TRANSLATIONAL RESEARCH



EGFR tyrosine kinase mutation testing in the treatment of non-small-cell lung cancer

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ABSTRACT

Background

Non-small-cell lung cancer (NSCLC) tumours with activating mutations of the epidermal growth factor receptor (EFGR) tyrosine kinase are highly sensitized to the effects of oral tyrosine kinase inhibitors such as gefitinib and erlotinib, suggesting the possibility of targeted treatment of NSCLC based on *EFGR* mutation status. However, no standardized method exists for assessing the *EGFR* mutation status of tumours. Also, it is not known if available methods are feasible for routine screening. To address that question, we conducted a validation study of methods used for detecting *EGFR* mutations in exons 19 and 21 at molecular laboratories located in five specialized Canadian cancer centres.

Methods

The screening methods were first optimized using cell lines harbouring the mutations in question. A validation phase using anonymized patient samples followed.

Results

The methods used at the sites were highly specific and sensitive in detecting both mutations in cell-line DNA (specificity of 100% and sensitivity of at least 1% across all centres). In the validation phase, we observed excellent concordance between the laboratories for detecting mutations in the patient samples. Concordant results were obtained in 26 of 30 samples (approximately 87%). In general, the samples for which results were discordant were also less optimal, containing small amounts of tumour.

Conclusions

Our results suggest that currently available methods are capable of reliably detecting exon 19 and exon 21 mutations of *EFGR* in tumour samples (provided that sufficient tumour material is available) and that routine screening for those mutations is feasible in clinical practice.

KEY WORDS

Non-small-cell lung cancer, epidermal growth factor receptor, genetic testing, gefitinib, erlotinib

1. INTRODUCTION

Non-small-cell lung cancer (NSCLC) is one of the leading causes of cancer death, with a 5-year survival of approximately 16% across all stages of the disease ¹. Cytotoxic polychemotherapy with platinum compounds and other antineoplastic agents has been the standard of care for several years for the treatment of newly diagnosed patients with advanced or metastatic NSCLC. Despite the incorporation of newer, "third-generation" cytotoxic agents such as gemcitabine, taxanes, vinorelbine, and irinotecan, the efficacy of classical cytotoxic chemotherapy appears to have reached a plateau. The various combinations have shown comparable efficacy in the treatment of NSCLC, and median survival remains poor at approximately 8–14 months ^{2–6}.

The concept of personalized targeted treatment has long been the goal of cancer therapy, whereby a patient would receive treatment tailored to the particular molecular mechanism or mechanisms driving their cancer biology. Achieving that goal has been a challenge, but there have been recent successes in targeted approaches: for example, with

imatinib mesylate in chronic myelogenous leukemia, with panitumumab and cetuximab in *KRAS* wild-type colorectal cancer, and with trastuzumab in breast cancer.

Non-small-cell lung cancer tumours with activating mutations of EFGR (the gene for epidermal growth factor receptor) demonstrate enhanced sensitivity to the effects of the oral tyrosine kinase inhibitors (TKIS) gefitinib and erlotinib 7-14. In a phase III trial in East Asian lung adenocarcinoma patients enriched for the occurrence of EFGR mutations, progression-free survival (PFS) was superior for patients treated with gefitinib compared with those treated with carboplatin-paclitaxel¹¹. The 12-month PFS rates were 24.9% with gefitinib and 6.7% with carboplatin-paclitaxel [hazard ratio (HR) for progression or death: 0.74; 95% confidence interval (CI): 0.65 to 0.85; p < 0.001]. The planned subgroup analysis indicated that in EFGR mutation-positive patients, PFS was significantly longer with gefitinib than with carboplatin-paclitaxel (median: 9.5 months vs. 6.3 months; HR for progression: 0.48; 95% CI: 0.36 to 0.64; *p* < 0.001). By contrast, for EFGR mutation-negative patients, PFS was significantly longer with carboplatin-paclitaxel than with gefitinib (median: 5.5 months vs. 1.5 months; HR: 2.85; 95% CI: 2.05 to 3.98; p < 0.001). Compared with carboplatin-paclitaxel, gefitinib also produced significant increases in objective response rate, quality of life, and lung cancer symptoms. Kobayashi et al. observed similar results in a study involving patients with EFGR mutation-positive stage IIIB/IV NSCLC⁹. An interim analysis showed significantly higher response rates with gefitinib than with carboplatin-paclitaxel (74.5% vs. 29.0%, p < 0.001) and a longer PFs of 10.4 months compared with 5.5 months (HR: 0.357; 95% CI: 0.25 to 0.51; p < 0.001). A recent literature-based meta-analysis confirmed the clinical value of EFGR TKI therapy in patients with NSCLC and EFGR mutations¹⁵.

The finding of enhanced sensitivity of *EGFR* mutation–positive tumours to the oral TKIS, coupled with the availability of rapid and cost-effective methods for the detection of mutations in tumour tissue, offers the promise of using a tumour's *EGFR* mutation status to inform treatment decisions. The potential result is an increased likelihood that patients will receive optimal therapy for their NSCLC and be spared a course of therapy with no or significantly less benefit. For that promise to be realized, it is essential that available screening methods be capable of reliably and reproducibly detecting the mutations in patient tumour samples.

In current clinical practice, there is no standardized method for the detection of *EGFR* mutations in NSCLC tumour samples. Standard dideoxy

sequencing has been the "gold standard" (and arguably the primary method) for detecting somatic mutations in tissue samples. The method is exceedingly robust, but it is time-consuming and costly, and it has only moderate sensitivity in the somatic setting, requiring a sufficient quantity of tumour cells for accurate results ^{16,17}. The samples available for detection of somatic mutations in tumours are usually composed of mutant and wildtype DNA from tumour cells and wild-type DNA from nonmalignant cells specific to each anatomic site (including epithelial cells, hematopoietic cells, and stromal cells such as fibroblasts). In many cases, including in NSCLC, the amount of wild-type DNA greatly exceeds the amount of mutant DNA and may compete with it in the amplification process, making it difficult to detect and identify mutations present at extremely low concentration. Those problems emphasize the need for microdissection of the tumour area within a specimen and assessment of tumour cellularity.

The sensitivity limitations of standard dideoxy sequencing for detecting mutations (including *EGFR* mutations) have led to the development of more sensitive, less expensive, and faster polymerase chain reaction (PCR) methods, a number of which are applicable for routine clinical use ^{18–20}. These methods use various techniques to enrich mutant DNA relative to wild-type DNA to allow for accurate detection of mutations ¹⁹. Some of the methods have been coupled with real-time PCR readout to provide more rapid analysis and faster reporting ¹⁹.

The lack of a universally accepted method for clinical use raises the question of the level of concordance between various centres in their ability to accurately detect EGFR mutations in patient tumour samples, and whether routine screening for those mutations is feasible. To investigate those questions, the present study set out to determine the specificity and sensitivity of EGFR mutation assay methods used by the molecular laboratories at five specialized cancer centres in Canada, and in addition, their concordance in accurately identifying the mutation status of tumour tissues from patients with NSCLC. The assays had to detect exon 19 and exon 21 mutations, the two most common mutations in NSCLC. The study was divided into two phases: an optimization phase using cell lines, in which the laboratories refined their methodology; and then a validation phase using patient samples.

Before study initiation, it was decided that the level of detection of the mutations should be as sensitive as possible. The reasoning for that decision was twofold: first, that mutations not be missed, and second, that detection levels be similar to those in trials in which a clinical benefit from therapy with EFGR TKIS had been demonstrated (IPASS, OPTIMAL, iTARGET)^{9,11,21–23}. It was also decided to detect only mutations currently associated with a clinical benefit. Because mutations associated with resistance to an EFGR TKI had not been specifically reported in those trials and were not used as exclusion criteria, they were not included in the assay development.

1.1 Role of EGFR Activating Mutations in NSCLC

Before proceeding to describe the methods used and the results obtained, we here briefly review the role of EFGR tyrosine kinase activating mutations in NSCLC.

The most common activating mutations of *EGFR* observed in NSCLC occur in the intracellular tyrosine kinase domain. The mutations occur in exons 18–21 of the receptor, which code for key elements in and around the catalytic site of the enzyme^{24,25}. Two mutations account for more than 90% of *EGFR* mutations reported to date, namely

- in-frame deletions in exon 19 (deletions of 9, 12, 15, 18, or 24 nucleotides are normally observed), and
- a point mutation (L858R) in exon 21.

In vitro studies have confirmed that these mutations are transforming when introduced into a variety of cell lines, including fibroblasts and lung epithelial cells ^{10,26,27}.

The mechanism underlying the significantly enhanced sensitivity to the oral TKIS of NSCLC tumours with activating mutations has not been clearly established. One explanation may be that, for mutationpositive tumours, the EGFR mutation is the only (or the key) factor driving the malignant transformation. Consequently, eliminating it will inhibit tumour growth. This concept has been called "oncogene addiction" 28,29. It is proposed that the "oncogenic activation" (in this case, constitutive ligand-independent activation of EFGR) sustains a predominance of prosurvival pathways over pro-apoptotic pathways ²⁸. However, once the oncogenic activity is silenced (in this case, by the TKIS), the pro-apoptotic pathways predominate, leading to cell death 28,29. The data indicating that EGFR mutations in NSCLC appear to be practically mutually exclusive with other oncogenic mutations ³⁰ would tend to support the hypothesis of "oncogene addiction." The enhanced sensitivity to the TKIS of EFGR mutation-positive tumours may also be a result of these agents being more effective inhibitors of the mutated enzymes than the wild-type enzyme. It has been shown that gefitinib binds 20

times more tightly to the L858R mutant than to the wild-type enzyme 24 .

2. METHODS

2.1 Optimization Phase

2.1.1 Cell Lines

Three cell lines were used in the optimization phase. The human lung cancer cell lines HCC827 and H3255 were used as positive controls for the exon 19 deletion (E746-A750) and the exon 21 mutation (L858R) respectively. These cell lines were obtained from the American Type Culture Collection (ATCC: Manassas, VA, U.S.A.) and Bruce Johnson (CRL-2868, NCI-H3255). The human colorectal cancer cell line HT-29, also obtained from ATCC (catalog number HTB-38), served as the negative control for *EFGR* mutations.

The lung cancer cell lines were cultured in Iscove's modified Dulbecco's medium (catalog number 36150: StemCell Technologies, Vancouver, BC) with 15% fetal bovine serum. The negative control, HT-29, was cultured in ATCC-formulated McCoy's 5A medium (catalog number 30-2007) with 10% fetal bovine serum. Cell lines were incubated at 37°C and 5% CO_2 . After 90% confluence, all cells were collected by treatment with 0.25% trypsin–0.53 mmol/L ethylenediaminetetraacetic acid, and adjusted to a common concentration of 10⁶ cells per millilitre.

In the optimization phase of the study, samples containing selected ratios (by cell number) of the positive lung cancer cell lines and the negative colorectal control were prepared for testing. For HCC827 (exon 19 control), samples containing 0.1%, 1%, 10%, 25%, 50%, and 100% of HCC827 by volume were prepared. For H3255 (exon 21 control) samples containing 0.1%, 1%, 10%, and 50% of H3255 by volume were prepared.

Aliquots of the serially diluted cells were centrifuged to pellet the cells, and the pelletized cells were then shipped on dry ice to the participating laboratories for blinded evaluation of mutation status. A total of 11 test pellets were sent to each of the five participating laboratories. The total number of cells in each pellet was approximately 10⁶.

Each participating laboratory had to achieve a level of detection of 1% and a specificity of 100% to participate in the validation phase of the study.

2.2 Validation Phase

2.2.1 Tumour Samples

Formalin-fixed paraffin-embedded tissue samples from 30 lung adenocarcinomas (primary and metastatic sites)

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were analyzed. Cell blocks from cytology specimens and representative tumour samples from surgical specimens were included to allow for testing of a variety of specimens representative of clinical practice.

The samples were obtained from three of the participating centres. Ten tumour blocks containing tumour tissue with known EFGR mutation status (determined previously by direct sequencing) were selected from each of the three sites. Each site then prepared 5 sets of slides from each block, with a "set" consisting of 1 hematoxylin and eosin slide and 5 unstained slides. These sets were sent to one centre for distribution. To ensure blinding of the analysis during the validation phase, the centre in charge of distribution assigned random numbers to the sets of slides and distributed one set to the laboratory at each of the participating centres for testing. The personnel who assigned the random numbers at the distribution centre were not the same personnel who performed the testing, thus ensuring complete blinding of the samples. The code on the slides was broken only after all labs had submitted their data.

2.2.2 Histological Assessment of Tumour Samples

At each of the five laboratories, before the molecular analysis, the hematoxylin and eosin slide from each sample set was evaluated by a pathologist for tumour cellularity and for the presence of histologic features that might interfere with DNA quality (for example, necrosis, presence of bone suggesting prior decalcification, and so on). Tumour-rich areas were marked on the slide (see Figure 1) to guide tissue macrodissection from unstained slides.



FIGURE 1 Hematoxylin and eosin slide of sample 19 (metastatic lymph node) shows focal tumour cell infiltration surrounded by lymphocyte-rich areas that may dilute the signal for mutant sequences. The areas marked by circles are used to guide macrodissection for DNA isolation.

2.2.3 Assessment of EGFR Mutation Status

For DNA extraction and assessment of EFGR mutation status, each laboratory applied their existing or preferred methodology (Table I). For exon 19 mutation testing, four of the five laboratories used PCR plus fragment analysis ³¹. The fifth laboratory used the commercially available TheraScreen EFGR kit (DxS Limited, Manchester, U.K.). For the exon 21 mutation testing, two laboratories (labs 2 and 5) used PCR with restriction fragment length polymorphism (RFLP) plus fragment analysis; another two (labs 1 and 3) used real-time PCR methods; and lab 4 used the TheraScreen kit (as for exon 19). Labs 2 and 5 also conducted direct sequencing on the tumour samples in this phase of the study.

3. RESULTS

3.1 Optimization Phase (Cell Lines)

All of the methods used by the labs showed 100% specificity for both mutations (Table II). With respect to sensitivity, all five laboratories were able to achieve a level of detection of at least 1% for both mutations. Higher sensitivities of 0.1% were also

TABLE I	Assay methods used for the detection of EGFR mutations
at exons	19 and 21

	Lab ID	Mutation location					
		Exon 19	Exon 21				
1	Method	PCR and	FRET-PCR				
		fragment analysis ^a	(in-house method)				
	Sequencing	No	No				
2	Method	PCR and	PCR-RFLP and				
		fragment analysis ^a	fragment analysis				
	Sequencing	Yes	Yes				
3	Method	PCR and	TaqMan ^b RT-PCR				
5		fragment analysis ^a					
	Sequencing	No	No				
4	Method	TheraScreen ^c kit	TheraScreen ^c kit				
	Sequencing	No	No				
5	Method	PCR and	PCR-RFLP and				
		fragment analysis ^a	fragment analysis ^a				
	Sequencing	Yes	Yes				

^a Method from Pan *et al.*, 2005³¹.

^b Roche Molecular Systems, Pleasanton, CA, U.S.A.

^c DxS Limited, Manchester, U.K.

PCR = polymerase chain reaction; FRET = fluorescence resonance energy transfer; RFLP = restriction fragment length polymorphism.

Sample			Laboratory									
ID	Composition (%)		1 Exon		2 Exon		<u> </u>		4 Exon		5 Exon	
	HCC827	H3255	19	21	19	21	19	21	19	21	19	21
EFGR-1	100	0	+	_	+	_	+	_	+	_	+	_
EFGR-2	0	0.10	_	_	_	_	_	_	_	+	_	+
EFGR-3	25	0	+	_	+	_	+	_	+	_	+	_
EFGR-4	0	50	_	+	_	+	_	+	_	+	_	+
EFGR–5	10	0	+	_	+	_	+	_	+	_	+	_
EFGR-8	1	0	+	_	+	_	+	_	+	_	+	_
EFGR–9	50	0	+	_	+	_	+	_	+	_	+	_
EFGR-10	0	0	_	_	_	_	_	_	_	_	_	_
EFGR-11	0	10	_	+	_	+	_	+	_	+	_	+
EFGR-12	0.10	0	_	_	_	_	+	_	+	_	_	_
EFGR-13	0	1	_	+	_	+	_	+	_	+	_	+
Sensitivity (%)			1	1	1	1	0.10	1	0.10	0.10	1	0.10
Specificity (%)			100	100	100	100	100	100	100	100	100	100

TABLE II Results of the sensitivity and specificity testing in optimization phase for *EFGR* exon 19 and exon 21 mutations across the participating laboratories

+ = positive for the mutation; - = negative for the mutation.

obtained in some participating laboratories. Using the TheraScreen kit, lab 4 achieved a sensitivity of 0.1% for both mutations (Table II). Sensitivities of 0.1% were also achieved by lab 3 and lab 5 for the exon 19 and exon 21 mutations respectively (Table II).

3.2 Validation Phase (Tumour Samples)

Of the five sites, two used sequencing for verification, four used fluorescent PCR or PCR-RFLP, one used the ARMS kit (Quiagen, Toronto, ON), and one used real-time quantitative PCR for exon 21 (Table I). Table III presents the results of the validation testing. Concordant results were obtained for 26 of the 30 samples; the samples that yielded discordant results were 7, 14, 19, and 22 (Table II). The details pertaining to the discordant samples are discussed in the subsections that follow.

3.2.1 Sample 7

The consensus result for sample 7 (four of five labs, supported by sequencing) was "wild type." One lab, using the TheraScreen kit, reported a L858R mutation. The reason for this discordant result is not known, but possibilities include either a low percentage of the *EFGR* mutant being present, or a false positive result. The tumour cellularity in this case was estimated at approximately 50%.

3.2.2 Sample 14

The consensus result for sample 14 (four of five labs, supported by sequencing) was "wild type." However, one lab, using fragment analysis, reported this sample as an exon 19 deletion at low percentage. Again, that result might be attributed to the presence of a low level of the mutant being present or a false-positive result, because tumour cellularity was estimated to be approximately 40%.

3.2.3 Sample 19

The consensus for sample 19 was an exon 21 L858R mutation (four of five labs). One lab using fragment analysis and fluorescent PCR-RFLP, reported 2 mutations: an exon 19 deletion at a very low level, plus a clear exon 21 L858R mutation. The exon 19 mutation is most likely a false positive seen in a metastatic lymph node with a small amount of tumour and with the presence of a large number of lymphocytes (Figure 1). Low-level positives should be confirmed and should present at a level that is reproducible in the data analysis (threshold cut-offs should be in place).

3.2.4 Sample 22

Three of five labs reported wild-type *EGFR*; the other two labs reported exon 19 deletion (using fragment analysis and the TheraScreen kit). Because of the presence of focal lymphocyte aggregates, tumour

Sample ID	Consensus result	Laboratory						
		1	2	3	4	5		
1	Exon 21 pos					√		
2	Exon 21 pos	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark		
3	Exon 21 pos	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark		
4	WT	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark		
5	Exon 19 pos		\checkmark	\checkmark	\checkmark	\checkmark		
6	Exon 21 pos		\checkmark	\checkmark	\checkmark	\checkmark		
7	WT		\checkmark	\checkmark	L858R	\checkmark		
8	Exon 21 pos		\checkmark		\checkmark	\checkmark		
9	Exon 19 pos		\checkmark	\checkmark	\checkmark	\checkmark		
10	Exon 19 pos		\checkmark	\checkmark	\checkmark	\checkmark		
11	Exon 21 pos			\checkmark		\checkmark		
12	Exon 19 pos		\checkmark	\checkmark	\checkmark	\checkmark		
13	Exon 21 pos		\checkmark	\checkmark	\checkmark	\checkmark		
14	WT		Exon 19 pos		\checkmark	\checkmark		
15	Exon 19 pos		\checkmark		\checkmark	\checkmark		
16	Exon 19 pos		\checkmark		\checkmark	\checkmark		
17	WT		\checkmark	\checkmark	\checkmark	\checkmark		
18	Exon 21 pos		\checkmark		\checkmark	\checkmark		
19	Exon 21 pos		Exon 19 pos	\checkmark	\checkmark	\checkmark		
			Exon 21 pos					
20	Exon 21 pos		\checkmark	\checkmark	\checkmark	\checkmark		
21	Exon 19 pos		\checkmark	\checkmark	\checkmark	\checkmark		
22	WT		\checkmark	Exon 19 pos	Exon 19 pos	\checkmark		
23	WT	\checkmark	\checkmark	\checkmark	\checkmark	eq/neg (<1%)		
24	Exon 19 pos	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark		
25	Exon 21 pos	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark		
26	Exon 19 pos	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark		
27	Exon 19 pos	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark		
28	Exon 21 pos	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark		
29	Exon 19 POS	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark		
30	Exon 21 pos	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark		

TABLE III Results of validation study with tumour samples

POS = positive; EQ = equivocal.

cellularity might be lower than estimated, and when coupled with a low percentage of the mutant being present, might have led to the discordant results. In addition, one lab using sequencing identified an exon 19 L747P mutation at 10% (TTA \rightarrow CCA), which was most probably an artifact because it was not reproducible by the sequencing of an independent PCR product ³².

4. DISCUSSION AND CONCLUSIONS

The present study was conducted as part of an initiative to establish *EFGR* mutation testing centres across Canada in anticipation of the approval of gefitinib for use in first-line therapy for NSCLC patients with *EFGR* mutation—positive advanced disease. The goal of the study was, first, to use DNA obtained from cell lines to evaluate the specificity and sensitivity of the methods that would be used by the laboratories for the assay of *EFGR* exon 19 and 21 mutations, and second, to use tumour samples from NSCLC patients to validate the utility and concordance of those methods for detecting mutations. The assay methods used by the sites consisted primarily of laboratory-developed methods; a commercial kit was used by only one laboratory. For the detection of the exon 19 mutation, four of the five labs applied the same methodology—namely, PCR followed by fragment analysis³¹. The other site used the commercially available TheraScreen EFGR kit. A wider variety of methods was used for the detection

of the exon 21 mutation, with two sites using PCR-RLFP plus fragment analysis, two sites using real-time PCR, and one site using the TheraScreen kit.

The results of the assay optimization phase showed that all methods used at the sites were highly specific and sensitive in detecting *EFGR* mutations in exon 19 and exon 21 in cell-line DNA. Specificities of 100% were obtained for both mutations, with sensitivities of at least 1% across all the labs. The lab using the TheraScreen kit was able to achieve consistently higher sensitivities of 0.1% for both mutations.

In the validation phase of the study, excellent concordance between the laboratories was observed despite the varying methodologies used at some sites. Concordant results were obtained for 26 of 30 samples—that is, almost 87%. In general, the samples showing discordant results were of poorer quality, often containing very small amounts of tumour.

The results of this study also demonstrated that exon 19 analysis seems more prone to false-positive results (deletion at low percentage), and therefore thresholds to distinguish true positives from negatives need to be clearly established in the lab when data are being analyzed. Results that appear to be at the cut-off for a positive should be repeated and processed in duplicate. For laboratories performing DNA extraction from unstained slides, it is mandatory that a strict lab protocol be implemented for obtaining contaminant-free, unstained slides through thorough cleaning of microtomes, microtome blades, and slide baths. When the testing laboratory permits the use of unstained slides received directly from peripheral hospitals for DNA testing (including for EFGR), specific instructions should be given to the laboratories to ensure that a proper protocol is in place to avoid contamination, thereby reducing the likelihood of a false-positive result. Familiarity with commonly observed artifacts that are often seen in poor-quality samples ($G \rightarrow A$ or $C \rightarrow T$ sequencing artifacts) will aid in avoiding the miscalling of mutations.

The laboratories involved in the present study are located in specialized cancer centres and are highly experienced in somatic mutation detection. The results of this study suggest that, when performed by experienced laboratories, the currently available methods reported here for detecting *EFGR* mutations in exons 19 and 21 are sufficiently sensitive and accurate enough to inform treatment decisions regarding the use of TKIS such as gefitinib and erlotinib. The format of this study provides a framework for using a network approach to collaborate on assay development for the facilitation of accurate and reliable detection of mutations by multiple labs. Such an approach enables the dissemination of specialized testing to cancer centres with appropriate laboratory infrastructure and facilitates informed treatment of NSCLC.

5. CONFLICT OF INTEREST DISCLOSURES

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