Targeted Next-generation Sequencing of Circulating Biomarkers in Non-Small Cell Lung Cancer (NSCLC)

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Abstract— **In NSCLC, *EGFR* mutations are highly actionable; activating mutations (p.L858R, exon 19 deletions) confer sensitivity to tyrosine kinase inhibitors (TKIs) while p.T790M mutation confers resistance to TKIs. As an alternative to invasive methods of patient mutational profiling (tumour biopsy, fine needle aspiration cytology), variants detected from blood plasma samples of advanced lung adenocarcinoma patients were characterised using next-generation sequencing (NGS). This is in accordance with promising research into liquid biopsy and high throughput sequencing. The sensitivity and specificity of three assays in detecting *EGFR* mutations were evaluated and compared: (i) quantitative polymerase chain reaction (qPCR) assay developed in-house, (ii) NGS AmpliSeq Lung and Colon Panel assay, and (iii) NGS Oncomine Lung cfDNA assay. With patient tumour biopsies as the gold standard, the Oncomine assay was shown to attain the highest sensitivity (>60%) across all three *EGFR* mutations while the AmpliSeq assay had the greatest specificity (100%). NGS also enabled the detection of non-*EGFR* variants (AmpliSeq: n=11; Oncomine: n=12) across 11 genes due to its high throughput capabilities. In the use of liquid biopsy, cell-free DNA (cfDNA) was shown to be more useful biomarkers than circulating tumour cells (CTCs). The merits of NGS in observing and enhancing patient treatment were validated with the detection of variants such as *BRAF* p.V600E with existing or developing targeted therapies. When liquid biopsy sequencing results were compared with corresponding clinical status derived from tumour biopsy, additional p.T790M mutants were detected across all three assays. Alongside observations on monitoring of *EGFR* mutations within three sets of serial samples, the potential for NGS to be used as a non-invasive tool for monitoring of patient treatment response to EGFR TKIs was demonstrated.**

Next-generation sequencing; cell-free DNA; liquid biopsy; non-small cell lung cancer (NSCLC); circulating tumour cells

I. INTRODUCTION

Lung cancer is the most common cancer worldwide and the leading cause of cancer deaths, with non-small cell lung cancer (NSCLC) making up 85% of lung cancers [1], [2]. Research in recent years has uncovered and focused on the effect of various genetic alterations on therapeutic responses [3]. As such, the diagnosis and treatment of NSCLC have experienced a shift over the years, moving away from standardised chemotherapy to personalised therapies targeted at molecular anomalies specific to a patient's tumour.

Epidermal growth factor receptor (EGFR) mutations in NSCLC represent one of the most actionable mutations in cancer genetics, and have a relatively high incidence in Asian NSCLC patients in particular [4], [5]. In fact, certain *EGFR* mutations have been found to confer progression-free survival benefits to tyrosine kinase inhibitors (TKIs) over standard chemotherapy [6]. TKIs generally compete with the ATP binding site of the catalytic domain of the tyrosine kinase in order to interfere with the signalling pathways that are responsible for cell proliferation and cell survival. In contrast to conventional chemotherapy which does not distinguish effectively between rapidly dividing normal cells and tumour cells, TKIs present a more targeted mode of therapy which may preclude toxic side effects arising from indiscriminate targeting of cells in chemotherapy [7].

Structurally, EGFR comprises an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. Exons 18 to 21 encode a portion of the tyrosine kinase domain. Activating mutations within these exons such as exon 19 deletions and L858R mutations are known to confer sensitivity to TKIs; exon 19 deletion results in a deletion of five amino acids, while L858R mutation results in amino acid arginine being substituted for leucine at position 858. On the other hand, T790M mutation which confers resistance against TKIs occurs within exon 20 results in an amino acid substitution (threonine to methionine) at position 790 on the *EGFR* gene [4], [8], [9].

Conventional methods of extracting patient samples in order to investigate these actionable genetic alterations generally involve extraction via either tumour biopsies, or fine needle aspiration cytology [10]. These methods are invasive, and limited to the area of the tumour where the samples are taken. There is thus a growing body of research into non-invasive diagnostic methods of liquid biopsies where circulating biomarkers such as cell-free DNA (cfDNA) are detected and sequenced [11]. cfDNA comprises small fragments of DNA released from cells that are undergoing apoptosis or necrosis in primary or metastatic tumours [11]. Shed by these primary or metastatic tumours into the bloodstream, cell-free tumour DNA fragments contain vital information about the tumour. In addition, circulating tumour cells (CTCs) in patient peripheral

blood also present a viable source of tumour cells that can be assessed non-invasively [12].

Currently, investigation into such vital tumour information rely on methods such as real-time PCR which is highly sensitive in the detection of small amounts of DNA due to the selectivity of primers used, and is able to quantify the mutation level within the patient sample [13]. However, it can only detect specific known mutations, and cannot be conducted on multiple alleles at one time. On the other hand, next-generation sequencing (NGS) is an emerging method of high speed and high throughput, revolving around sequencing via synthesis. NGS is thus effective in capturing large amounts of vital tumour information, conferring advantages in terms of the scope and speed of sequencing [14]. More recently, molecular barcoding has been proposed to further improve on the sensitivity of NGS. In these assays, only amplified DNA targets that are identical to the original sequence will have unique barcodes tagged onto them [15]. Sequences without these barcodes are not read during the run, reducing chances of amplification bias and PCR errors arising from the amplification step. This novel approach to PCR enrichment increases the sensitivity of the detection of specific mutations.

In this project, cfDNA and CTCs isolated from the blood plasma of NSCLC patients were examined and characterised in order to determine their *EGFR* mutation status. This is investigated through conventional and emerging approaches i.e. quantitative polymerase chain reaction (qPCR), and Ion Torrent NGS, comprising the AmpliSeq Lung and Colon panel assay, and the Oncomine Lung cfDNA assay which incorporates the aforementioned molecular barcoding technique. This project aims to elucidate the efficacy of current and emerging approaches in the detection of actionable variants in NSCLC such as *EGFR* mutations. The use of high throughput methods such as NGS can also help to identify the presence of other mutations that may be relevant in shaping patients' clinical profiles, and their subsequent treatment.

II. Materials and Methodology

A. Study subjects

All 29 patients and 20 healthy volunteers provided written informed consent before blood samples were collected. All patients had advanced lung adenocarcinoma. 28 patients had known *EGFR* mutations from their lung biopsies. EGFR testing was not done in one patient. The healthy volunteers were recruited in the community setting and their health statuses were self-reported in a questionnaire provided.

B. Extraction and purification

Whole blood was collected in EDTA tubes and spun down at 3000rpm for 10 minutes to separate the plasma from the blood cells. Plasma was stored at -80°C until it was processed. CfDNA was then extracted and purified from 1-4mL of plasma samples using the Circulating Nucleic Acids Kit (Qiagen, Germany), with some modifications to manufacturer's protocol.

C. Circulating tumour cell (CTC) isolation

CTCs were isolated using the microsieve as previously described in N. A. Mohamed Suhaimi *et al* [12]. Briefly, 3mL of blood was passed through the microsieve at a flow rate of 0.5mL/min. After 3 washes with PBS, the sieve was removed and stored at -80°C. DNA was then extracted from the cells trapped on the sieve using the QIAamp DNA micro kit (Qiagen).

D. Quantitative PCR (qPCR) for mutation detection

A qPCR method was developed and validated in-house for the detection of *EGFR* mutations down to 0.2%. 3 major *EGFR* mutations can be detected by this method: p.E746_A750delELREA (exon 19), p.T790M (exon 20), and p.L858R (exon 21). Methods and primers used are proprietary and details are hence not included in this report. Briefly, DNA targets were amplified and mutation-specific primers were used in a qPCR reaction to specifically amplify mutant sequences. Mutant allelic fractions were determined by comparing the cycle threshold (CT) values obtained with that of standards prepared using lung cancer cell line DNA.

E. Quantification of cfDNA

Quantification of the extracted cfDNA was conducted using qPCR in duplicate, with the use of TaqMan Fast Advanced Mastermix (Applied Biosystems, Foster City, CA) and optimised probe for the B-actin gene (Hs03023880_g1). Using the Viia7 machine (Applied Biosystems, Life Technologies), qPCR was used due to the low levels of cfDNA present in blood plasma which renders conventional modes of DNA sensitivity testing such as the NanoDrop Spectrophotometer inaccurate.

F. AmpliSeq library preparation

Libraries were prepared from extracted cfDNA according to the protocol provided by manufacturers, using the Ion AmpliSeq Colon and Lung Cancer Research Panel v2.0 (Life Technologies, Austin, TX) which comprises 92 pairs of primers in a single pool covering 22 genes. As a means of quality check and quantification, the libraries were run on the Agilent 2100 Bioanalyser High Sensitivity Chip according to the protocol provided by manufacturers.

G. Oncomine library preparation

Libraries were prepared from extracted cfDNA using the Oncomine cfDNA Lung panel which covers 11 genes and >150 hotspots. Library preparation was conducted according to the protocol provided by the manufacturer (Life Technologies, Austin, TX), with some modifications made. The barcoded libraries then underwent a round of quantification and quality checking using the Ion Library Quantitation Kit (Life Technologies, Austin, TX). Serial dilutions of the *Escherichia coli* (*E.coli*) DH10B control library were prepared according to the protocol. For the dilution of the sample libraries, 1:100 and 1:1000 dilutions were used. The quantitation runs were conducted using the ViiA 7 machine.

H. Next-generation Sequencing (NGS) and NGS analysis

The AmpliSeq and Oncomine libraries were then loaded onto separate Ion Proton P1 chips that were run on the Ion Torrent Proton Sequencer, having prepared the chips on the Ion Chef System prior to the run. Data from the NGS run was analysed using Ion Reporter, where "Hotspot Minimum Allele Frequency" and "SNP Frequency" were further reduced to 0.005 and 0.01 respectively. Called variants were then further visualised using the Integrative Genomic Viewer (iGV), and cross-referenced with the COSMIC database before tabulation.

III. Results and Discussion

Clinical profiles of patients derived via tumour biopsy were used as the standard against which detection of mutations in patients using qPCR and the two NGS assays were measured. From the clinical status, of the 29 NSCLC patients, 20 patients have exon 19 deletions, eight patients have L858R. Of these, five have a concomitant T790M reported. Activating EGFR mutations in exon 19 and exon 21 are mutually exclusive. Serial samples from three patients (002, 019, and 130) were analysed. All 52 samples were analysed by qPCR and AmpliSeq. A subset of these (n=23) were analysed by Oncomine. For AmpliSeq and Oncomine respectively, mean sequencing coverage of 26347 and 116530 were attained.

A. Detection of EGFR mutations from cfDNA

TABLE I.
NUMBER OF EACH TYPE OF *EGFR* MUTATION DETECTED THROUGH QPCR AND NGS IN CFDNA

Mutation	**Tumour biopsy**	**No. of patients detected / no. of patients with mutation**		
		qPCR (Total: 29)	*AmpliSeq (Total: 29)*	*Oncomine[a] (Total: 16)*
EGFR exon 19 deletion[b]	20	10 / 15[c]	12 / 22	9 / 13
EGFR p.T790M	5	12 / 6	8 / 6	11 / 5
EGFR p.L858R	8	7 / 9	6 / 9	4 / 4

a. Not all patient samples from qPCR and AmpliSeq were used in the Oncomine Lung cfDNA Assay.
b. All variants of exon 19 deletion are considered here. The exact variant of exon 19 deletion is unreported in 2 patients.
c. Only patients with specifically delE746-A750 are considered since the primers are only specific to this particular deletion.

As reported in **Table I**, the number of patients detected to have a specified *EGFR* mutation via qPCR and AmpliSeq assay was comparable across the three *EGFR* mutations. Conversely, the Oncomine Lung cfDNA assay consistently detected a greater proportion of patients with each specific *EGFR* mutation reported via tumour biopsy. Detection via NGS appeared to uncover additional mutations previously unreported by tumour biopsy (AmpliSeq: n=2 for ex19del, n=6 for p.T790M; Oncomine: n=6 for p.T790M; where n is the number of patients with said mutation). Across both NGS assays, there seemed to be a relatively high incidence of p.T790M mutations (visually represented in Figure 4B) which were unreported by tumour biopsy. Similarly, qPCR also detected additional *EGFR* p.T790M mutations (n=8). No additional L858R mutants were reported.

It would appear that there is a relatively high level of concordance with the clinical status of the patients across qPCR and the two NGS assays (**Table I**). In particular, the Oncomine assay appears to be particularly sensitive, reaffirming the claim that it has a low detection threshold for mutations present even at 0.1% frequency [16]. However, the fact that the assays picked up on additional mutations may be indicative of a certain degree of a false positive rate, and that these mutations were false positives that could have arisen from non-specificity of primers and erroneous reads for qPCR and NGS assays respectively. Yet, the high incidence of additional p.T790M mutations specifically across all three assays suggests otherwise. Furthermore, the detection of additional p.T790M mutations have been reported in other studies too; it is possible that due to lapse in time between the tumour sample and the blood sample being taken, additional p.T790M mutations might have developed as a result of acquired resistance against certain TKIs [17].

Conversely, the detection of additional exon 19 deletions from the two NGS assays may have varying reasons; considering the sensitivity of the Oncomine assay, it can be reasoned that the exon 19 deletions detected could have fallen below the detection limits of the other two assays. Such a discrepancy could also be attributed to the nature of the samples used, where the cfDNA used in the liquid biopsy approach would have been shed from multiple sites on the tumour, leading to greater heterogeneity in its mutational profile as compared to the tumour biopsy. The latter could explain the fact that additional exon 19 deletions were also detected from the AmpliSeq assay.

B. Monitoring of serial samples in cfDNA

TABLE II.
MONITORING NUMBER AND FREQUENCY OF MUTATIONS WITHIN SERIAL PATIENT SAMPLES USING THE "LIQUID BIOPSY" APPROACH.

ID	**Date**	**Time between samples (months)**	**Clinical status (tumour biopsy)**	**Mutation**	**Frequency (%)**		
					qPCR	*AmpliSeq*	*Oncomine*
019	May-16	5.1	Unspecified exon 19 deletion	p.E746_A750delELREA	0.5	0.66	2.1
				p.T790M	UD[d]	UD	0.07
019	Oct-16		Unspecified exon 19 deletion	p.E746_A750delELREA	<0.5	UD	1.13
				p.T790M	UD	UD	0.16
002	Sep-15	5.6	Unspecified exon 19 deletion	p.Leu747_Ala750delinsPro	NA[e]	19.5	Not sequenced via Oncomine (due to limited sample)
				p.T790M	0.5-5	7	
002	Mar-16		Unspecified exon 19 deletion	p.T790M	<0.5	UD	
130	Sep-15	4.57	p.T790M; p.L858R	p.T790M	>5	5	3.35
				p.L858R	>5	20	15.16
				Ex18 p.E709K	NA	17	19.79
130	Feb-16		p.T790M; p.L858R	p.T790M	UD	UD	1.64

d. Undetected
e. Not applicable as qPCR does not cover this variant of exon 19

The detection of additional mutations was also apparent in serial patient samples, where two samples were taken from the same patient at two different time-points. These patients were receiving 3rd generation tyrosine kinase inhibitors (TKI) that specifically targets the TKI-resistant mutation T790M. In sample 019, all three assays were able to accurately detect the *EGFR* exon 19 deletion, as per the tumour biopsy in the first time-point; the Oncomine assay detected an additional *EGFR* p.T790M mutation at a very low frequency of 0.07% (**Table II**). In comparison to the first sample, the Oncomine assay appears to indicate an increase in the frequency of the *EGFR* p.T790M mutation within the serial sample. In sample 002, the unknown *EGFR* mutation reported from tumour biopsy was detected only by the AmpliSeq assay. *EGFR* p.T790M mutation was detected in its serial sample as well, although a decrease in the frequency from 0.5-5% to <0.5% was observed from qPCR (**Table II**). Similarly, in the third sample 130, there appears to be a reduction in the frequency of *EGFR* p.T790M mutation from 3.35% in the first sample to <1.64% in its serial sample via the Oncomine assay; qPCR and the AmpliSeq assay were unable to detect any p.T790M in the serial sample (**Table II**). The *EGFR* p.L858R mutation previously detected in the first sample was also absent from the serial sample, despite being reported in the corresponding tumour biopsy.

From **Table II**, an additional p.T790M mutation was detected in one of the sets of serial samples (019) via the Oncomine assay, where the increase in the frequency of the mutation presents a possible increase in resistance towards TKIs. Conversely, the other two sets of serial samples (130 and 002) showed a reduction in the frequency of p.T790M mutation (**Table II**). The lowered p.T790M mutation may be a result of treatment with AZD9291, which has been shown in a study conducted by Ku et al., 2016 to deplete EGFR T790M protein; it is possible that the use of AZD9291 is correlated with a change at the DNA level that leads to such depletion. NGS assays thus present themselves as non-invasive tools for the monitoring of patient disease progression, and a rudimentary reflection of patient response to treatment.

C. Verification of cfDNA controls' statuses

Preliminary findings from qPCR conducted indicate that *EGFR* mutations were detected in the controls (n=8), similarly detected at low levels in the Oncomine assay. The mutations detected in the controls could be false positives since they were present at minute frequencies. Cross-contamination of samples, PCR errors and erroneous reads could have contributed to false positives in qPCR and the Oncomine assay respectively. Controls in the Oncomine assay also had low molecular coverage, which affected the limit of detection.

A significant factor possibly affecting the false positive rate of the Oncomine assay would be the cfDNA loading amount for each control. With a lower amount of cfDNA loaded, in the preparation of the Oncomine libraries, amplification bias could have occurred, leading to false calls for the variants. Thus, to further increase the specificity of the Oncomine assay, sufficient input cfDNA appears to be a key requirement; further studies should be conducted in order to determine a more definitive threshold.

D. Comparison between qPCR and NGS based on cfDNA

TABLE III. COMBINED 2X2 TABLES SHOWING POSITIVES AND NEGATIVES (TRUE AND FALSE) AMONG *EGFR* MUTATIONS ACROSS THREE ASSAYS.

		qPCR			AmpliSeq			Oncomine		
***EGFR* mutations**	*Detection*	*Clinical status*		*Total*	*Clinical status*		*Total*	*Clinical status*		*Total*
		+	-		+	-		+	-	
Exon19del	Positive (+)	10	2	12	10	0	10	9	1	10
	Negative (-)	5	35	40	12	30	42	4	9	13
	Total	15	37	52	22	30	52	13	10	23
p.L858R	Positive (+)	7	1	8	6	0	6	4	1	5
	Negative (-)	2	42	44	3	43	46	1	17	18
	Total	9	43	52	9	43	52	5	18	23
p.T790M[f]	Positive (+)	3	6	9	10	0	10	5	4	9
	Negative (-)	3	40	43	12	30	42	0	14	14
	Total	6	46	52	22	30	52	5	18	23

f. p.T790M was detected in samples whereby the mutation was not reported by the clinics. The total number of samples with p.T790M detected are as follows: qPCR: 12; AmpliSeq: 8; Oncomine: 11

TABLE IV. COMPARISON OF SENSITIVITY AND SPECIFICITY ACROSS QPCR AND THE TWO NGS ASSAYS.

***EGFR* mutation**	qPCR		AmpliSeq		Oncomine	
	Sensitivity (%)	*Specificity (%)*	*Sensitivity (%)*	*Specificity (%)*	*Sensitivity (%)*	*Specificity (%)*
Exon19del	66.7	94.6	45.4	100	69.2	90.0
p.L858R	50.0	87.0	33.3	100	100	77.8
pT790M	77.8	97.7	66.7	100	80.0	94.4

The rate of concordance with the clinical status and detection of mutations in the controls contribute to the sensitivity and specificity of the assays in the detection of *EGFR* mutations. As reported in **Table III**, the AmpliSeq assay has no false positives throughout all three *EGFR* mutations. The AmpliSeq assay thus appeared to perform the best in terms of specificity, with 100% true negative rate across all three mutations (**Table IV**). However, qPCR and the Oncomine assay largely have a significant number of false positives for the p.T790M mutation (n=6 and n=4 respectively) (**Table III**). As a result, as seen in **Table IV**, specificity dipped below 90% for qPCR and the Oncomine Assay. In terms of sensitivity, the Oncomine assay consistently had the greatest performance (>60%) in the detection of all three *EGFR* mutations (**Table IV**). Conversely, the other two assays, particularly the AmpliSeq assay, appear to be unable to detect a significant proportion of the true positives (**Table III** and **IV**).

The Oncomine assay was shown to have the highest sensitivity (**Table IV**). However, it has a relatively lower specificity compared to AmpliSeq which has the best performance. Instead of using each method in silos, a complementary approach can be taken. For specific, known mutations, qPCR can be used to verify the mutations detected via the Oncomine assay; for novel, non-specific variants, the two NGS assays can be used to complement each other. Although the Oncomine assay may have lower specificity, thresholds can be determined from the controls to further filter

off false variant calls. Nevertheless, NGS assays still offers advantages such as identification of unspecified *EGFR* exon 19 deletions, as well as other *EGFR* mutations and non-*EGFR* variants, which qPCR is unable to achieve.

E. Evaluation of EGFR mutations detected from CTCs

TABLE V. *EGFR* MUTATIONS DETECTED FROM CTCS

ID	Clusters observed? (Y/N)	Mutation detected in CTC DNA by AmpliSeq / frequency (%)			Mutation detected in cfDNA by qPCR / frequency (%)	
		Exon19del	*p.L858R*	*p.T790M*	*Exon19del / p.L858R*	*p.T790M*
001	Y	0.0175	0	0.0573	UD	UD
002	Y	0.0258	0.00997	0.07278	NA	0.5-5
003	Y	0.0111	0.012	0.0585	NA	UD
006	N	0.0383	0.0267	0.0385	>5	UD
009	N	0.0156	0.0125	0.0305	>5	>5
14	N	0	0.0137	0.0774	>5	UD
130	N	0.0293	0.0111	0.0639	>5	>5
131	Y	0.0375	0.0134	0.0734	>5	UD
144	N	0	0	0.0864	0.5-5	UD
148	N	0	0.0681	0.0979	0.5-5	0.5-5
151	N	0	0	0.0526	<0.5	<0.5

We isolated CTC DNA from 11 patients who either have high levels of *EGFR* mutation detected in their cfDNA, or from whom we detected clusters of cells trapped on the microsieve in a separate experiment. We analysed the CTC DNA for *EGFR* mutations by qPCR and no *EGFR* mutations were detected in all samples.

To further confirm the absence of *EGFR* mutations in CTC DNA, as well as to probe for additional mutations e.g. TP53 that may be present, we subjected CTC DNA to NGS. Table V shows the levels of *EGFR* mutations detected by NGS by aligning the reads in iGV. All of them fall below the threshold of detection. Also, no additional mutations were found in all the samples. This indicates that the CTCs are either present at levels below the threshold of detection for qPCR and NGS, or insufficient CTCs could be captured in the first place. In that sense, CTCs appear to be less reliable biomarkers compared to cfDNA.

F. Non-EGFR variants detected in cfDNA

Apart from *EGFR* mutations, other variants were also reported in patient cfDNA samples via the AmpliSeq and Oncomine assays, as shown in **Table VI**. These variants were reported only if they had a corresponding ID within the Catalogue of Somatic Mutations in Cancer (COSMIC) database. From the AmpliSeq assay, common mutations that were detected were *TP53* (total *TP53* mutations: n=9; p.P72S: n=5, p.R249G: n=1, p.P72H: n=1, p.Gln136Glu: n=1, p.D207G: n=1) and *FBXW7* (p.R278*: n=2). *DDR2* p.L595V (n=1), *ERBB4* p.T244R (n=1) and *STK11* p.E57fs*7 (n=1) were also detected through the AmpliSeq assay. From the Oncomine assay, in decreasing order of incidences, common mutations detected were *KRAS* (total *KRAS* mutations: n=7; p.Q61H: n=4, p.G13D: n =2, p.G12D: n =1), *BRAF* (total *BRAF* mutations: n=6; p.V600E: n=6), and *TP53* (total *TP53* mutations: n=6; p.R158L: n=2, p.H179R: n =1, p.R249S: n= 1, p.C242F: n=1, p.R282W: n= 1). *PIK3CA* p.E545K (n=1), *NRAS* p.G13D (n=1) and *ALK* p.F1174L (n=1) were also detected.

Interestingly, from the Oncomine assay, there were also incidences where *KRAS* and *BRAF* mutations were detected in *EGFR* mutants (EGFR mutant with KRAS mutations: n=3, EGFR mutant with BRAF mutations: n=3, EGFR mutant with KRAS and BRAF mutations: n=3). Among the samples that underwent both assays, with regards to detection of non-EGFR variants, the Oncomine assay detected a significantly greater number (n=21) compared to AmpliSeq (n=3). In addition, the only mutation that appeared to be detected across both assays was *KRAS* p.Q61H (n=1).

The high incidence of *TP53* mutations detected across both NGS assays is in accordance with various studies which utilise NGS as a tool for high throughput mutation detection, where *TP53* mutations are said to be one of the common variants found in lung cancer patients [19]. A study conducted by Kosaka et al., 2009 suggests a correlation between TP53 mutations and shorter survival time; high throughput detection of *TP53* mutations via NGS may thus present a means of predicting patient disease prognosis.

Some of the other common mutations detected via the two NGS assays are also known to have clinical relevance in the form of existing or developing targeted therapies. Studies have indicated that NSCLC tumours with *BRAF* p.V600E mutation were responsive to vemurafenib or dabrafenib [21], [22]. *BRAF* p.V600E generally results in a mutant BRAF protein that has been observed to possess increased kinase activity which has been implicated in decreased sensitivity to EGFR TKIs. Similarly, *KRAS* mutations have been suggested to confer decreased sensitivity to EGFR TKIs, although the fact that *KRAS* mutants are generally *EGFR* wild-types could be the contributing factor to such a treatment response instead. *KRAS* mutations detected in our assay (p.G123D and p.G12D) make up the majority of KRAS mutations, although development of successful inhibitors and subsequent clinical trials are still ongoing [23].

In addition, the fact that these mutations (*KRAS* and *BRAF*) were detected in EGFR mutants was unexpected as such an observation opposes the general consensus that *KRAS*/*BRAF* mutations rarely, if at all, co-exist with *EGFR* mutations. Although KRAS has become a clear marker to preclude EGFR TKIs in other cancers such as colorectal cancer, the same impact has still not been completely established for lung cancer. Further validation of detected *KRAS*/*BRAF* variants should be conducted to verify their presence in EGFR mutants; this might prove an interesting finding, at the very least, to aid in the understanding of mutations within NSCLC.

Other reported variants such as *STK11*, *ALK*, *PIK3CA*, *ERBB4*, *NRAS* and *DDR2* are less commonly reported in lung adenocarcinoma, for which there may only be preliminary evidence of targeted therapies.

TABLE VI. COMPARISON OF NON-*EGFR* VARIANTS DETECTED IN SAMPLES VIA AMPLISEQ AND ONCOMINE ASSAY.

Sample ID	AmpliSeq		Oncomine	
	Mutation	*COSMIC ID*	*Mutation*	*COSMIC ID*
159	*KRAS* p.Q61H	COSM554	*KRAS* p.Q61H	COSM554
012	*TP53* p.P72S (missense)	COSM44018	*BRAF* p.V600E	COSM476
			KRAS p.Q61H	COSM554
025	ND		*KRAS* p.Q61H	COSM554
166			*BRAF* p.V600E	COSM476
			KRAS p.G13D	COSM532
015			*BRAF* p.V600E	COSM476
019-161008			*BRAF* p.V600E	COSM476
			TP53 p.R158L	COSM10714
018			*TP53* p.H179R	COSM10889
013			*TP53* p.R249S	COSM10817
130-160216	ND[g]		*TP53* p.C242F	COSM10810
			TP53 p.R282W	COSM10704
130-150929			*NRAS* p.G13D	COSM532
136			*KRAS* p.G12D	COSM521
144	*TP53* p.P72S (missense)	COSM44018	*ALK* p.F1174L	COSM28055
			PIK3CA p.E545K	COSM763
019-160505	ND		*BRAF* p.V600E	COSM476
			KRAS p.Q61H	COSM554
			KRAS p.G13D	COSM532
			TP53 p.R158L	COSM10714
152	*FBXW7* p.R278* (nonsense; novel)	COSM22971	*BRAF* p.V600E	COSM476
001	*FBXW7* p.R278* (nonsense; novel)	COSM22971		
	DDR2 p.L595V (missense; novel)	COSM94126		
003	*ERBB4* p.T244R (missense; novel)	COSM48368		
	TP53 p.R249G (missense)	COSM10668		
	STK11 p.E57fs*7 (frameshift del)	COSM21212		
002	*TP53* p.P72H (missense)	COSM45985		
014	[h]*TP53* p.Gln136Glu	COSM11166; COSM43767		
157	*TP53* p.D207G	COSM45519		
	TP53 p.P72S (missense)	COSM44018		
007	*TP53* p.P72S (missense)	COSM44018		
131	*TP53* p.P72S (missense)	COSM44018		

g. Not detected
h. p.Q136* (nonsense); p.Q136E (missense)

IV. CONCLUSION

In summary, the sensitivity and specificity of qPCR and the two NGS assays were evaluated, whereby the Oncomine assay was shown to achieve the highest sensitivity. Further guidelines and thresholds have to be determined in order to ensure that its specificity can be on par with the other two assays. One recommendation would be to ensure a sufficient loading cfDNA amount in order to reduce false variant calls. Between cfDNA and CTCs, however, cfDNA still seems to be a more useful biomarker. Nevertheless, the Oncomine and AmpliSeq assay were validated as tools for the targeted sequencing of *EGFR* mutations which are highly actionable in NSCLC; a small-scale study with three sets of serial samples demonstrates the possibility of NGS being used as a non-invasive means of monitoring disease progression and patient treatment response. The Oncomine assay also presents a possible new perspective to the hitherto-accepted consensus that *KRAS* and *BRAF* mutations do not exist in *EGFR* mutants; further validation with digital PCR is required.

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