ORIGINAL ARTICLE

A Pan-Canadian Validation Study for the Detection of *EGFR* T790M Mutation Using Circulating Tumor DNA From Peripheral Blood

Shamini Selvarajah, PhD,[a,b] Sophie Plante, MSc,[c] Marsha Speevak, PhD,[a,b] Andrea Vaags, PhD,[a,b] Darren Hamelinck, MSc,[a] Martin Butcher, MSc,[d] Elizabeth McCready, PhD,[e,f] Daria Grafodatskaya, PhD,[e,f] Normand Blais, MD,[g] Danh Tran-Thanh, MD,[g] Xiaoduan Weng MD,[g] Rami Nassabein, MD,[g] Wenda Greer, PhD,[h] Ryan N. Walton, MPH,[i] Bryan Lo, MD,[j] Doug Demetrick, MD,[k,l,m,n] Stephanie Santos, BSc,[o] Bekim Sadikovic, PhD,[o,p,q] Xiao Zhang, BSc, MSc,[r] Tong Zhang, MD,[s] Tara Spence, PhD,[t] Tracy Stockley, PhD,[b,s] Harriet Feilotter, PhD,[r,t] Philippe Joubert, MD, PhD[c,*]

[a]*Department of Laboratory Medicine and Genetics, Trillium Health Partners, Mississauga, Ontario, Canada*
[b]*Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada*
[c]*Institut Universitaire de Cardiologie et de Pneumologie de Québec-Université Laval, Québec, Quebec, Canada*
[d]*Department of Oncology, McMaster University, Hamilton, Ontario, Canada*
[e]*Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada*
[f]*Hamilton Regional Laboratory Medicine Program, Hamilton Health Sciences, Hamilton, Ontario, Canada*
[g]*Centre Hospitalier de l'Université de Montréal, Montréal, Quebec, Canada*
[h]*Queen Elizabeth II Health Sciences Center, Department of Pathology, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada*
[i]*AstraZeneca Canada, Mississauga, Ontario, Canada*
[j]*Department of Pathology and Laboratory Medicine, The Ottawa Hospital, Ottawa, Ontario, Canada*
[k]*Department of Pathology and Laboratory Medicine, University of Calgary, Calgary, Alberta, Canada*
[l]*Department of Oncology, University of Calgary, Calgary, Alberta, Canada*
[m]*Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta, Canada*
[n]*Department of Medical Genetics, University of Calgary, Calgary, Alberta, Canada*
[o]*Molecular Genetics Laboratory, Molecular Diagnostics Division, London Health Sciences Centre, London, Ontario, Canada*
[p]*Department of Pathology and Laboratory Medicine, Western University, London, Ontario, Canada*
[q]*Verspeeten Clinical Genome Centre, London Health Sciences Centre, London, Ontario, Canada*
[r]*Laboratory Genetics, Kingston Health Sciences Center, Kingston, Ontario, Canada*
[s]*Department of Clinical Laboratory Genetics, University Health Network, Toronto, Ontario, Canada*
[t]*Department of Pathology and Molecular Medicine, Queen's University, Kingston, Ontario, Canada*

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***Corresponding author.**

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Address for correspondence: Philippe Joubert, MD, PhD, Institut Universitaire de Cardiologie et de Pneumologie de Québec-Université Laval, 2725 chemin Sainte-Foy, C2259, Québec, QC, Canada, G1V4G5.
E-mail: Philippe.Joubert@criucpq.ulaval.ca

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ABSTRACT

Introduction: Genotyping circulating tumor DNA (ctDNA) is a promising noninvasive clinical tool to identify the *EGFR* T790M resistance mutation in patients with advanced NSCLC with resistance to EGFR inhibitors. To facilitate standardization and clinical adoption of ctDNA testing across Canada, we developed a 2-phase multicenter study to standardize T790M mutation detection using plasma ctDNA testing.

Methods: In phase 1, commercial reference standards were distributed to participating clinical laboratories, to use their existing platforms for mutation detection. Baseline performance characteristics were established using known and blinded engineered plasma samples spiked with predetermined concentrations of T790M, L858R, and exon 19 deletion variants. In phase II, peripheral blood collected from local patients with known *EGFR* activating mutations and progressing on treatment were assayed for the presence of *EGFR* variants and concordance with a clinically validated test at the reference laboratory.

Results: All laboratories in phase 1 detected the variants at 0.5 % and 5.0 % allele frequencies, with no false positives. In phase 2, the concordance with the reference laboratory for detection of both the primary and resistance mutation was high, with next-generation sequencing and droplet digital polymerase chain reaction exhibiting the best overall concordance. Data also suggested that the ability to detect mutations at clinically relevant limits of detection is generally not platform-specific, but rather impacted by laboratory-specific practices.

Conclusions: Discrepancies among sending laboratories using the same assay suggest that laboratory-specific practices may impact performance. In addition, a negative or inconclusive ctDNA test should be followed by tumor testing when possible.

Keywords: Non–small cell lung cancer; Plasma ctDNA testing; Liquid biopsy; EGFR T790M variant

Introduction

Approximately 75 % of patients with NSCLC have advanced disease (stage III and IV) at diagnosis.[1,2] In addition, the intrinsic aggressive behavior of lung cancer, as illustrated by a death rate of 5 % to 7 % monthly after initial diagnosis, emphasizes the necessity of rapid access to diagnosis and treatment for the management of patients.[3]

Patients with NSCLC harboring certain variants in the *EGFR* gene are eligible for treatment with EGFR tyrosine kinase inhibitors (TKIs) as first-line systemic therapy. Certain variants that occur in the tyrosine kinase domain of *EGFR*, such as deletions in exon 19 and the point mutation L858R, activate the kinase activity by a ligand-independent mechanism, leading to increased cell survival, proliferation, invasion, and metastasis. Treatment with TKIs results in dramatic antitumor activity in a subset of patients with NSCLC with these sensitizing variants. Patients whose tumors initially respond to EGFR TKIs inevitably reveal disease progression, typically within a year of starting treatment.[4] The most common mechanism of acquired resistance is the acquisition of the T790M variant in the *EGFR* kinase domain, which occurs in approximately 60 % of tumors resistant to first-generation and second-generation EGFR TKIs. This mutation sterically hinders the binding of first- and second-generation TKIs to the adenosine triphosphate (ATP) binding site while the affinity of EGFR for ATP is increased. Thus, the downstream signaling is not inhibited and disease progression occurs.[4,5] Selective and efficient third-generation TKIs have been developed as a means of overcoming this limitation. These compounds selectively target activating *EGFR* mutations and the T790M resistance mutation through the formation of a covalent bond to the C797 residue in the ATP-binding site of mutant *EGFR*.[6–8]

In patients with advanced NSCLC with *EGFR* T790M, variants can be detected in tumor tissue or cell-free circulating tumor DNA (ctDNA) extracted from plasma.[9–11] Current testing recommendations for *EGFR* T790M detection include identification of the variant in cell-free DNA (cfDNA) of patients exhibiting clinical progression, owing to challenges such as tumor inaccessibility, tumor heterogeneity,[12] and patient morbidity[13] to obtain tumor tissue. Depending on the platform used, the sensitivity and specificity of *EGFR*-T790M detection from ctDNA vary from 50 % to 80 % and 90 % to 100 %, respectively, when compared with tumor biopsy,[14,15] so subsequent testing of the tumor specimen is recommended if the plasma result is negative.[16,17] Diverse plasma ctDNA technologies in both commercial and academic laboratories are in routine or emerging use. However, routine liquid biopsy testing in Canada is still in the early steps of implementation.[18] In addition to being less invasive, advantages of ctDNA testing include faster processing compared with using tumor tissue, and the inclusion of genetic material from multiple sites, which might reflect tumor heterogeneity more adequately.[19]

Tumor testing for targetable genetic alterations in NSCLC is standard-of-care in most hospitals in Canada. But although ctDNA testing for the T790M variant has an

established role in the clinical management of patients with NSCLC, technical challenges have limited the availability of this testing modality. Challenges include the paucity of positive cases to serve as controls in validation cohorts, and the multiplicity of available assays. Despite the availability of the required molecular platforms in many hospitals, there is the need to assess the metrics of the assays for this testing modality during validation. To facilitate standardization and clinical adoption of ctDNA testing across Canada, a multicenter RING study to explore the development and validation of *EGFR*-T790M variant detection using plasma ctDNA testing was undertaken. We compared various methodologies in this analytical and clinical validation study to evaluate plasma T790M testing.

Materials and Methods

Experimental Design

This study was divided into an analytical validation phase (phase 1a and 1b); and a clinical validation phase (phase 2). The aim of phase 1 was analytical validation of the platforms being evaluated by the participating laboratories, which included next-generation sequencing (NGS) (Illumina TruSight Tumor 15 [TST15], Illumina; ThermoFisher Oncomine Lung cfDNA assay, Thermo Fisher Scientific), the QX200 AutoDG droplet digital polymerase chain reaction (ddPCR) system (Bio-Rad systems), the Entrogen ctEGFR Mutation Detection kit, Cobas *EGFR* Mutation Test version 2 (EntroGen), and UltraSEEKTM Lung Panel for the MassARRAY System (Agena Bioscience). Phase 2 encompassed the clinical validation by a subset of the participating sites from phase 1. A total of nine Canadian institutions participated in phase 1 and seven continued onto phase 2. The platforms, assays, and cfDNA extraction kits used by each of the sites are provided in Table 1. Reference laboratory testing was provided by the Division of Clinical Laboratory Genetics, University Health Network for all phases, as the liquid biopsy assay had already been clinically validated in this laboratory.[20] The reference laboratory method was ddPCR using the Bio-Rad Assay with custom integrated DNA Technologies primers and probes.

Phase 1: Platform Assessment and Analytical Validation

In phase 1a, laboratories were provided with commercial plasma samples (Horizon Discovery, Cambridge, United Kingdom), which consisted of human genomic DNA derived from cancer cell lines fragmented to 160 base pairs, with levels of 0.05 %, 0.5 %, and 5 % of *EGFR* variants (L858R, T790M, E746-A750del, T790M/L858R mixture), and one 0 % mutant (100 % wild-type) control. These were processed according to technical guidelines provided by the manufacturer. When possible, the ctDNA was isolated using locally established protocols at each site (Table 1). In phase 1b, the laboratories were provided with five commercially derived reference standards, though they were blinded to the identity of the variants and corresponding mutant allele frequencies. These samples were processed similarly to those in phase 1a. The primary objective of phase 1 was to enable the laboratories to choose the platform that best fits their need, and to determine the quality metrics and run parameters for each platform to obtain clinically relevant analytical sensitivity and specificity.

Phase 2: Clinical Validation

Patients were enrolled from participating sites in accordance with each site's standard process for identifying patients eligible for testing of *EGFR* T790M. Institutional ethics approval was obtained for all participating sites. Patients' consent was taken before the initial blood draw. Each participating site collected whole blood samples in DNA Streck tubes (10 mL each), with two tubes being retained for in-house ctDNA extraction and analysis, and two tubes sent to the reference laboratory for parallel clinical testing and reporting. The methods for Cobas (Roche Diagnostics, Indianapolis, IN), ddPCR, Entrogen detection kit, and NGS (Illumina TST15, ThermoFisher Oncomine Lung) assays of plasma circulating T790M variant are described in the Appendix. Laboratory 1 collected the validation data from all sites and collated it with the clinical molecular reports provided by the reference site (Fig. 1).

The sensitizing variants (exon 19 deletions and L858R) were used as a marker for the presence of ctDNA in the samples. The results from each center were compared with those reported by the reference laboratory at the conclusion of the study to determine concordance with the reference center findings. Results were reported as follows: (1) "detected" when the T790M variant was identified; (2) "undetected" if the sensitizing variant was detected but T790M was not, or if the sensitizing mutation was not tested; (3) and "inconclusive" if both the T790M and sensitizing variant were undetectable.

In addition, patient recruitment by laboratory 1 included peripheral blood samples and a corresponding tumor biopsy or a cytology specimen. Because of different institutional research ethics board policies in the other six sites participating in phase 2, a subsequent tumor or cytology specimen was obtained only after a negative or inconclusive result by the reference laboratory, and if a further biopsy was possible, which was aligned with the standard-of-care at the time in these centers.

Table 1. Testing Sites, Platforms Used, and Study Phase Participation

Laboratory #	Platform Used	Assays	Phase 1	Phase 2	cfDNA Extraction Kit Used
1	Droplet digital PCR	Bio-Rad EGFR assay and IDT (Integrated DNA Techonologies) designed primers and probes	X	X	QIAamp circulating nucleic acid kit
2	NGS (S5)	ThermoFisher Oncomine Lung cfDNA assay	X	X	QIAamp circulating nucleic acid kit
3	NGS	IlluminaTruSight Tumor 15 - TST15 panel	X	X	QIAamp circulating nucleic acid kit
4	Real-time PCR (Cobas 4800)	Cobas EGFR mutation test v2	X	X	Cobas cfDNA sample preparation kit
5	Real-time PCR	Entrogen ctEGFR mutation detection kit	X	X	Nucleosnap kit for plasma DNA
6	Real-time PCR	Entrogen ctEGFR mutation detection kit	X	X	QIAamp circulating nucleic acid kit
7	Real-time PCR (Cobas 4800)	Cobas EGFR Mutation test v2	X	X	Cobas cfDNA sample preparation kit
8	Real-time PCR (Cobas 4800)	Cobas EGFR mutation test v2	X		Cobas cfDNA sample preparation kit
9	Mass array	UltraSEEK lung panel	X		QIAamp circulating nucleic acid kit
Reference laboratory	Droplet digital PCR	Bio-Rad EGFR assay and IDT designed primers and probes	X (1b only)	X	QIAamp circulating nucleic acid kit

#, number; cfDNA, cell-free DNA; IDT, integrated DNA Technologies; NGS, next-generation sequencing; PCR, polymerase chain reaction.

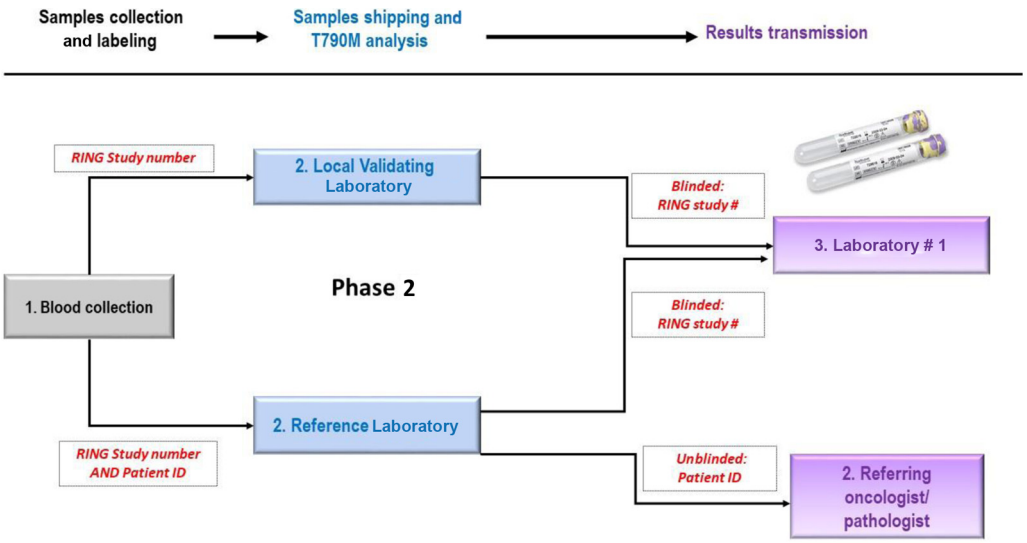

Figure 1. Phase 2 specimen collection, analysis, and results from transmission workflow. In phase 2, peripheral blood was collected in Streck tubes from patients with previously identified sensitizing *EGFR* mutations and who were exhibiting signs of clinical progression. Two Streck tubes were used for in-house validation of the platforms being evaluated by the participating labs, whereas another two tubes were shipped to the reference laboratory for routine clinical testing. A RING study number was also generated for each patient in the validating laboratory and sent along with the test requisition and specimens to the reference laboratory. For the duration of the validation, each patient specimen in the validating laboratory only had the RING study number for identification, which accompanied the subsequent results of the validation to laboratory 1 for data collation. On completion of testing by the reference laboratory, clinical reports were issued to the referring oncologist. At the same time, results were also sent to laboratory 1 (with only the corresponding RING study number) for collation with results from the validating laboratory. #, number; ID, identification document.

Table 2. Phase 1a Results

Phase 1a Samples	Mutation	Mutant Allelic Frequency, %	Laboratory 1 (ddPCR)	Laboratory 2 (NGS - ThermoFisher Oncomine)	Laboratory 3 (NGS - Illumina TST15)	Laboratory 4 (Cobas EGFR Mutation Test v2)	Laboratory 5 (Cobas EGFR Mutation Test v2)
Sample 1	EGFR T790M	0.05	None detected	None detected	None detected	None detected	None detected
Sample 2	EGFR T790M	0.50	T790M	T790M	T790M	T790M	T790M
Sample 3	EGFR T790M	5	T790M	T790M	T790M	T790M	T790M
Sample 4	EGFR L858R	0.05	L858R	L858R	None detected	None detected	None detected
Sample 5	EGFR L858R	0.50	L858R	L858R	L858R	L858R	L858R
Sample 6	EGFR L858R	5	L858R	L858R	L858R	ΔE746-A750+L858R	ΔE746-A750+L858R
Sample 7	EGFR ΔE746-A750	0.05	None detected	None detected	None detected	ΔE746-A750	ΔE746-A750
Sample 8	EGFR ΔE746-A750	0.50	ΔE746-A750	ΔE746-A750	ΔE746-A750	ΔE746-A750	ΔE746-A750
Sample 9	EGFR ΔE746-A750	5	ΔE746-A750	ΔE746-A750	ΔE746-A750	ΔE746-A750	ΔE746-A750
Sample 10	EGFR T790M/L858R	0.05	L858R	T790M	None detected	None detected	None detected
Sample 11	EGFR T790M/L858R	0.50	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M
Sample 12	EGFR T790M/L858R	5	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M
Sample 13	EGFR Wild-type	0	None detected	None detected	None detected	None detected	None detected

Phase 1a Samples	Mutation	Mutant Allelic Frequency, %	Laboratory 6 (Entrogen ctEGFR Mutation Detection kit)	Laboratory 7 (Entrogen ctEGFR Mutation Detection kit)	Laboratory 8 (Cobas EGFR Mutation Test v2)	Laboratory 9 (UltraSEEK Lung Panel)
Sample 1	EGFR T790M	0.05	None detected	None detected	T790M	None detected
Sample 2	EGFR T790M	0.50	T790M	T790M	T790M	T790M
Sample 3	EGFR T790M	5	T790M	T790M	T790M	T790M
Sample 4	EGFR L858R	0.05	L858R	None detected	None detected	None detected
Sample 5	EGFR L858R	0.50	L585R	L858R	L858R	L858R
Sample 6	EGFR L858R	5	L5858R	L858R	ΔE746-A750+L858R	L858R
Sample 7	EGFR ΔE746-A750	0.05	None detected	None detected	ΔE746-A750	None detected
Sample 8	EGFR ΔE746-A750	0.50	ΔE746-A750	ΔE746-A750	ΔE746-A750	ΔE746-A750
Sample 9	EGFR ΔE746-A750	5	ΔE746-A750	ΔE746-A750	ΔE746-A750	ΔE746-A750
Sample 10	EGFR T790M/L858R	0.05	L858R+T790M	None detected	None detected	None detected
Sample 11	EGFR T790M/L858R	0.50	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M
Sample 12	EGFR T790M/L858R	5	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M
Sample 13	EGFR Wild-type	0	None detected	None detected	None detected	None detected

ctEGFR, circulating tumor EGFR; ddPCR, droplet digital polymerase chain reaction; NGS, next-generation sequencing; TST15, TruSight Tumor 15.

Results

Analytical Validation With Commercial Controls (Phase 1a and 1b)

The results and details for the analytical validation using the commercial controls (Horizon Discovery) are summarized in Tables 2 and 3. In phase 1a, the variants and allele fractions in each of the 13 samples were disclosed to the laboratories. These were used to determine the limit of detection (LOD) and to evaluate false-positive and false-negative rates for three variants: the sensitizing ΔE746-A750 and L858R variants, and the resistance T790M variant.

All three sites validating the Cobas assay (Roche Diagnostics) (laboratories 4, 7, and 8) were able to detect the T790M variant at 0.50 % and 5 %, respectively, though only laboratory 8 was able to detect the variant at 0.05 % in sample 1 (Table 2). The L858R variant at allele frequencies of 0.5 % and 5.0 % were detected by all three labs, though none were able to detect it at 0.05 %. Interestingly, all three laboratories using the Cobas platform reported detecting the ΔE746-A750 variant in sample 6, which was known to harbor only the L858R variant at 5 %. Because none of the other platforms detected the ΔE746-A750 variant in this sample, and personal communication with the manufacturer of the controls confirmed that only the L858R variant was present, the ΔE746-A750 variant was presumed to be a false-positive call. After these results, the centers using the Cobas modified the sensitivity parameters for this specific variant. For the remaining controls, the Cobas detected the ΔE746-A750 single variant and T790M+L858R compound variants at allele frequencies of 5 % and 0.5 %, but not at 0.05 %.

Of the two sites validating the Entrogen kit, both sites were able to detect the T790M, L858R, and ΔE746-A750 single variants and compound T790M+L858R variants at 5 % and 0.5 %. One site was also able to detect the L858R single variant and compound T790M+L858R variants at 0.05 % allele frequency. Both the NGS platforms (Illumina TST15 and ThermoFisher Oncomine lung cfDNA panels) performed comparably, being able to detect the single T790M, L858R, ΔE746-A750, and compound L858R+T790M variants at 0.5 % and 5 %. The UltraSEEKTM mass array platform was able to detect the single and compound variants at 0.5 % and 5.0 %, but not at 0.05 %. The droplet digital PCR platform was able to detect the single T790M and ΔE746-A750 at 0.5 % and 5 % allele frequencies, whereas L858R (single and compound with T790M) was also detected at 0.05 %. No variants were detected in the 100 % wild-type (0 % mutant) control (sample 2016-10) by any of the platforms. Collectively for phase 1a, the 0.05 % control specimens were detected as positive only for certain variants and by some platforms. Inconsistencies were observed among similar platforms for the same variant at this low allele frequency.

In phase 1b, five blinded controls samples were assayed. The results obtained are detailed in Table 3. Briefly, all platforms were able to detect the T790M variant, either alone or as a compound with L858R at 0.5 % and 5 % allele frequencies. Similar to phase 1a, no variants were detected in the *EGFR* wild-type by any of the platforms. On the basis of the capability for all the platforms to consistently identify the tested *EGFR* variants at 0.5 %, the LOD for all assays were set to 0.5 % by the local validating labs, before proceeding to the clinical validation in phase 2. This LOD was also in line with the detection threshold of the ddPCR platform used by the reference laboratory (0.5 %).

Clinical Validation (Phase 2)

Concordance of ctDNA Results With Reference Center. Seven of the nine sites went on to enroll patients for phase 2 of the study. Collectively, 156 patients from the seven sites consented to participate in the RING study. Of these, 16 samples failed quality control metrics and were therefore not processed as part of the clinical validation. In total, 140 samples were included in phase 2. Table 4 details the *EGFR* T790M positivity rate per center. To analyze the concordance among the results reported by the reference and participating centers, all undetectable and inconclusive results were considered as negative results. Of the 140 patients whose plasma was processed for testing, the reference laboratory identified an *EGFR* T790M variant in 32 samples (22.9 %). Overall, the concordance rate of the results reported by the participating laboratories with that of the reference laboratory was high (97.9 %), with only three discordant results collectively (Table 5), coming from participating laboratories using either the real-time PCR-based Entrogen or Cobas assay (Roche Diagnostics). In two cases, the reference laboratory called a T790M variant, which was not detected by the participating laboratory; in the other, the participating laboratory called a T790M variant that was not detected by the reference laboratory. In addition, of the laboratories that did not detect a T790M variant, the sensitizing *EGFR* variant (L858R or exon 19 deletions) was detected in only 27 % of these cases in which the primary variant was tested for.

Because some centers used the same assay approach (e.g., Cobas, Entrogen, and Amplicon-based NGS), data were pooled by platform type to assess performance (Table 6). A concordance rate of 100 % with the reference laboratory was observed for the ddPCR platform (laboratory 1), and the amplicon-based NGS panels (laboratories 2 and 3). A lower concordance rate was observed for the Roche Cobas

Table 3. Phase 1b Results

Phase 1b Samples	Variant Allelic Frequency, %	Mutation	Laboratory 1 (ddPCR)	Laboratory 2 (NGS - ThermoFisher Oncomine)	Laboratory 3 (NGS - Illumina TST15)	Laboratory 4 (Cobas EGFR Mutation Test v2)	Laboratory 5 (Cobas EGFR Mutation Test v2)
Sample 1	0.50	EGFR T790M	T790M	T790M	T790M	T790M	T790M
Sample 2	5	EGFR T790M	T790M	T790M	T790M	T790M	T790M
Sample 3	0.50	EGFR T790M/L858R	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M
Sample 4	5	EGFR T790M/L858R	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M
Sample 5	0	EGFR Wild-type	None detected	None detected	None detected	None detected	None detected
Phase 1b Samples	Variant Allelic Frequency, %	Mutation	Laboratory 6 (Entrogen ctEGFR Mutation Detection kit)	Laboratory 7 (Entrogen ctEGFR Mutation Detection kit)	Laboratory 8 (Cobas EGFR Mutation Test v2)	Laboratory 9 (UltraSEEK Lung Panel)	Reference Center
Sample 1	0.50	EGFR T790M	T790M	T790M	T790M	T790M	T790M
Sample 2	5	EGFR T790M	T790M	T790M	T790M	T790M	T790M
Sample 3	0.50	EGFR T790M/L858R	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M
Sample 4	5	EGFR T790M/L858R	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M
Sample 5	0	EGFR Wild-type	None detected	None detected	None detected	None detected	None detected

ctEGFR, circulating tumor EGFR; ddPCR, droplet digital polymerase chain reaction; NGS, next-generation sequencing; TST15, TruSight Tumor 15.

(laboratories 4 and 7) and Entrogen assay (laboratories 5 and 6) at (95.8 %) and (89.5 %), respectively.

Comparison of ctDNA Results With Tumor Biopsy. Of the 52 patients enrolled by laboratory 1, a total of 39 had results from both ctDNA and tumor testing. Of these, 13 were T790M positive by both testing modalities, 10 samples were positive (for T790M) by tissue and negative by liquid, and 14 samples were negative for T790M by both tissue and ctDNA testing. No sample was negative by tissue and positive by liquid. These results were used to calculate the concordance with the reference laboratory. For the remaining phase 2 participating laboratories (laboratories 2–7), a tumor biopsy was obtained only when the ctDNA analysis yielded a negative or inconclusive result and when the patient was amenable for further tumor biopsy. The concordance between tissue and liquid biopsy testing for each laboratory is provided in Supplementary Table 1. Collectively, 25 of the 69 samples (36.2 %) with negative liquid biopsy results subsequently tested positive by tissue testing. Of note, the liquid biopsy result from the reference laboratory was used to assess the concordance.

Serial ctDNA Testing. In a small number of patients from laboratory 1, second (n = 11) and third (n = 2) liquid biopsies were tested after inconclusive results with the initial blood draw and when patients could not undergo subsequent tissue biopsies. Of these 13 patients, three (23.1 %) had a positive T790M result after a second (n = 2) or a third (n = 1) blood draw. The interval of time between the additional sample ranged between 13 and 329 days (mean = 106 d) and was 69 and 137 days in positive patients.

Discussion

Plasma ctDNA represents a promising alternative to tissue biopsy for the assessment of tumor mutational status while avoiding challenges such as tumor inaccessibility, tumor heterogeneity, and patient comorbidities. It can also serve as a personalized biomarker to monitor minimal residual disease and predict response to therapy. Despite these obvious benefits and clinical utility, ctDNA analysis has not yet been translated into routine molecular diagnostics in Canada. This, in part, may be because of the unknown dynamics of tumor shedding and inherent technical challenges of assessing potentially low levels of ctDNA, and the lack of consensus with respect to preanalytical and analytical procedures.

Here, we present a multicenter collaboration in which we leveraged platforms already being used by

Table 4. Phase 2 *EGFR*-T790M Positivity Rate Per Center

Local Laboratory #	# Cases	# Positive Cases (Local Laboratory)	# Positives Cases (Reference Laboratory)	[a]% Positive Cases
Laboratory 1 (ddPCR)	52	13	13	25.0
Laboratory 2 (NGS – Oncomine Lung cfDNA)	10	2	2	20.0
Laboratory 3 (NGS – Illumina TST15)	35	8	8	22.9
Laboratory 4 (Cobas)	8	3	3	37.5
Laboratory 5 (Entrogen)	12	5	4	33.3
Laboratory 6 (Entrogen)	7	0	1	14.3
Laboratory 7 (Cobas)	16	0	1	6.3
Total	140	31	32	22.9

[a]Percent positive cases were based on cases detected by reference laboratory.
#, number; cfDNA, cell-free DNA; ddPCR, droplet digital polymerase chain reaction; NGS, next-generation sequencing; TST15, TruSight Tumor 15.

participating laboratories for molecular profiling to facilitate the adoption and implementation of ctDNA testing in routine molecular testing. Our findings with both commercially available ctDNA reference material harboring *EGFR* variants and clinical specimens revealed that laboratories could consistently detect a 0.5 % variant allelic frequency.

As evidenced from the results, all NGS systems tested were able to consistently detect the T790M variants in plasma for samples that were positive from the reference laboratory. In addition to the excellent sensitivity and specificity, NGS allows a broad survey of multiple clinically important targets in more patients at one time compared with single target assays, which is an important consideration as the number of clinically relevant genes and targets in NSCLC continues to grow. The drawback, however, with NGS platforms is that the increased sensitivity requires increasing the coverage or read depth for each sample necessitating fewer samples per sequencing run. Inevitably, this translates to increased costs.

In the nonsequencing space, the ddPCR platform evaluated by laboratory 1 also displayed excellent sensitivity and specificity. The reliability of ddPCR in the detection and quantification of rare mutant alleles has been reported elsewhere, and this approach has been proposed to have a higher sensitivity compared with NGS.[21] In addition to lower-cost reagents, it has an easier set-up process, faster turnaround time, and does not require complex informatics for analysis. Also favorable to laboratories with low test volume is the ability to test one patient at a time without the need for batching. However, the applicability of ddPCR is limited to known variants and to those variants for which primers are available. The former limitation makes it impossible to identify other resistance mechanisms in patients progressing after first-line TKI therapy. The latter makes it difficult to determine if sufficient ctDNA was available for testing, as the rare primary sensitizing variants without available primers cannot be concurrently tested as a marker for adequate tumor shedding. In addition, multiplexing comes with challenges such as varying efficiency of individual assays, different primer annealing temperatures, possible oligonucleotide cross-dimerization, and accurate separation of fluorescent signals from a limited number of reporter dyes with overlapping emission spectra. However, it is possible to develop discriminatory multiplex ddPCR assays that enable very rapid and cost-effective monitoring for a limited number of variants in serial plasma samples, especially after target variants are identified using broader sequence profiling of tumor tissue or a baseline ctDNA sample.

Table 5. Phase 2 Concordance Rate of Local Testing Laboratory With Reference Laboratory

Local Laboratory #	# Cases	# Concordant Cases	% Concordance
Laboratory 1 (ddPCR)	52	52	100.0
Laboratory 2 (NGS – Oncomine Lung cfDNA)	10	10	100.0
Laboratory 3 (NGS – Illumina TST15)	35	35	100.0
Laboratory 4 (Cobas)	8	8	100.0
Laboratory 5 (Entrogen)	12	11	91.7
Laboratory 6 (Entrogen)	7	6	85.7
Laboratory 7 (Cobas)	16	15	93.8
Total	140	137	97.9

#, number; cfDNA, cell-free DNA; ddPCR, droplet digital polymerase chain reaction; NGS, next-generation sequencing; TST15, TruSight Tumor 15.

Table 6. Phase 2 Concordance Rate (With Reference Laboratory) by Platform

Platform	# Cases	# Concordant Results	% Concordance
ddPCR	52	52	100.0
NGS	45	45	100.0
Cobas (qPCR)	24	23	95.8
Entrogen (qPCR)	19	17	89.5

#, number; ddPCR, droplet digital polymerase chain reaction; NGS, next-generation sequencing; qPCR, quantitative polymerase chain reaction.

More traditional PCR-based assays (Roche Cobas and Entrogen ctEGFR kit) revealed a robust performance during the analytical validation, though concordance with the reference center was lower compared with the NGS and ddPCR platforms. Both platforms missed detecting the T790M variant in one patient. In each case, the variant was identified by the reference laboratory. In addition, laboratory 5, which used the Entrogen kit, also detected the T790M in the plasma of a patient in which the variant was not identified by the reference laboratory. It was not possible to establish whether these cases represented false-positive or false-negative results owing to the limited samples available. Regardless, the high concordance rate reported in our study is in line with previous studies.[10,22,23]

It was also noted that the overall positivity rate observed for laboratory 7 (Cobas) was lower compared with other participating labs, and the one positive case identified by the reference laboratory was not identified by laboratory 7. In addition, several samples from this laboratory that were tested by the reference laboratory were reported as inconclusive owing to failure to detect ctDNA, which was determined by evaluating for the sensitizing *EGFR* mutation as a proxy. Some of the factors, which could result in low ctDNA yield include improper blood collection, prolonged storage of the Streck tubes at less than ambient temperatures, insufficient training/unfamiliarity with the assay, and recruitment of patients very early during disease progression when ctDNA levels were lower than the assay LOD. Because of the limited number of specimens tested, it was not possible to identify the source of the discrepancy.

Clinical Utility of Liquid Biopsy to Detect the EGFR-T790M Resistance Mutation

EGFR-T790M detection in the plasma varied between 6.3 % and 37.5 % (average of 22.9 %) of all samples (Table 4), consistent with the literature.[9] The variable detection rates may be owing to the low patient numbers at some centers, and the variable clinical progression patterns after first or second-generation TKIs. For example, in some patients, acquired T790M variant could be detected in ctDNA before radiological progression.[24] Eligibility for this validation study required stage IIIB or IV advanced *EGFR*-mutant NSCLC and along with clinical signs of progression. However, no specific predetermined criteria were used to define clinical progression. In addition, because the presence of ctDNA has been found to correlate with tumor burden, high metabolism, and the number and size of the metastatic lesions,[25,26] and degree of vascular invasion[27] variability in these parameters would be expected to impact the detection rate of ctDNA *EGFR*-T790M. This variability may partly explain a proportion of patients in whom neither the T790M nor sensitizing variants were detected in the plasma. Whereas these variants may have been present at below the lower LOD (i.e., <0.5 %) in some patients, it is also likely that some had tumors that were ctDNA nonshedders. Of note, in a small number of patients from laboratory 1, second and third liquid biopsies were tested after inconclusive results with the initial blood draw and when patients could not undergo subsequent tissue biopsies. Of these patients, 23.1 % had a positive T790M result with the subsequent draws. Although these results were obtained in a limited number of patients from one laboratory, the findings reveal the dynamic process of ctDNA tumor shedding in the blood and suggest that it may be prudent to retest patients in whom the activating *EGFR* variant was not detected in the initial blood draw, and who are not eligible for subsequent invasive tissue biopsies.

Altogether, 69 patients (63.9 %) underwent repeat tumor biopsy after a negative or inconclusive result for liquid biopsy testing. A total of 25 of these patients (36.2 %) subsequently tested positive for T790M in their tumor biopsy (Supplementary Table 1), which is in line with the 50 % to 60 % rate reported in the literature.[28,29] This highlights key considerations regarding T790M variant testing in patients progressing on first-line EGFR TKIs. Because of limitations associated with tumor heterogeneity and the invasive nature of tissue biopsies, evidence-based best practice guidelines recommend liquid biopsy testing first to determine T790M status.[30] However, recent studies have uncovered a complex interplay that determines ctDNA release kinetics involving not only apoptosis and necrosis but also senescence.[31] This may explain why ctDNA levels do not always correlate strongly with tumor burden and suggests that these factors should be considered when analyzing ctDNA as a subset of patients with low ctDNA shed may still be at risk of clinical progression. Therefore, a negative or inconclusive liquid biopsy test should be followed by tumor testing when possible.

In conclusion, plasma ctDNA is found to be a viable option for identifying patients who would benefit from

TKI therapy for resistance mutations. Limited ctDNA yield and complex kinetics introduce a risk of false-negative results, even with sensitive and well-validated molecular detection methods. To that end, this project allowed the participating centers to develop rigorous validation protocols and data from a considerable number of clinical samples to submit as evidence to support clinical reimbursement by provincial authorities.

CRediT Authorship Contribution Statement

Shamini Selvarajah: Validation, Original draft preparation.

Sophie Plante: Project administration, Data curation, Formal analysis.

Marsha Speevak, Andrea Vaags, Elizabeth McCready, Daria Grafodatskaya, Normand Blais, Danh Tran-Thanh, Wenda Greer, Bryan Lo, Doug Demetrick, Bekim Sadikovic: Writing–reviewing and editing.

Darren Hamelinck, Xiaoduan Weng, Rami Nassabein, Stephanie Santos, Xiao Zhang, Tara Spence: Investigation.

Martin Butcher: Data curation.

Ryan N. Walton: Project administration, Writing–reviewing and editing.

Tong Zhang: Project administration.

Tracy Stockley: Resources, Writing–reviewing and editing.

Harriet Feilotter Philippe Joubert: Conceptualization, Funding acquisition, Writing–reviewing and editing.

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Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *JTO Clinical and Research Reports* at www.jtocrr.org and at https://doi.org/10.1016/j.jtocrr.2021.100212.

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