Concordance of Targeted Sequencing from Circulating Tumor DNA and Paired Tumor Tissue for Early Breast Cancer

Chi-Cheng Huang  
Taipei Veterans General Hospital

Yi-Fang Tsai  
Taipei Veterans General Hospital

Chun-Yu Liu  
Taipei Veterans General Hospital

Pei-Ju Lien  
Taipei Veterans General Hospital

Yen-Shu Lin  
Taipei Veterans General Hospital

Ta-Chung Chao  
Taipei Veterans General Hospital

Chin-Jung Feng  
Taipei Veterans General Hospital

Yen-Jen Chen  
Taipei Veterans General Hospital

Jiun-I Lai  
Taipei Veterans General Hospital

Han-Fang Cheng  
Taipei Veterans General Hospital

Bo-Fang Chen  
Taipei Veterans General Hospital

Chih-Yi Hsu  
Taipei Veterans General Hospital

Jen-Hwey Chiu  
Taipei Veterans General Hospital

Ling-Ming Tseng  
Taipei Veterans General Hospital

Research Article
Abstract

Purpose

In this study we evaluated the concordance of targeted sequencing between paired circulating tumor DNA (ctDNA) and matched tumor samples from early breast cancers treated with curative intention. Most studies for liquid biopsy were performed for advanced disease, and we reported the scenario of early breast cancer.

Materials and Methods

The study VGH-TAYLOR comprised a subgroup of early-stage breast cancer. Molecular profiling was performed for both fresh-frozen paraffin-embedded (FFPE) tumor tissue and plasma using the Oncomine Comprehensive Assay v3 and the Oncomine Breast cfDNA Assay v2, respectively. Common genes interrogated by both platforms were identified, and concordance between paired targeted sequencing results from the same individual was reported.

Results

A total of 612 patients underwent liquid biopsy; 239 (39%) of which reported at least one mutation. Among 246 early-stage patients assayed for both ctDNA and matched tumor, cfDNA assay detected 73 (29.6%) and comprehensive assay detected 201 (81.7%) breast cancers with at least one alteration (c2 test, p = 0.001). Sixty-seven (25.6%) were tested positive for both platforms, while cfDNA and comprehensive assay detected additional 10 (4%) and 138 (56%) cases, respectively. The most prevalent mutant genes were TP53 (68.3%) and KRAS (53.5%), while PIK3CA (39.4%), AKT1 (45.9%) and ERBB2 (17.1%) mutations constituted biomarkers for FDA-approved therapeutics.

Conclusion

Only one-quarter of breast cancers were concordant between tumor and liquid biopsy from the same subject. Early-stage breast cancer might shed fewer ctDNA from tumor and compromise detectability of liquid biopsy. Our study showed that tumor tissue should be the source of actionable mutation detection for early breast cancers.

Introduction

Circulating tumor DNA (ctDNA) refers to DNA fragment shed from primary tumor in the blood. The phenomenon of ctDNA is not totally understood. Current research shows that it may result from apoptosis, necrosis, or active secretion of tumor cells (1–2). Once detected, ctDNA can be sequenced with genetic variants revealed. As ctDNA may reflect the entire tumor genome immediately, it has gradually
gained attention in recent years for potential clinical application. For example, analysis of ctDNA may be a good tool for early detection of molecular residual disease, assessment of treatment response, and monitoring of disease progression, thus potentially improving cancer patients’ outcomes (3–5). In addition, ctDNA may also be an effective biomarker for non-invasive surrogate of tumor burden, further widening the clinical applications and directing to personalized therapy (6).

Liquid biopsy refers to obtaining plasma samples by drawing blood. Samples can be taken and examined at different time points to monitor changes in tumors during treatment. Analysis of ctDNA obtained through liquid biopsy may greatly change the detection, treatment, and monitoring of cancers (7). For patients with solid tumor (such as patients with advanced breast cancer), because it is often impossible to obtain metastatic samples for analysis by direct biopsy or surgical excision, liquid biopsy is particularly attractive due to its non-invasive nature.

In this study, we evaluated the concordance of targeted sequencing between ctDNA and paired tumor tissue from early-stage breast cancer patients scheduled for curative surgery with or without adjuvant therapy. Most studies for liquid biopsy were conducted for advanced disease, and we focused on targeted sequencing for early breast cancer. If high concordance was observed, pre-operative liquid biopsy could serve as a non-invasive surrogate for variants doomed to be revealed from tumor after definite surgery. On the other hand, poor concordance might limit the clinical application of liquid biopsy for early-stage breast cancer.

**Materials and methods**

The study VGH-TAYLOR: Comprehensive precision medicine research on the heterogeneity of Taiwanese breast cancer patients, consisting of three years of enrollment and approximately four years of follow-up has been published elsewhere (8). Breast cancer patients were assigned into Group 1A: planned to received surgery as the first-line treatment and followed by adjuvant therapy, Group 2: planned to receive neoadjuvant therapy as the first-line treatment and followed by surgery, and Group 3: diagnosed with de novo and treatment naive stage IV breast cancer, or stage IV breast cancer with recurrence beyond three years after surgery. In current study, we focused on subpopulation of early-stage breast cancer patients (Group 1 and 2) only.

Molecular profiling and potential biomarkers were determined using the Oncomine Comprehensive Assay v3 from fresh-frozen paraffin-embedded (FFPE) tissues and the Oncomine Breast cfDNA Assay v2 from plasma as the form of liquid biopsy. Tumor-only sequencing results of the VGH-TAYLOR study using the comprehensive assay had also been reported (9–10). The Oncomine Comprehensive Assay is a targeted sequencing panel using FFPE samples, including 161 cancer-relevant genes and types of mutation detected such as frameshift, missense, synonymous, single nucleotide variant (SNV), insertion/deletion (Indel), and copy number variation (CNV). The Oncomine Breast cfDNA Assay detects breast cancer-derived cell free DNA (cfDNA) including hotspot genes (~152 hotspots) such as **AKT1**, **EGFR**, **ERBB2**, **ERBB3**, **ESR1**, **FBXW7**, **KRAS**, **PIK3CA**, **SF3B1** and **TP53**, as well as CNVs from **CCND1**, **ERBB2** and **FGFR1**.
Common genes interrogated by both platforms were identified, and concordance between paired targeted sequencing results from the same subject was reported (Fig. 1). Liquid biopsy was carried out at the time of cancer diagnosis, while tumor tissue was obtained from definite surgery (Group 1A) or tumor biopsy (Group 2).

The extraction of ctDNA was performed by the QIAamp circulating nucleic acid kit (QIAGEN, Hilden, Germany). The thawed plasma was centrifuged at 16,000 g for 10 minutes at 4°C. Five mL supernatant of centrifuged plasma was transferred into a 50 mL tube with 500 µL QIAGENE Proteinase K and 4 mL Buffer ACL without carrier RNA. The mixture was pulse-vortexed for 30s then incubated at 60°C for 30 mins. Nine mL Buffer ACB was added into the lysate and mixed well by pulse-vortexing for 15-30s. The mixture was incubated for 5 mins on ice, then applied into the QIAamp Mini column and drawn through the column by vacuum pump for DNA capture. The captured DNA was washed by 600 µL Buffer ACW1, 750 µL ACW2, and 750 µL ethanol (96–100%) sequentially, and the column was centrifuged at 20,000 g for 3 mins to remove the remained wash buffers. The column was incubated at 56°C for 10 mins to dry the membrane completely. Buffer AVE (50 µL) was applied on to the dry membrane to elute cfDNA. The eluted DNA was collected into the collection tube by centrifugation at 20,000 g for 1 min. The amount of ctDNA harvested was quantified by the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA). Target enrichment sequencing of ctDNA was carried out with the standard procedures provided by the manufacturer.

Library generation followed the standard protocols: 2–3 libraries (depending on the required read depth) were multiplexed for templating on the Ion OneTouch 2 System and subsequently sequenced on the Ion PGM System using the Ion 318 Chip Kit. Libraries were constructed using the Oncomine Breast cfDNA Assay v2 and the Oncomine Comprehensive Assay v3. Variant detection was performed by the Torrent Variant Caller plugin on Torrent Suit Software or Ion Reporter Software. Additional annotations for actionability and OncoPrinter visualization were carried out using the OncoKB database and ESMO Scale for Clinical Actionability of molecular Targets (ESCAT) criteria (11–13).

Results

The study evaluated the concordance of targeted sequencing between paired ctDNA and tumor samples from a cohort of early breast cancers scheduled for curative therapy in Taiwan. The primary outcomes were actionable mutations determined by genetic profiling of Taiwanese breast cancers using the next generation sequencing (NGS) assay. We reported the mutational landscape of 612 patients (full VGH-TAYLOR cohort) interrogated with the Oncomine Breast cfDNA assay; 239 out of 612 patients reported at least one mutation (39%, Fig. 2). TP53 constitutes the most common variants (79%), followed by PIK3CA (28%) with other mutations took place less than 5% of the study population.

Among 246 early-stage breast cancer patients (Group 1A and Group 2 from the VGH-TAYLOR study) assayed for both ctDNA and tumor tissue, the cfDNA assay detected 73 (29.6%, Fig. 3) and comprehensive assay detected 201 (81.7%, Fig. 4) breast cancers with at least one variant (c2 test, p =
0.001). Sixty-seven (25.6%) were tested positive for both liquid and tissue assay, while the cfDNA and comprehensive assay detected additional 10 (4%) and 138 (56%) cases, which were not identified by the other platform. Table 1 details the distributions of called variants among the 10 common targeted genes.

The most prevalent mutant genes from liquid biopsy and tissue samples, collectively, were TP53 (68.3%, n = 168) and KRAS (53.5%, n = 131), both were well-known cancer driver genes. From breast cancer actionability, PIK3CA (ESCAT Tier IA) were reported in 39.4% (n = 97), AKT1 mutation (ESCAT Tier IIB) in 45.9% (n = 113) and ERBB2 mutation (ESCAT Tier IIB) in 17.1% (n = 42) of early-stage Taiwanese breast cancers (Table 1).

Table 1. Distributions of variants from common genes (n=6) between cell free DNA (cfDNA) and the tumor tissue-based comprehensive assay.

<table>
<thead>
<tr>
<th>Gene</th>
<th>cfDNA</th>
<th>Tumor tissue</th>
<th>Both detectable</th>
<th>Both non-detectable</th>
<th>Total affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT1</td>
<td>1</td>
<td>113</td>
<td>1</td>
<td>133</td>
<td>113 (45.9%)</td>
</tr>
<tr>
<td>ERBB2</td>
<td>0</td>
<td>42</td>
<td>0</td>
<td>204</td>
<td>42 (17.1%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>4</td>
<td>120</td>
<td>3</td>
<td>115</td>
<td>131 (53.5%)</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>15</td>
<td>93</td>
<td>1</td>
<td>149</td>
<td>97 (39.4%)</td>
</tr>
<tr>
<td>SF3B1</td>
<td>3</td>
<td>14</td>
<td>1</td>
<td>230</td>
<td>16 (6.5%)</td>
</tr>
<tr>
<td>TP53</td>
<td>65</td>
<td>146</td>
<td>43</td>
<td>78</td>
<td>168 (68.3%)</td>
</tr>
</tbody>
</table>

**Discussion**

Circulating tumor DNA is free DNA bound to proteins in plasma that originates from tumor, and tumor derived DNA may only be a small minority of total cell free DNA present in plasma. Next generation sequencing has been developed for the detection of ctDNA in clinical trials, with potentialities of selecting therapies in metastatic settings, interrogating clonal evolution, and monitoring of therapy in both metastatic and early settings (14). Most studies for ctDNA in breast cancer have been conducted for advanced/metastatic setting, while in this study we tried to answer the question that whether pre-operative ctDNA testing could serve as a non-invasive surrogate for variants which were to be identified from tumor tissue after surgery if the high concordance was observed.

Based on the results of 246 early-stage Taiwanese breast cancers, only one-quarter of patients were tested positive for both the cfDNA and comprehensive assay from the same subject, indicating that assay-specific sensitivity inevitably resulted in a diagnostic discrepancy of targeted sequencing. In addition, the source of nucleic acid for NGS experiments mattered especially for an early-stage disease setting. The plausible explanation came from the fact that fewer ctDNA spillage from tumor was expected for early breast cancer patients treated with curative intention, which might compromise detectability from liquid biopsy.
In order to enhance the sensitivity of liquid biopsy, a technology called "cancer personalized profiling by deep sequencing" (CAPP-Seq) has been developed in the past decade, which can achieve high sensitivity with personalized profiling by deep sequencing (15–17). For example, in non-small cell lung cancer, CAPP-Seq had shown that the amount of ctDNA was highly correlated with tumor volume as well as residual disease, and was advocated as being able to forecast the effect of treatment earlier than imaging examinations (18). Although our study design was not sophisticated enough to adopt CAPP-Seq, the limit of detection (LOD) parameters of the cfDNA assay in current study are listed as below; SNVs/short indels: LOD down to 0.1% allele frequency (AF) could be achieved with a sensitivity of > 80% and specificity of > 98% and TP53 whole-target SNVs/indels: 0.5% AF (looking at all bases within amplicons); CNV targets: detection as low as 1.4-fold change can be achieved (19). Consequently, LOD should not be the excuse for discordance between liquid biopsy and tumor tissue sequencing.

The most prevalent mutations with both platforms combined were TP53 (68.3%) and KRAS (53.5%), both were well-known cancer driver genes. Although both were currently not targetable for breast cancer, TP53 is a gene very commonly mutated in both clonal hematopoisis and tumors and is rarely representative of the germline Li-Fraumeni syndrome; consequently, routine germline testing may not be necessary for most patients with somatic TP53 mutations (20). KRAS mutations are observed across a variety of cancer entities while recent advent of the KRAS(G12C) inhibitor render KRAS-mutant tumors druggable (21–22). For contemporary breast cancer actionability, PIK3CA mutation is a biomarker for the FDA-approved PI3Ka inhibitor alpelisib, AKT1 mutation is indicated for agents such as capivasertib (AZD5363) and ipatasertib, and ERBB2 mutation for tyrosine kinase inhibitor neratinib (23–27). Our study did ascertain the value of targeted sequencing for breast cancer, even at an earlier disease setting.

It's not a coincidence that the guideline update for biomarkers suggested that testing for PIK3CA mutations should use samples from tumor tissue or ctDNA in plasma to determine eligibility for treatment with alpelisib plus fulvestrant, a selective estrogen receptor degrader. If no mutation is found in ctDNA, testing in tumor tissue, if available, should be used as this will detect a small number of additional patients with PIK3CA mutations (28). Although PI3K inhibitors are indicated for late-stage hormone receptor positive, human epidermal growth factor receptor II (HER2) negative breast cancers and liquid biopsy detection rate was assumed to be much higher for advanced disease, it was found that patients from the SOLAR-1 trial displayed low concordance in PIK3CA mutations between ctDNA and tumor tissue (56%) and FDA-approved labeling recommends a reflex tissue testing when PIK3CA mutation is not detected from liquid biopsy (29). Despite detection rate of liquid biopsy (PCR or NGS) was much lower than that of tumor tissue (34% versus 60%), liquid biopsy was still suggested as the starting point for mutation testing as liquid biopsy may represent the most recent sample during tumor evolution.

Cancer treatment is rapidly evolving towards personalized medicine with targeted therapy corresponding to molecular alterations that lead to tumor growth. Therefore, proper assessment of dominant variants driving tumor evolution is warranted when deciding which patients are eligible for specific treatments. The way to obtain enough and the most recent tumor tissue for molecular profiling is quite challenging. Therefore, liquid biopsy through ctDNA has been proposed as an alternation for tumor slices in the past.
In this study, we expanded the use of liquid biopsy to early breast cancer, which can be used to detect molecularly metastatic or minimally residual disease pre- and post-operatively, indicating aggressiveness not readily detectable by contemporary pathological and clinical staging system. The genetic variants identified with ctDNA are postulated to associate with treatment response and discover the earliest aura of recurrence. In current study, the low concordance between tumor tissue and liquid biopsy did not surprise us too much given the early-stage disease setting. It deserves notice, however, that there were still a few cases with identified variants from ctDNA only, and these patients should be followed up longitudinally to understand the meaning and prognostication of ctDNA-positive/tumor tissue-negative phenomenon.

There were some limitations of the study. First, interrogated genes were not identical between comprehensive and cfDNA assay. Unlike targeted sequencing assays delivered by Foundation Medicine (Cambridge, MA) or Illumina (San Diego, CA) which deliver almost the same region of interest between tumor sequencing and liquid biopsy, targeted genes for the Oncomine cfDNA Assay were far less than one-tenth of the Comprehensive counterpart. Second, liquid biopsy was performed per-operatively while tumor tissues were obtained from definite surgery (Group 1A) or diagnostic tissue biopsy (Group 2). Difference sources of liquid and tissue biopsy might result in a time-lag about 2 to 4 weeks and fluctuations in ctDNA during this time interval could not be completely ruled out. Third, the prospective manner of the VGH-TAYLOR study needed longer follow up to see the prognostication of targeted sequencing, especially for those cases with ctDNA-only alterations.

**Declarations**

**Acknowledge**: The authors would like to thank the Melissa Lee Cancer Foundation and the Taiwan Clinical Oncology Research Foundation for their help during the study period, as well as technical support from Ms Abbie PF Wu. This study contained materials presented at poster sessions during the Taipei International Breast Cancer Symposium (TIBCS) 2021 during Oct 30-31, 2021 at Taipei and San Antonio Breast Cancer Symposium (SABCS) 2022 during Dec 6-10, 2022 at SA, TX as well as an oral presentation during the Oncomine World 2023: A Virtual NGS Education Meeting at Mar-30, 2023.

**Ethical Approval**

This study had been reviewed and approved by the Internal Review Board of the Taipei Veterans General Hospital and followed the Declaration of Helsinki. All participants signed the informed consent before enrollment and all authors reviewed and approved the final version of submission.

**Competing interests**

All authors declare that there is no conflict of interest.

**Authors’ contributions**
CCH drafted the manuscript. YFT, CYL, PJL, YSL, TCC, CJF, YJC, JIL, HFC and BFC involved in patient enrollment and sample collection. CYF took the responsibility for pathological examinations, JHC assisted in data analysis and LMT approved the final submission.

Funding

This study was sponsored exclusively by the Yung-Lin Health Foundation and National Science and Technology Council grant NSCT111-2314-B-075-063-MY3.

Availability of data and materials

All data have been included in the main text, tables and figures of the study. Individual targeted sequencing raw data such as FASTQ, BAM and VCF files are restricted for access for protection of individual genetic information. Further requirements for these data can be made with the permission of the corresponding author with additional review from the Internal Review Board.

References


**Figures**

**Figure 1**
Molecular profiling and potential biomarkers were determined using a comprehensive next generation sequencing (NGS) panel from fresh frozen paraffin-embedded (FFPE) tissues and a breast cell free DNA (cfDNA) assay from plasma.

**Figure 2**

Mutational landscape of the whole VGH-TAYLOR cohort (n=612) with the Oncomine Breast cell free DNA (cfDNA) assay. Group 1A: surgery followed by adjuvant therapy, Group 2: Neoadjuvant therapy followed by surgery, Group 3-1: de novo stage IV, Group 3-2: late recurrence (beyond 3 years) after curative surgery.

**Figure 3**

Oncoprinter of circulating tumor DNA (ctDNA) targeted sequencing from 246 early breast cancers. Seventy-three patients reported at least one mutation, which were also the denominator when calculating the proportion of affected cases with each gene.
Figure 4

Oncoprinter of tumor tissue targeted sequencing from 246 early breast cancers. Two hundreds and one patient reported at least one mutation, which were also the denominator when calculating the proportion of affected cases with each gene.