Liquid biopsy for detection of EGFR T790M mutation in nonsmall cell lung cancer: An experience of proficiency testing in Taiwan

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Abstract

Background: The use of liquid biopsy to detect epidermal growth factor receptor (EGFR) T790M mutation in nonsmall cell lung cancer (NSCLC) is a promising method to screen patients eligible for third-generation EGFR inhibitors. Proficiency testing (PT) programs involving liquid biopsy are currently lacking. In this study, we conducted a PT program to assess the quality assurance of liquid biopsy tests for detecting EGFR T790M mutation in molecular pathology laboratories in Taiwan.

Methods: Whole blood samples (2 mL) with various concentrations of the EGFR T790M mutation were prepared and analyzed in six participating laboratories using their clinically validated assays.

Results: For circulating cell-free DNA (cfDNA) isolation, three of the six participating laboratories used the cobas cfDNA Sample Preparation Kit, and three used the QIAamp Circulating Nucleic Acid Kit. For testing platforms, two of the six participating laboratories used mass spectrometry, three used the cobas EGFR mutation test, and one used a laboratory-developed test. There was 100% concordance in detection of all the given concentrations of EGFR T790M mutation between the participating laboratories and different testing platforms. The testing platforms used by all participating laboratories could successfully detect EGFR T790M mutation to an expected frequency of 1%.

Conclusion: In this first PT program using liquid biopsy in Taiwan, local clinical laboratories were suitably equipped and proficient in the use of cfDNA to test for the EGFR T790M mutation. Establishing a routine PT system to ensure the reliability and accuracy of liquid biopsy in clinical practice in Taiwan would be helpful.

Keywords: EGFR T790M; Liquid biopsy; Nonsmall cell lung cancer; Proficiency testing

1. INTRODUCTION

Lung cancer is the most common cause of cancer-related deaths worldwide, and is classified into nonsmall cell lung cancer (NSCLC) and small cell lung cancer.\textsuperscript{1} NSCLC accounts for approximately 80% to 85% of all lung cancer cases, and is further classified into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.\textsuperscript{2} Epidermal growth factor receptor (EGFR) mutations are more frequently found in women, never smokers, patients with East Asian ethnicity, and those with the adenocarcinoma subtype.\textsuperscript{3,4} In Taiwan, more than 60% of patients with lung adenocarcinoma harbor EGFR mutations.\textsuperscript{4} EGFR tyrosine kinase inhibitors (EGFR-TKIs) have been approved by the US and Taiwan Food and Drug Administration (FDA) as first-line treatment for patients with advanced or metastatic NSCLC and sensitizing EGFR mutations. Even though most patients initially respond to EGFR-TKI treatment, almost all patients ultimately develop acquired resistance to EGFR-TKIs after a progression-free period of 9 to 14 months. The most common mechanism of acquired resistance is the emergence of the T790M mutation on exon 20 of the EGFR gene, which is observed in approximately 50% of resistant cases.\textsuperscript{5-7} Third-generation EGFR-TKIs such as osimertinib (AZD9291), CO-1686 (rociletinib), HM61713, ASP8273, EGF816, and PF-06747775 have been developed to overcome EGFR T790M resistance through a significant increase in targeted potency.
for mutant than for wild-type EGFR protein. Osimertinib (AZD9291, TAGRISSO) was recently approved by the US FDA as the standard of care for metastatic EGFR T790M mutation-positive NSCLC patients with disease progression after first-line EGFR-TKI treatment. Testing for the EGFR T790M mutation is therefore necessary to identify patients who may benefit from new EGFR-TKI treatment.

Although rebiopsy is the gold standard procedure to obtain tumor tissue for molecular analysis, some problems may be encountered in clinical practice. First, there may not be accessible tumors for rebiopsy, especially in patients who have received previous treatment. Second, rebiopsy is an invasive approach and usually risky for patients who are weak due to tumor recurrence. Third, rebiopsy may not represent the entire entity of a tumor due to tumor heterogeneity, particularly in tumors with heterogeneously expressed EGFR T790M mutation. Liquid biopsy refers to isolation of circulating cell-free DNA (cfDNA) or circulating tumor cells from patients’ peripheral blood, and it is a noninvasive method to examine genetic alterations in tumor cells. Compared to tumor biopsy, liquid biopsy may be a more feasible approach for early diagnosis of cancer, assessing prognosis, monitoring tumor burden, and identifying acquired resistance during treatment. Therefore, cfDNA for EGFR T790M testing is now considered as an alternative approach for patients in whom it may not be possible to perform tissue rebiopsy.

Due to the low abundance of tumor-derived cfDNA in the blood, several testing platforms have been used to analyze EGFR mutations in plasma, such as the cobas EGFR mutation test, therascreen EGFR amplification refractory mutation system (ARMS), droplet digital PCR (ddPCR), BEAMing dPCR assay, mass spectrometry, and next generation sequencing. However, the sensitivity and specificity of detection vary between these testing platforms. Considering that the accuracy of EGFR T790M testing using liquid biopsy is critical for monitoring the development of acquired resistance and subsequent management of NSCLC, the aim of this study was to evaluate a proficiency testing program to detect the T790M mutation using cfDNA.

2. METHODS

2.1. Sample preparation for PT

Relevant concentrations of Multiplex I cfDNA Reference Standards (Horizon Diagnostics, Cambridge, UK) were spiked into 15 mL of healthy human whole blood to obtain different concentrations of the EGFR T790M mutation with percentages of 0%, 1%, 2.5%, and 5%. Freshly prepared blood samples (2 mL) were immediately aliquoted into DNA BCT tubes (Streck, Inc.) and shipped to each participating laboratory. Before submission, PT samples were subjected to between-run extraction methods or nucleic acid quantification methodologies used in each laboratory. For the results of EGFR T790M mutation detection, all of the participating laboratories identified that sample A contained no EGFR T790M, and that samples B, C, and D contained 1%, 2.5%, and 5% of EGFR T790M, respectively (Table 4).

2.2. Validation of PT samples

Before submission, PT samples were subjected to between-run and within-run validation using the ddPCR platform. Briefly, blood samples were centrifuged at 1500 g for 10 minutes to isolate plasma for cfDNA extraction using QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocols. The quality and quantity of extracted cfDNA were assessed using Qubit fluorometer (Thermo Fisher Scientific). A total ddPCR reaction mixture of 20 μL containing 2× Master Mix (Bio-Rad Laboratories, Inc.), 20× primers, and TaqMan Probe (Applied Biosystems Life Technologies), nuclease-free water and DNA template, and 70 μL of droplet generation oil were subsequently loaded into the droplet generator cartridge. The cartridge was then placed into the droplet generator for emulsification to produce individual droplets. The emulsified sample was then transferred to a 96-well PCR plate for PCR amplification, after which the plate was placed on a QX200 droplet reader (Bio-Rad) to analyze the droplets in each well. The PCR data were further analyzed using QX200 software version 1.7.4.0917 (Bio-Rad Laboratories, Inc.).

### Table 1

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Mutation frequency expected, %</th>
<th>Mutation frequency detected, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>0.97</td>
</tr>
<tr>
<td>C</td>
<td>2.5</td>
<td>2.34</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>3.63</td>
</tr>
</tbody>
</table>

ddPCR = droplet digital PCR; EGFR = epidermal growth factor receptor; PT = proficiency testing.

### Table 2

<table>
<thead>
<tr>
<th>Platform</th>
<th>No.</th>
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<tbody>
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<td>PNA-MALDI-TOF MS</td>
<td>2</td>
</tr>
<tr>
<td>cobas EGFR mutation test v2</td>
<td>3</td>
</tr>
<tr>
<td>ARMS-based technology</td>
<td>1</td>
</tr>
</tbody>
</table>

ARMS = amplification refractory mutation system; EGFR = epidermal growth factor receptor; PNA-MALDI-TOF MS = peptide nucleic acid and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

3. RESULTS

Six molecular pathology laboratories participated in this PT program to detect the EGFR T790M mutation using cfDNA. With regards to the testing platforms (Table 2), two laboratories used PNA-MALDI-TOF MS, which is a combination of peptide nucleic acid (PNA) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with a detection limit of 0.1%. Three laboratories used the cobas EGFR mutation test v2 (cobas Roche Diagnostic), which is a real-time PCR-based technology, and one laboratory performed a laboratory-developed test (LDT) in which the ARMS was used to amplify the mutation fragments and the high resolution capillary electrophoresis (QIAxcel Advanced System, Qiagen) was used to analyze mutation alleles (unpublished data). Among the six participating laboratories, the average amount of cfDNA extracted was 727.4 ng (range, 17.8 to 2050 ng) from sample A, 891.9 ng (range, 49.3 to 1850 ng) from sample B, 671 ng (range, 24.2 to 1850 ng) from sample C, and 923.3 ng (range, 24.7 to 1850 ng) from sample C (Table 3). The total amount of cfDNA extracted from the PT specimens varied between the participating laboratories, which may have been due to the different extraction methods or nucleic acid quantification methodologies used in each laboratory. For the results of EGFR T790M mutation detection, all of the participating laboratories identified that sample A contained no EGFR T790M, and that samples B, C, and D contained 1%, 2.5%, and 5% of EGFR T790M, respectively (Table 4). Taken together, the testing platforms used by all of the participating laboratories could successfully detect the EGFR T790M mutation to an expected frequency of 1%, and the variation in cfDNA concentration measured across the laboratories did not seem to influence the results.
EGFR T790M mutation, respectively. These results suggest test and BEAMing dPCR, with the sensitivity of 82% and 87% high concordant rate of >90% between cobas EGFR mutation

cordance, demonstrating that the molecular laboratories in Taiwan are suitably equipped and proficient to test for the EGFR T790M mutation in liquid biopsy. The presence of cfDNA in human blood was first described in 1948 by Mandel and Métais. Cancer patients have an elevated level of cfDNA compared to healthy individuals, which may be due to the release of tumor-related cfDNA by tumor cells through various cell physiological events such as apoptosis, necrosis, and secretion.

Moreover, a cross-platform comparison showed that digital and nondigital approaches (digital PCR, and the therascreen EGFR ARMS-PCR) in the detection of EGFR mutations using cfDNA in patients with NSCLC, and demonstrated a concordance rate of 60% to 90% compared with the results using tissue specimens. Moreover, a cross-platform comparison showed that digital approaches (digital PCR and BEAMing dPCR) had a higher sensitivity than nondigital approaches (cobas EGFR Mutation test and therascreen EGFR ARMS-PCR) in the detection of EGFR T790M mutation using cfDNA. However, a subsequent assessment of 72 baseline plasma samples showed that there was a high concordant rate of >90% between cobas EGFR mutation test and BEAMing dPCR, with the sensitivity of 82% and 87% for EGFR-sensitizing mutations, and 73% and 81% for the EGFR T790M mutation, respectively. These results suggest that both digital and nondigital approaches are feasible methods to analyze EGFR mutations using cfDNA. In addition, the US FDA has recently approved cobas EGFR mutation test as a companion diagnostic tool for TAGRiSSO (osimertinib).

In this study, the testing platforms used in the participating laboratories included MALDI-TOF MS, cobas EGFR Mutation Test v2, and an in-house LDT method. Except for the in-house assay, these methods have been previously demonstrated to have sufficient sensitivity and specificity for detection of the EGFR T790M mutation in plasma. Our results showed that both commercial and in-house methods were accurate to detect the EGFR T790M mutation even at a 1% frequency of mutation allele. The amount of cfDNA extracted from the PT specimen varied among the laboratories. It has been reported that some procedure factors before cfDNA extraction may influence the final yield of cfDNA, including the type of collecting tubes, centrifugation, storage and thawing temperature, extraction reagents, and DNA quantification methods. In addition, several studies have found that after venipuncture, the concentration of cfDNA increases over time prior to blood processing due to the release of genomic DNA from blood cells. In this study, all of the PT specimens were collected using DNA BCT tubes, which have been demonstrated to provide better cfDNA preservation at room temperature compared to other types of tubes. However, the delay in blood processing, if present, may also have caused an artificial increase in cfDNA concentration.

Another possible reason contributing to the variation among laboratories may be due to the different DNA quantification methods used in this study. A variety of methods for the quantification of cfDNA have been reported, including fluorescent and spectrophotometric measurements as well as qPCR. In this study, two of the six participating laboratories used the fluorescent measurement and four used the spectrophotometric measurement. The spectrophotometric measurement such as NanoDrop through direct measuring UV absorbance at A260 was the most common method used for nucleic acid quantification. However, this method cannot distinguish between double-strand DNA (dsDNA) and single-strand DNA (ssDNA) or RNA, and may be interfered with other factors such as PCR primers, dNTP, and aromatic organic compounds. In contrast, the fluorescent measurement using fluorescent dye selectively bound to dsDNA for quantification, for example, Qubit Fluorometer, can specifically determine the amount of dsDNA. However, the concentration determined by fluorescent measurements may decrease with the level of DNA fragmentation and denaturation. Since cfDNA has a rapid turn-over time and is frequently of low abundance and highly fragmented in blood, further optimization and standardization of preanalytical phases are crucial for cfDNA testing in clinical practice.

To the best of our knowledge, this is the first PT study of liquid biopsy in Taiwan and also worldwide. The results of this study indicate that most of molecular pathology laboratories in Taiwan are quality assured to perform EGFR T790M mutation testing using cfDNA. This study also raises awareness of the importance of quality assurance regarding liquid biopsy and further PT programs should be periodically conducted to monitor the competence of clinical laboratories in providing liquid biopsy services.

**Table 3**

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>Quantification method</th>
</tr>
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<tbody>
<tr>
<td>cobas cfDNA extraction kit</td>
<td>Spectrometry</td>
</tr>
<tr>
<td>QiAamp circulating nucleic acid kit</td>
<td>Fluorometer</td>
</tr>
<tr>
<td>cobas cfDNA extraction kit</td>
<td>Spectrometry</td>
</tr>
<tr>
<td>cobas cfDNA extraction kit</td>
<td>Spectrometry</td>
</tr>
<tr>
<td>QiAamp circulating nucleic acid kit</td>
<td>Spectrometry</td>
</tr>
</tbody>
</table>

**Table 4**

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>EGFR T790M expected, %</th>
<th>EGFR T790M found</th>
<th>EGFR T790M unfound</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0 (0%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>6 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>C</td>
<td>2.5</td>
<td>6 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>6 (100%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

EGFR = epidermal growth factor receptor.
ACKNOWLEDGMENTS

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