirubicin and cyclophosphamide (TEC) was performed before the mastectomy and continued adjuvant chemotherapy and radiotherapy after the surgery. After that treatment, the patient was treated with endocrine therapy. In Oct 2017, pleural effusion, peritoneal effusion and liver multiple occupancies were observed in the patient. No cancer cell was found in the pleural effusion, but invasive adenocarcinoma was found in the biopsy of liver tissue, suggesting the liver metastasis. Her-2 negative in the metastasis tissue was confirmed by both IHC and FISH tests. After a treatment of paclitaxel combined with capecitabine, the liver metastases were reduced, and some tumor cells were identified in the pleural effusion. In Mar 2018, pleural effusion increased a lot and trastuzumab was added into the chemotherapy. Two month later, the pleural effusion disappeared, and tumor partial remission was much more obviously observed compared to the effect of chemotherapy only. A 16-month progression-free survival (PFS) was recorded for Patient ID3 until the manuscript was finished (Figs. 3 and 4).

Changes of CTC and ctDNA in patient ID3 before and after the treatment

CTCs and mutations of ctDNA (57 gene panel) were detected at first diagnosis and after the trastuzumab related treatment in patient ID3 (Fig. 4). At the first diagnosis, 7 CTCs/7.5 ml blood were identified, a variation of TP53 R248W and amplification of FGFR1 were observed in the plasma. After the trastuzumab related treatment, neither CTC in blood nor gene alternation in plasma was found in this patient (Table 2, Figs. 3 and 4).

Discussion

With the development of oncology in recent years, diagnosis and treatment of breast cancer have been continuously improved, and the mortality rate shows a downward trend. However, breast cancer is a heterogeneous disease with high incidence, easy to recur and metastasis, which leads to that accurate diagnosis and treatment is still a hot and difficult point of research [20]. The intra-tumor heterogeneity of breast cancer is manifested in spatial and temporal, and individual tumors in one patient have different subpopulations of cancer cells in distant regions [21]. Circulating tumor cells (CTCs) have the same characteristics of primary or metastatic lesions. Some studies [20, 22, 23] have proved that the tumor genome heterogeneity and micrometastase can be found by CTC whole genome sequencing, and it is feasible to conduct tumor drug guidance according to the characteristics of CTC genome. In our study, genomic HER2 characteristics of CTC in Patient ID1 and Patient ID2 were consistent with tissue test results, and targeted therapy was in line with expectations. However, HER2 status of CTC in Patient ID3 is

not consistent with tissue, but targeted therapy is effective, which shows that our results are consistent with the reports.

Key driver mutations such as mutations of TP53 and PIK3CA, amplifications of MYC, CCND1, and HER2 usually take place in primary tumor cells [24, 25]. Some gene alternation presented by WGS analysis of CTC, like CNV gains of HER2 gene in patient ID1 and ID2 which were consistent with the result of IHC detection for tissue, and TP53 R248W in patient ID3 which was consistent with the result of ctDNA detection, are potentially come from the primary tumors. Additional driver mutations or amplifications during the tumor progression may lead to further clonal diversity in primary or metastasis tumors and treatment resistance[26]. Numerous studies have shown that discordance rates of HER2 between primary and recurrent or metastatic tumors is 8–16% respectively [27–29]. In our study, CNV gains of HER2 gene was identified in the DNA of CTC but in primary and liver metastasis tumor, which implied that HER2 CNV gained in another metastatic tumor that is not discovered at that moment. What's more, no HER2 alternation was presented in ctDNA, which implied that the potential metastasis was too tiny to be identified clinically. Although increased pleural effusion suggested pulmonary metastasis, no tumor cell was found in pleural effusion sample. Heterogeneity in tumors poses a severe challenge to the diagnosis and prognosis of diseases. The heterogeneity of breast cancer is the main cause of many treatment failures. For the patient ID3, pleural effusion was still in the state of disease progression after chemotherapy. However, this pleural abnormality was released, enumeration of CTC and the burden of ctDNA in patient ID3 were decreased after the original regimen combined with trastuzumab treatment. Trastuzumab is an effective target drug for HER2-positive breast cancer [30, 31]. Therefore, we speculate the existence of micrometastasis in the lung but not clinically detected. HER2-positive CTCs may be associated with lung metastasis, which ultimately leads to the ineffectiveness of previous chemotherapy.

In the investigation of patient ID3, no abnormal of HER2 was observed in the tumor tissue detected by IHC and FISH, membrane expression checking of CTC, or ctDNA sequencing. Only the WGS analysis of CTC revealed both the CNV gains and mutations of the HER2 gene. HER2 detection on tissues is commonly used as a criterion for targeting therapy in the clinic. However, due to the heterogeneity of tumors and the defects of detection techniques, the detection results are inaccurate. Likewise, the detection of Her-2 protein expression on CTC cell membranes also has problems considering the epithelial-mesenchymal transition [32, 33]. The ctDNA assay is limited by the design of the assay panel, and the tumor signal is diluted and eventually prone to false negative [34, 35]. Previous studies pointed that when traditional tissue biopsies are difficult to obtain, CTCs sequencing may provide an alternative method for comprehensive genome studies to analyze tumor heterogeneity and obtain optimal targets for therapeutic [15, 23], which was consistent with our results.

Conclusions

This is the first time we have discovered an advanced breast cancer patient with a HER2 gene amplification/mutation in the single CTC by WGS but not histologically expressed in the tumor tissue. We also validated the efficacy of patients treated with trastuzumab for HER2 gene mutations in clinical

cases, which was consistent with previous research reports. In view of the small number of cases in our study, the heterogeneous CTC single-cell sequencing of breast cancer needs more investigation. CTC sequencing makes us further understand the heterogeneity of breast cancer, permits the non-invasive and repeated accurate monitoring of therapeutic response and tumor progression, helps us to make therapeutic decisions and predict the outcome, which ultimately achieves personalized molecularly guided cancer treatment.

List Of Abbreviations

Circulating tumor cells (CTCs); whole genome sequencing (WGS); immunohistochemistry (IHC); centromere of chromosome 8 (CEP8); next generation sequencing (NGS); quantitative PCR (qPCR); Disease free survival (DFS); progression-free survival (PFS);

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee Shanghai Pudong Hospital (No. W2001), and all study participants provided written informed consent prior to study enrollment.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Conceptualization, YL and BY; Methodology, YL.; Software, XJ; Validation, MY, HZ. and LW; Formal Analysis, QY; Investigation, HY; Resources, BZ, XX; Data Curation, QY; Writing – Original Draft Preparation, YL; Writing – Review & Editing, BY; Funding Acquisition, YL and LW. All authors reviewed and approved the final manuscript.

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Disclosure of potential conflicts of interest

The authors have no potential conflicts of interest.

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Figures

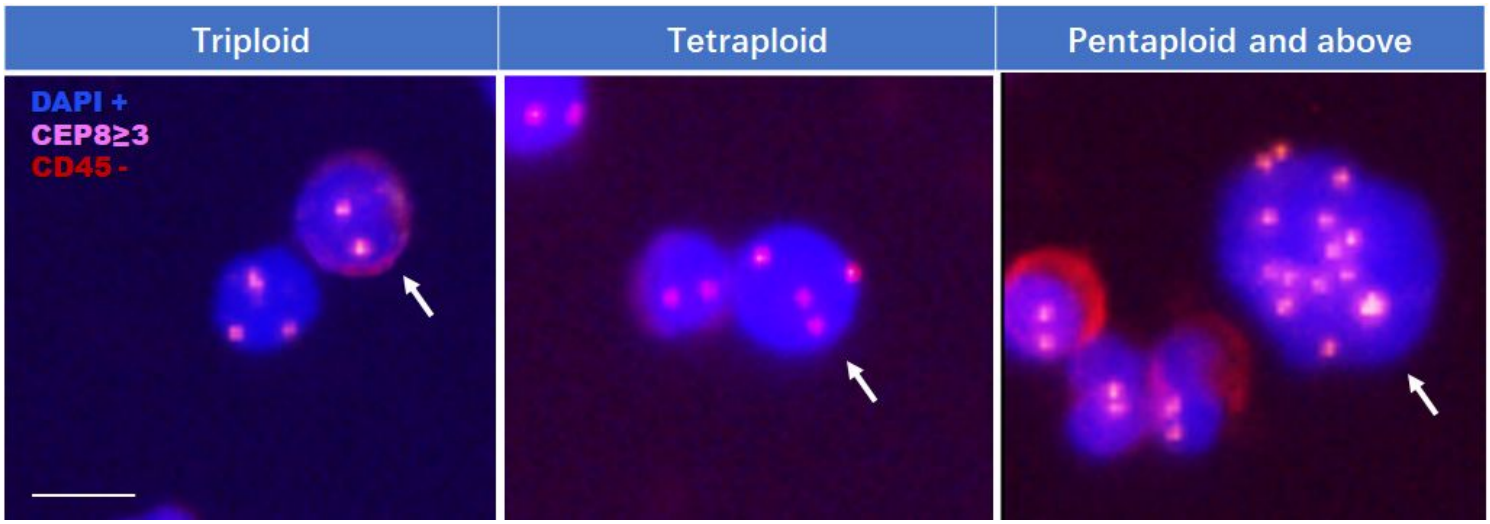
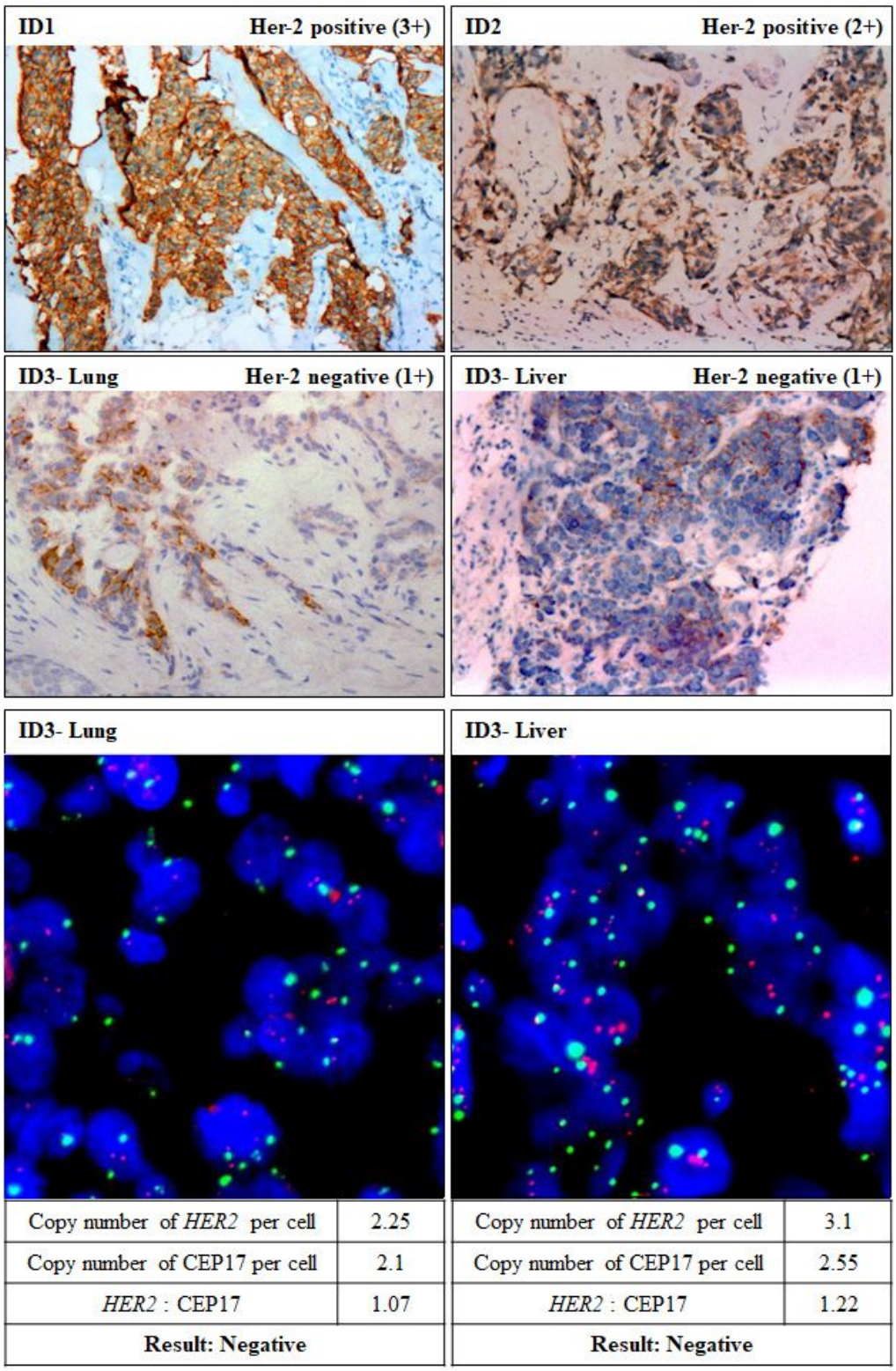


Figure 1

CTCs in patients with breast cancer. White arrows showed the identified CTCs. Scale bar is 10 μm.



Copy number of *HER2* per cell	2.25	Copy number of *HER2* per cell	3.1
Copy number of CEP17 per cell	2.1	Copy number of CEP17 per cell	2.55
HER2 : CEP17	1.07	*HER2* : CEP17	1.22
Result: Negative		**Result: Negative**	

Figure 2

Her-2/HER2 status in the tissue of the 3 patients. The Her-2 protein expression in the primary tissue of patient ID1, ID2, and ID3, and in the liver metastasis tissue of patient ID3 were detected by IHC test (upper). The copy number of HER2 gene in the primary and liver metastasis tissue of patient ID3 were detected by FISH test (lower).

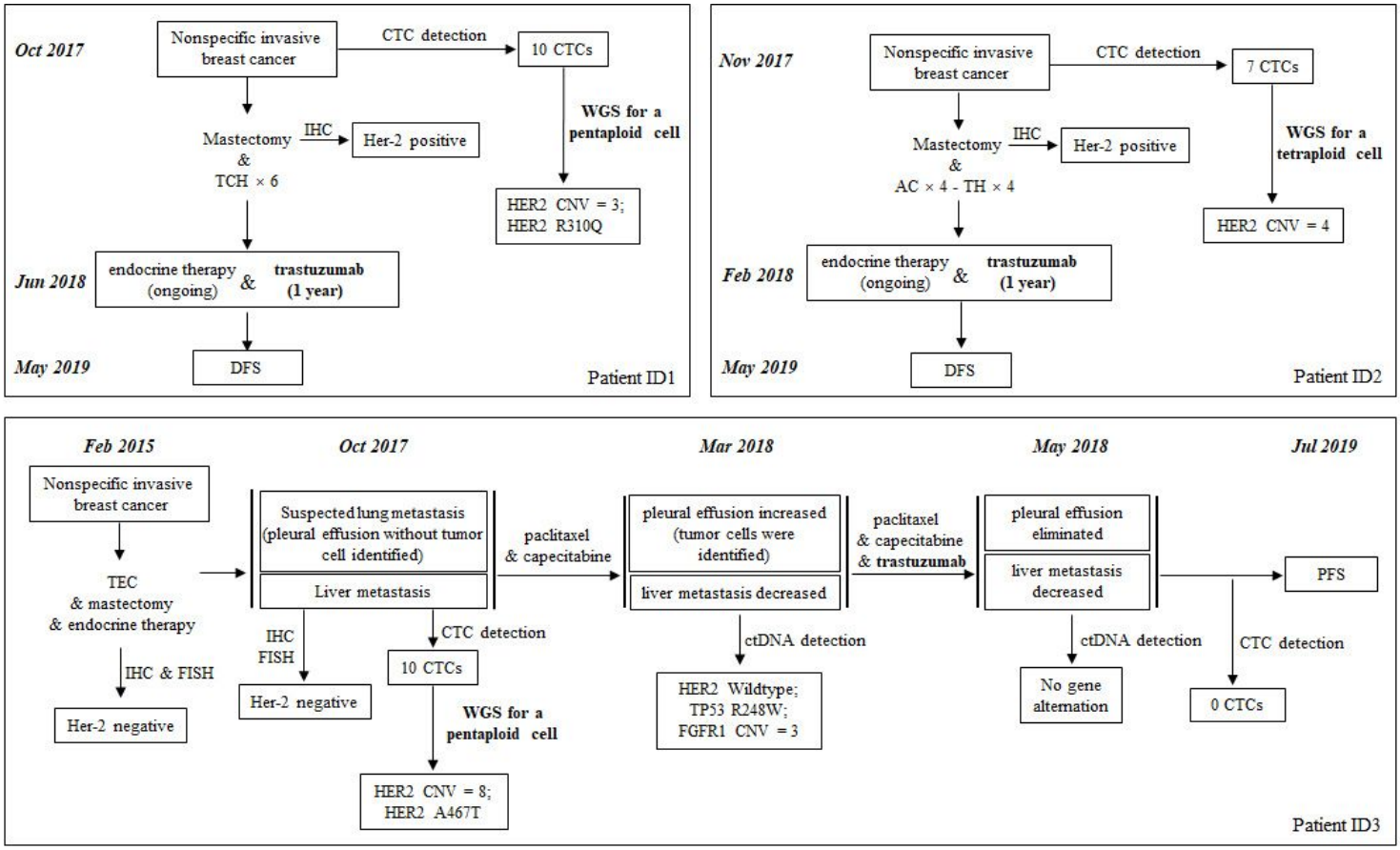


Figure 3

Clinical timelines of the 3 patients with breast cancer. The HER2 targeted drug trastuzumab were highlighted in bold type. DFS, disease-free survival; PFS, progression-free survival.

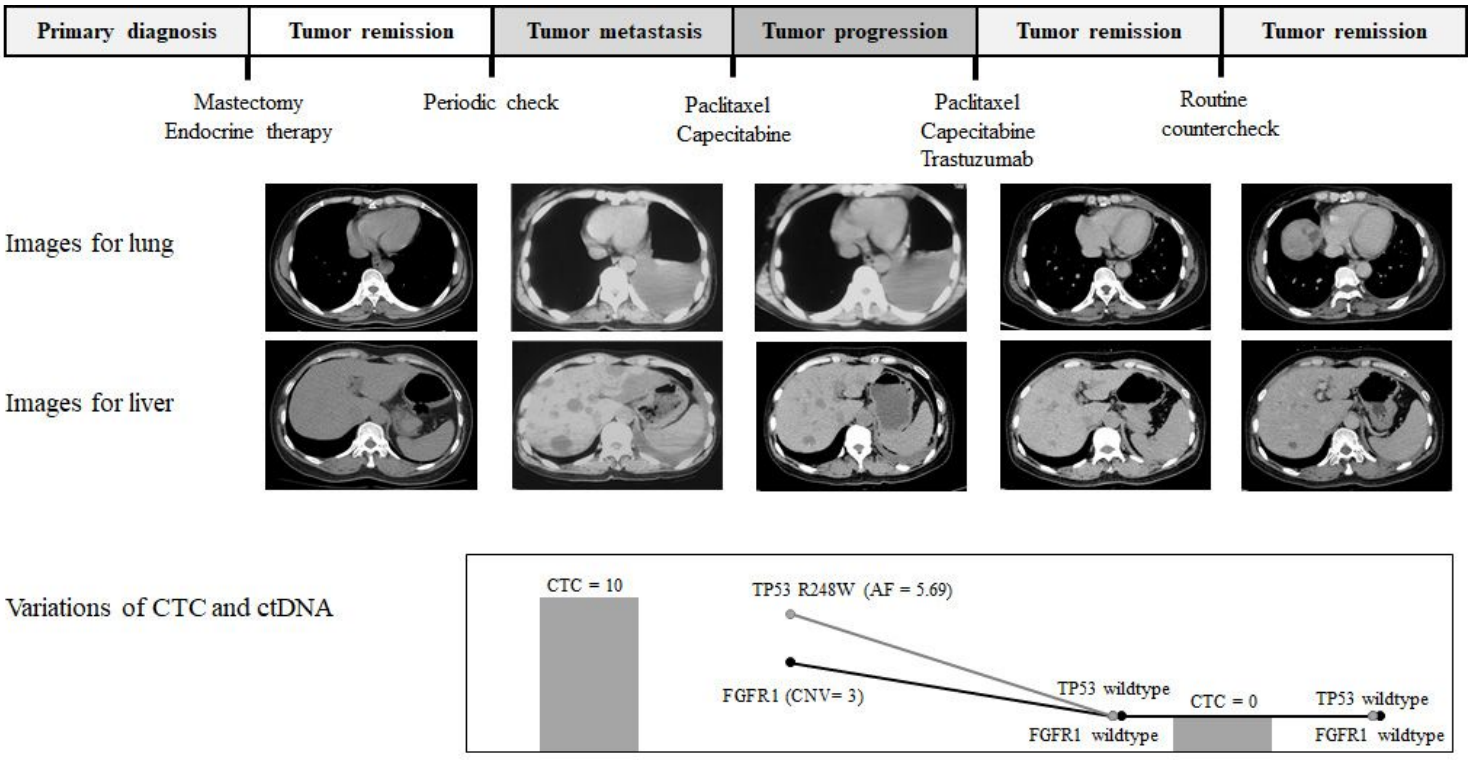


Figure 4

Variations of imaging and molecular biomarkers in Patient ID3 during a series of medical treatments. Treatment process is showed in the top. Images of the lung (upper) and liver (lower) metastasis are listed in the middle. Variations of CTC (grey column) and ctDNA (black line for CNV of FGFR1; grey line for mutation of TP53 R248W) are presented in the bottom.

Supplementary Files

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