Screening for activating *EGFR* mutations in surgically resected nonsmall cell lung cancer

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ABSTRACT: The clinical applicability of screening surgically resected nonsmall cell lung cancer (NSCLC) tumour tissue and serum for activating epidermal growth factor receptor (*EGFR*) mutation is unknown. Furthermore, the comparative accuracy of inexpensive *EGFR* mutation tests, mutant-enriched (ME)-PCR and high-resolution melt (HRM) has not been determined.

Lung tumour DNA from 522 surgically resected stage I–IV NSCLC and matched serum DNA from a subset of 64 subjects was analysed for *EGFR* mutations in exons 19 and 21 using ME-PCR and HRM. Additionally, 97 subjects had previous *EGFR* DNA sequencing data available for comparison.

ME-PCR and HRM detected *EGFR* mutations in 5% (27 out of 522) of tumour samples. Compared to DNA sequencing, ME-PCR had a sensitivity of 100% and specificity of 99%, while HRM had 100% sensitivity and specificity. Six subjects with *EGFR* mutation tumours had matched serum, where ME-PCR detected mutations in three samples and HRM in two samples. In the cohort of never-smoker subjects, those with *EGFR* mutated tumours had worse survival compared with wild-type tumours (30 versus 49 months; $p=0.017$).

ME-PCR and HRM have similar accuracy in detecting *EGFR* mutations but the prognostic implications of the mutations in resected NSCLC warrants further study.

KEYWORDS: Epidermal growth factor receptor, high-resolution melt analysis, mutant-enriched PCR, nonsmall cell lung cancer, prognosis, somatic mutations

Lung cancer is a global health problem, resulting in about 1 million deaths annually [1]. Nonsmall cell lung cancer (NSCLC) is the most common histological subtype, accounting for 80% of lung cancer diagnoses. The prognosis for NSCLC continues to remain poor, with a 5-yr survival of 15%. Even in early stage NSCLC (stages I and II), after patients undergo surgical resection of the tumour, recurrence of the primary tumour in stage I NSCLC occurs in 30–35% [2]. Platinum-based adjuvant chemotherapy has been shown to improve survival in stages II and IIIA, but the benefit in stage I patients is contentious [3].

Recently, in advanced NSCLC, the introduction of epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitors (TKIs) (gefitinib and erlotinib) has demonstrated improvements in clinical outcomes, particularly in subjects with mutant *EGFR* tumours [4–6]. A 15-bp deletion

involving amino acid residues leucine 747 to glutamic acid 749 in exon 19 (DEL) and a point mutation substituting arginine for leucine at codon 858 (L858R) in exon 21 account for 44 and 41%, respectively, of NSCLC *EGFR* mutations [7]. These mutations are associated with TKI responsiveness, occur in the ATP-binding pocket of the tyrosine kinase domain of *EGFR* and are referred to as “activating” mutations [8]. But while screening for activating *EGFR* mutations in advanced NSCLC has important clinical implications, the clinical utility of this strategy in early stage NSCLC subjects undergoing surgical resection is controversial, with studies providing conflicting results. While some studies have not identified a relationship between *EGFR* mutation status and overall survival [9–13], others suggest better survival among *EGFR* mutation subjects [6, 14]. Furthermore, recent studies have demonstrated that subjects with activating *EGFR* mutations may

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derive benefit, albeit nonsignificant, from adjuvant chemotherapy [9, 15]. Hence, it is important to evaluate the utility of screening for *EGFR* mutations in this population, since the presence of activating mutations may not only determine prognosis but may be predictive of TKI responsiveness, either as adjuvant therapy or in situations of tumour recurrence.

The classic method of detecting *EGFR* mutations is dideoxynucleotide sequencing. However, uptake of DNA sequencing for routine testing is impeded by relatively high cost, and the need for sophisticated equipment and technical expertise, which are often only available in specialised molecular pathology laboratories. Additionally, DNA sequencing cannot be used to accurately detect *EGFR* mutations in low tumour content samples which have <20% mutant allele content [16]. This would limit the applicability of DNA sequencing to test for *EGFR* mutations in instances such as tumour recurrence, in which it is often challenging to obtain tumour tissue for mutation analysis. Hence, there is growing interest in testing serum samples from NSCLC subjects for *EGFR* mutations [17]. Recently, novel molecular methods have been developed to accurately detect *EGFR* mutations in NSCLC tumour tissue and low tumour content samples [16]. Two such methods are mutant-enriched (ME)-PCR and high-resolution melt (HRM). ME-PCR uses restriction enzymes to selectively digest wild-type *EGFR* alleles and amplify mutant alleles [18]. ME-PCR can detect *EGFR* mutations in tissue and serum samples [19]. However, ME-PCR requires manual handling and can be subject to false positive results. Conversely, HRM does not require manual handling due to a closed tube format and is more time efficient than ME-PCR. HRM has >90% sensitivity and specificity in tumour tissue [20], but the diagnostic accuracy of HRM in serum samples has not been evaluated.

In this single-centre retrospective study, we evaluated surgically resected lung tumour tissue from 522 subjects with primary NSCLC, for the presence of *EGFR* exon 19 DEL and exon 21 mutations using ME-PCR and HRM. We also studied matched serum samples to determine the feasibility of using ME-PCR and HRM for the detection of *EGFR* mutations in a subset of 64 subjects. The relationship between *EGFR* mutation status and clinico-pathological factors, including overall survival after resection of primary NSCLC, was also evaluated.

MATERIALS AND METHODS

Samples

Fresh frozen surgically resected tumour tissue was obtained from subjects who donated remnant tissue samples to The Prince Charles Hospital, Brisbane, Australia between 1992 and 2009. Whenever possible, matched blood samples from the subjects were collected. DNA was extracted from the serum in the blood samples. The study was approved by the human research ethics committees at The Prince Charles Hospital and The University of Queensland, Brisbane, Australia. Additional detail on study samples is provided in the online supplementary material.

Dilution assays

Dilution studies were performed using American Tissue Cell Collection (Manassas, VA, USA) lung cancer cell lines, H1650 and H1975, which harbour a DEL and L858R mutation, respectively [21]. H1650 and H1975 DNA was mixed with

human female genomic DNA (Promega, Sydney, Australia) (*EGFR* wild type) in 50%, 10%, 5%, 2.5% and 0.5% dilutions.

ME-PCR assay

We found it necessary to optimise the original ME-PCR assays [18] to suit the equipment, conditions and reagents used in our laboratory. *EGFR* exon 19 DEL experiments consisted of PCR amplification followed by digestion by *Mse*I. PCR amplification was performed using ATCCCAGAAGGTGAGAAAGA TAAAATTC (forward primer, exon 19-S1) and CTGAGGT TCAGAGCCATGGA (reverse primer, exon 19-S1). The final product was analysed using 12% polyacrylamide gel electrophoresis where the 88-bp band represented the DEL allele and the 100-bp band represented the wild-type allele.

EGFR exon 21 L858R experiments were performed using two rounds of PCR amplification interspersed with digestion steps using *Msc*I and *Sau*96I, respectively. PCR amplification was performed using CAGCCAGGAACGTACTGGTGA (forward primer, exon 21-S1), CGCAGCATGTCAAGATCACAGAT (forward primer, exon 21-S2) and TCCCTGGTGTGAGAAAAT GCT (reverse primer, exon 21-AS1). The final products were analysed using 4% agarose gel electrophoresis where the 99-bp band represented the L858R allele and the 130-bp band represented the wild-type allele. Additional detail on the method and the modifications from the original ME-PCR method are provided in the online supplementary material.

HRM analysis

HRM methods and primers were adapted from published protocols [20]. The primers for *EGFR* exon 19 forward are: GTGCATCGCTGGTAACATCCA; and reverse: AAAGGTGG GCCTGAGGTTCA; and for *EGFR* exon 21 forward: CTCAC AGCAGGGTCTTCTCTG; and reverse: TGGCTGACCTAAAG CCACCTC. Each experiment was run with an *EGFR* wild-type control (human female genomic DNA) and a mutation-positive control sample. Normalised and difference curves were used to interpret the HRM results. Samples revealing skewed curves similar to the positive controls were deemed to harbour mutations. Additional detail on the method is provided in the online supplementary material.

DNA sequencing

DNA sequencing of *EGFR* exons 19 and 21 was available for 97 of the 522 subjects (part of a prior study) [11]. Separately, DNA sequencing was also used to confirm the mutation-positive samples identified by either ME-PCR or HRM. Bidirectional sequencing was performed at the Australian Genome Research Facility (Brisbane, Australia) using the HRM forward and reverse primers.

Statistical analysis

We used SPSS (Chicago, IL, USA) statistical software, version 17, for analysis. Fisher's exact test was applied to assess the relationship between *EGFR* mutation status and clinico-pathological categorical characteristics. A two-tailed p-value <0.05 was considered statistically significant. The censored 5-yr overall survival time after surgical resection was estimated using the Kaplan-Meier method and survival differences were analysed using the log-rank test.

RESULTS

Subject characteristics

We evaluated 522 resected lung tumour samples and a subset of 64 matched serum samples. There were 345 males and 177 females, 487 current or former smokers and 35 never-smokers. There were 294 subjects with adenocarcinoma, 161 subjects with squamous cell carcinomas, 32 subjects with large cell carcinoma and 35 subjects with adenosquamous carcinoma. Pathological staging revealed that 259 (49%) subjects had stage I, 140 (27%) subjects had stage II, 108 (21%) subjects had stage III and 15 (3%) subjects had stage IV NSCLC. Non-Aboriginal (inferred to be Caucasian) ethnicity was reported in 498 subjects, six subjects reported Asian ethnicity, two subjects reported Australian Aboriginal ethnicity and 16 subjects did not report their ethnicity. The clinico-pathological characteristics of the study subjects are listed in table 1.

Dilution studies of ME-PCR and HRM

We evaluated the DEL and L858R mutant allele detection threshold of ME-PCR and HRM by serially diluting cell line DNA containing heterozygous *EGFR* mutations with wild-type DNA (fig. 1). For ME-PCR, DEL and L858R mutations were detected in the 50%, 10%, 5%, 2.5% and 0.5% dilution mixtures. Using HRM, DEL mutations were detected in 50% and 10% mutant allele frequency, but not 5% or below, and L858R mutations were detected in 50%, 10%, 5% and 2.5%, but not 0.5% allele frequency.

Detection of *EGFR* mutations in tumour samples

In the 522 samples, *EGFR* mutations were detected in 27 samples (5%) by ME-PCR and HRM and 23 of these samples were *EGFR* mutation positive by both methods. Additionally, the two methods were concordant for *EGFR* wild-type in 491 tumour samples.

ME-PCR

ME-PCR detected 11 DEL mutations and 16 L858R mutations (table 2). Among the DEL mutation samples, DNA sequencing identified c.2235-2249 (p.E746-A750del) in five samples, p.L747-S752del in two samples, p.L747-E749del in one sample, and no DEL mutation was detected in three samples. In the 16 L858R mutation-positive samples, DNA sequencing identified the L858R in 15 samples, and no mutation could be detected in one sample.

HRM

HRM identified exon 19 DEL mutations in nine samples and exon 21 mutations in 18 samples (table 2). Among the DEL-positive samples, DNA sequencing identified c.2235-2249 (p.E746-A750del) in five samples, p.L747-S752del in two samples, p.L747-E749del in one sample, and no DEL mutation could be detected in one sample. In the exon 21 mutation-positive samples, DNA sequencing identified L858R in 14 samples, L861Q in one sample, R486K in one sample, P848L in one sample, and no exon 21 mutation in one sample.

Comparison of ME-PCR and HRM

The diagnostic accuracy of ME-PCR and HRM was determined by comparing the results against DNA sequencing in a cohort of 97 samples, where *EGFR* mutations were detected in 9% (nine

TABLE 1 Subject characteristics

Characteristics	Total	EGFR wild-type	EGFR mutation [#]
Subjects	522	496	26
Sex			
Male	345	337 (68)	8 (31)
Female	177	159 (32)	18 (69)
Age yrs	65 (33–85)	66 (33–85)	65 (30–82)
Ethnicity			
Caucasian	498	475 (95.5)	23 (88)
Asian	6	4 (1)	2 (8)
Indigenous	2	2 (0.5)	
Not stated/unknown	16	15 (3)	1 (4)
Smoking history			
Never	35	27 (5)	9 (35)
Former	64	62 (13)	2 (8)
Current	425	407 (82)	15 (57)
Median pack-yrs	44	40	18
Tumour size mm	40 (5–110)	40 (5–110)	32 (18–75)
Tumour differentiation			
Well to moderate	259	244 (49)	15 (58)
Poor	263	252 (51)	11 (42)
Histological diagnosis			
Adenocarcinoma	294	273 (55)	21 (81)
Squamous cell carcinoma	161	159 (32)	2 (8)
Large cell carcinoma	32	31 (6)	1 (4)
Adenosquamous carcinoma	35	33 (7)	2 (7)
Stage[*]			
IA, IB	259	255 (51)	6 (23)
IIA, IIB	140	127 (26)	11 (42)
IIIA, IIIB	108	99 (20)	9 (35)
IV	15	15 (3)	
Follow-up months	28 (1–215)	28 (1–215)	23 (1–103)
Survival status			
Alive	228	215 (43)	13 (50)
Dead	294	281 (57)	13 (50)

Data are presented as n, n (%) or median (range). EGFR: epidermal growth factor receptor. [#]: EGFR mutation status confirmed by direct DNA sequencing in all study subjects; ^{*}: pathological stage according to the American Joint Committee on Cancer staging criteria, sixth edition [22].

out of 97). ME-PCR had a sensitivity of 100% and specificity of 99%, and HRM had a sensitivity and specificity of 100%.

Detection of *EGFR* mutations in serum

In a subset of 64 subjects, *EGFR* mutation testing was performed on matched tumour tissue and serum. *EGFR* mutations were detected in six tumour samples (DEL in two samples and L858R in four samples) and *EGFR* wild-type in 58 tumour samples. In the matched serum samples of two DEL mutation tumour samples, ME-PCR and HRM identified the DEL mutation in only one of the samples. In the four L858R-positive tumour samples, ME-PCR detected the corresponding mutation in two matched serum samples and HRM detected the corresponding mutations in only one matched serum sample (table 3).

FIGURE 1. Determination of sensitivity for epidermal growth factor receptor (*EGFR*) exon 19 DEL and exon 21 L858R by high-resolution melt analysis (a and c) and mutation-enriched PCR assay (b and d). Adenocarcinoma cell lines containing *EGFR* exon 19 DEL (H1650) and exon 21 L858R (H1975) were mixed with pooled genomic DNA (*EGFR* wild-type) to produce dilutions of 50%, 10%, 5%, 2.5%, 0.5% and 0% (wild-type) dilutions. a) Difference plots obtained by high-resolution melting analysis identified 50% and 10% dilution samples as containing the DEL mutation. b) The bands of 88 bp indicate exon 19 DEL mutant alleles, and bands of 100 bp indicate wild-type alleles. The exon 19 DEL allele was detected in the 50%, 10%, 5%, 2.5% and 0.5% dilutions. c) Difference plots obtained by high-resolution melting analysis identified 50%, 10%, 5% and 2.5% dilution samples as containing the L858R mutation. d) The bands of 99 bp indicate exon 21 L858R mutant alleles, and bands of 130 bp indicate wild-type alleles. The exon 21 L858R allele was detected in the 50%, 10%, 5%, 2.5% and 0.5% dilutions.

Clinico-pathological parameters and survival analysis

We evaluated the relationship between the tumour *EGFR* mutation status, as determined by ME-PCR and HRM with clinico-pathological parameters. *EGFR* mutations were detected more frequently in never-smokers *versus* smokers (27% *versus* 3.5%; $p < 0.001$), females *versus* males (10% *versus* 2.3%; $p < 0.001$), adenocarcinoma histology *versus* non-adenocarcinoma histology (7% *versus* 2.5%; $p = 0.039$) and Asian *versus* non-Asian ethnicity (33% *versus* 5%; $p = 0.031$).

We also examined the relationship of clinico-pathological parameters, *EGFR* mutation status and overall survival at 5 yrs post-surgery. The follow-up duration was calculated from the date of surgical resection. The two variables that were associated with survival differences were tumour stage ($p < 0.001$) and differentiation ($p = 0.005$), while there were no survival differences between adenocarcinoma and squamous cell carcinoma histological subtypes ($p = 0.272$) or between the < 65 yrs and > 65 yrs age groups ($p = 0.837$) (fig. 1, online supplementary material). There was a trend towards better survival among

never-smokers compared to ever-smokers, but this difference did not reach statistical significance (44 months *versus* 39 months; $p = 0.158$). The median survival time for subjects with *EGFR*-mutated tumours was 34 months compared with 45 months for subjects with *EGFR* wild-type tumours; however, this difference was not significant ($p = 0.966$) (fig. 2). In never-smokers, *EGFR* mutation status was associated with worse survival compared to wild-type status (30 months *versus* 49 months; $p = 0.017$), while there was no difference in survival between smokers with *EGFR* mutant and wild-type tumours (43 months *versus* 39 months; $p = 0.400$) (fig. 2).

DISCUSSION

Currently, a clinico-pathological model, *i.e.* the tumour node metastases (TNM) system, remains the most reliable predictor of prognosis in surgically resected NSCLC [23]. Recent molecular NSCLC genomic studies have provided refinements to the TNM prognosis models [24]. However, these methods have not yet been demonstrated to predict therapeutic responsiveness and have not been validated in prospective studies. Conversely,

TABLE 2 Characteristics of patients with epidermal growth factor receptor (*EGFR*) mutations

Case	Age yrs	Ethnicity	Sex	Stage	Histology diagnosis	Tumour differentiation	Smoking status	ME-PCR	HRM	DNA sequencing
29	82	Caucasian	F	IIB	AC	Moderate	Never	L858R	Ex21	c.2573T>G
82	69	Caucasian	F	IB	SqC	Well	Current	L858R	Ex21	c.2573T>G
215	59	Caucasian	F	IIIA	LCC	Poor	Current	L858R	Ex21	c.2573T>G
241	51	Caucasian	F	IB	AC	Well	Current	L858R	Ex21	c.2573T>G
277	77	Caucasian	F	IIA	AC	Poor	Never	L858R	Ex21	c.2573T>G
286	36	Caucasian	M	IIB	AC	Poor	Never	L858R	Ex21	c.2573T>G
322	62	Caucasian	M	IIIB	AC	Moderate	Current	L858R	Ex21	c.2573T>G
489	70	Caucasian	F	IIB	AC	Poor	Current	L858R	Ex21	c.2573T>G
535	62	Asian	F	IIIA	ACSqC	Moderate	Current	L858R	Ex21	c.2573T>G
569	69	Caucasian	F	IIIB	AC	Moderate	Never	L858R	Ex21	c.2573T>G
680	75	Caucasian	M	IB	AC	Moderate	Current	L858R	Ex21	Wild-type
747	73	Caucasian	M	IA	AC	Moderate	Current	L858R	Ex21	c.2573T>G
823	78	Caucasian	M	IIB	AC	Poor	Former	L858R	Ex21	c.2573T>G
883	69	Caucasian	F	IIIB	AC	Moderate	Never	L858R	Ex21	c.2573T>G
928	75	Caucasian	F	IB	AC	Moderate	Current	L858R	Ex21	c.2573T>G
420	50	Caucasian	M	IIIB	SqC	Poor	Current	Wild-type	Ex21	c.2522G>A
500	68	Caucasian	F	IIB	AC	Moderate	Current	Wild-type	Ex21	c.2582T>A
646	57	Caucasian	M	IA	AC	Poor	Current	Wild-type	Ex21	c.2543C>T
27	50	Caucasian	F	IIIB	AC	Moderate	Never	DEL	DEL	E746-A750del
93	66	Asian	M	IIB	AC	Moderate	Never	DEL	DEL	L747-E749
418	61	Caucasian	M	IIA	AC	Moderate	Current	DEL	DEL	E746-A750del
499	59	Caucasian	F	IIB	AC	Poor	Never	DEL	DEL	L747-S752del
599	77	Caucasian	F	IIB	AC	Poor	Current	DEL	DEL	E746-A750del
605	77	Caucasian	F	IB	AC	Moderate	Never	DEL	DEL	L747-E749
633	59	Caucasian	F	IIIA	ACSqC	Poor	Former	DEL	DEL	E746-A750del
711	72	Not stated/unknown	F	IIB	AC	Moderate	Current	DEL	DEL	E746-A750del
781	54	Caucasian	M	IIB	AC	Poor	Current	Wild-type	DEL	Wild-type
764	62	Caucasian	F	IIIB	AC	Poor	Current	L858R	Wild-type	c.2573T>G
66	63	Caucasian	M	IA	AC	Moderate	Current	DEL	Wild-type	Wild-type
532	70	Caucasian	M	IIIA	AC	Moderate	Current	DEL	Wild-type	Wild-type
625	68	Caucasian	M	IB	AC	Well	Current	DEL	Wild-type	Wild-type

ME: mutant-enriched; HRM: high-resolution melt; F: female; M: male; AC: adenocarcinoma; SqC: squamous cell carcinoma; LCC: large cell carcinoma; ACSqC: adenosquamous cell carcinoma. *EGFR* mutation status was confirmed by direct DNA sequencing in all study subjects.

TABLE 3 Comparison of detection of epidermal growth factor receptor (*EGFR*) mutations in matched serum and tumour tissue

Serum	Tumour [#]	
	Mutation	Wild-type
Total	6	58
ME-PCR		
Mutation	3	0
Wild-type	3	58
HRM		
Mutation	2 [†]	0
Wild-type	4 ⁺	58

ME: mutant-enriched; HRM: high-resolution melt. [#]: *EGFR* mutation status determined by ME-PCR, HRM and DNA sequencing; [†]: both of the samples were also *EGFR* mutation positive by ME-PCR; ⁺: three of the samples were also *EGFR* mutation positive by ME-PCR.

determining the *EGFR* mutation status of advanced NSCLC tumours can be applied to predict the response to TKIs and standard chemotherapy, a strategy that has recently been validated in prospective clinical studies [4, 5]. While contemporary clinical practice consists of screening subjects with advanced NSCLC for *EGFR* mutations, the benefit of adopting a similar practice in the management of subjects with surgically resected NSCLC remains to be established.

In the management of subjects with surgically resected NSCLC and the current era of TKIs, one approach may be to screen for *EGFR* mutations only in subjects with recurrence of the primary malignancy. However, such a strategy implies that TKIs will not be utilised as adjuvant therapy to prevent recurrence of the primary malignancy. Hence it may be more beneficial to screen for *EGFR* mutations in the surgically resected tumour tissue, in order to determine prognosis, and in those subjects with an adverse risk profile to treat with adjuvant TKI or chemotherapy, in order to improve their outcomes. In this study, we found that when considering all

FIGURE 2. Kaplan–Meier survival curves of survival data with follow-up duration of 5 yrs after surgical resection of primary nonsmall cell lung cancers (total n=522). Censored values indicate the last known follow-up time for those subjects still alive after surgical resection. a) Subjects with wild-type epidermal growth factor receptor (*EGFR*) tumours versus mutant *EGFR* tumours. b) Never-smokers versus smokers. c) Never-smokers with mutant *EGFR* tumours versus never-smokers with wild-type *EGFR* tumours. d) Smokers with mutant *EGFR* versus smokers with wild-type *EGFR* tumours. *EGFR* mutation status in never-smokers was associated with significant survival differences. p-values are from the log-rank test.

subjects with surgically resected NSCLC, *EGFR* mutation status was not prognostic of overall survival. This is consistent with previously reported data [10, 11, 13]. Interestingly, studies that have demonstrated a survival benefit in *EGFR* mutation subjects after surgical resection have conflicting results [6, 14]. While MARKS *et al.* [6] noted a survival benefit at 5 yrs post-surgery on univariate and on multivariate analyses, a subsequent study by LIU *et al.* [14] was able to find a median survival benefit at 3 yrs post-surgery but not at 5 yrs, and on multivariate analyses but not univariate analyses. The survival benefit in the studies by MARKS *et al.* [6] and LIU *et al.* [14] may be partly explained by a higher proportion of never-smokers in the cohort of subjects with *EGFR* mutations, 90% and 47% respectively, compared with 35% in our study. However, we also found that *EGFR* mutation status is associated with worse prognosis in never-smokers but not among smokers. This interesting finding requires further investigation in a prospective study with a larger sample size of never-smokers. If validated, this observation implies that this group of subjects may be candidates for adjuvant therapy with *EGFR* TKIs. Our findings are concordant with the growing evidence that molecular changes in never-smokers with lung cancer are characteristically different from

those occurring in smokers with lung cancer [25]. This is of major clinical significance globally since 25% of lung cancer cases worldwide occur in never-smokers [25]. Consequently, screening never-smokers with surgically resected NSCLC for *EGFR* mutations may not only be prognostic but may also assist in planning adjuvant treatment.

There are currently several methods for performing *EGFR* mutation testing on NSCLC subject samples, with DNA sequencing being the current gold standard test [16]. However, the cost and technical challenges of DNA sequencing mandate that clinical *EGFR* mutation testing is performed in only a small proportion of NSCLC subjects. Our study demonstrates that ME-PCR and HRM are accurate and can be considered viable alternatives to DNA sequencing in the detection of *EGFR* mutations in tumour tissue. Either of these relatively inexpensive methods could make routine clinical testing for *EGFR* mutations practical in all NSCLC patients, particularly in resource-scarce communities, where the incidence of lung cancer is increasing [1].

The *EGFR* mutation status of 97 subjects from our study cohort has been reported previously [11], in which nine (9.3%)

subjects were *EGFR* mutation positive. The difference in frequency of *EGFR* mutation rates between the 97 subjects and the 522 subjects in the current study can be explained by the enriched population of never-smokers in the earlier study (34% versus 7%). The current study is a more accurate representation of the general NSCLC patient population in our institution. We found mutation prevalence of 5% by screening available cases resected for NSCLC at our Australian centre. This is somewhat lower than earlier studies reporting mutations of 10–16% [7, 26], each with differing inclusion criteria for enrolment. However, our data is consistent with these other studies in that higher mutation frequencies were observed in never-smokers (33%), subjects of Asian ethnicity (33%), females (10%) and those with adenocarcinoma histology (7%). Therefore, the relatively low mutation frequency may simply reflect the predominance in our subject group of Caucasian male smokers, who accounted for 64% of the study cohort and in whom the mutation frequency was 2%.

In real-life clinical scenarios, the amount of primary tumour tissue available for molecular testing is often limited and, hence, a clinically applicable test should ideally also be able to detect mutations in blood samples. Cell free DNA contained in blood is most likely released from either necrotic tumour cells and/or DNA from circulating tumour cells. Recent studies have found that *EGFR* mutations in the serum of NSCLC subjects are also predictive of responsiveness to TKIs [27]. In two out of six subjects in our study, HRM was also able to detect the same mutation in both serum and tumour tissue. ME-PCR confirmed the findings in the two serum samples. This finding suggests that, similar to ME-PCR [19], HRM may also be a useful screening test for *EGFR* mutations in serum samples. The role of screening serum for *EGFR* mutations in subjects with surgically resected NSCLC, in whom there is usually adequate tumour tissue available for mutation testing, may be in detecting recurrence or residual disease and monitoring response to adjuvant therapy with TKIs in cases of *EGFR* mutation-positive tumours.

In our study, we have screened only for the most common activating *EGFR* mutations, *i.e.* DEL and L858R. There is a growing catalogue of *EGFR* mutations in NSCLC which have considerably less frequency than the DEL and L858R mutations [28]. Screening tests are designed to detect only known mutations, as compared to DNA sequencing, which can detect both known and unknown mutations. Since we have screened only for the most common activating mutations, it is conceivable that we have overlooked other mutations, albeit less common, which may have prognostic implications in surgically resected NSCLC. Another shortcoming of our study is the disproportionately small number of subjects with matched serum and tumour tissue (12%). This limits our ability in making firm conclusions about the reliability and accuracy of ME-PCR and HRM in detecting *EGFR* mutations in serum samples. We are overcoming this limitation by modifying our biobank collection protocols to ensure that matched serums are collected from all patients who undergo surgical resection of NSCLC.

In summary, our study suggests that, although the presence of activating *EGFR* mutations is not prognostic in smokers, it is associated with worse survival in never-smoker subjects with

surgically resected NSCLC. Confirmatory studies will need to be performed, particularly in a large cohort of never-smoker subjects. Consequently, we are planning a large-scale prospective screen for *EGFR* mutations in surgically resected NSCLC, consisting of never-smokers and smokers, to validate the findings of our current study. We have also demonstrated that ME-PCR and HRM are reliable alternatives to DNA sequencing in the detection of activating *EGFR* mutations in tumour tissue. Additionally, HRM is comparable to ME-PCR, a highly sensitive method, in being able to detect *EGFR* mutations in tissue and serum. Since ME-PCR and HRM are both technically simple and high-throughput methods, they can be conveniently established in most pathology laboratories, thus enabling *EGFR* mutation testing to be more readily incorporated into clinical practice than is possible with DNA sequencing. Such a strategy will enable inexpensive routine testing for *EGFR* mutations in both surgically resected and advanced NSCLC subjects and will contribute to the delivery of personalised cancer prognosis and treatment plans.

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STATEMENT OF INTEREST

None declared.

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