An Alternative Approach to Determining Therapeutic Choices in Advanced Non-small Cell Lung Carcinoma (NSCLC): Maximizing the Diagnostic Procedure and the Use of Low-Volume Lung Biopsies

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Background: Accurate mutational analysis, especially epidermal growth factor receptor (EGFR) mutations, of diagnostic biopsies from all Asian NSCLC patients is crucial to their clinical management, but faces problems. Here, we explore, within usual hospital constraints, the practicalities of incorporating mutational analysis in every newly diagnosed case of NSCLC, namely, maximizing tissue acquisition during the diagnostic procedure and determining the maximum quantity and quality of DNA sequence data available from these biopsies.

Methods: Sixty-eight Chinese patients were enrolled. Thirty-five underwent surgical resections for early-stage tumors. Thirty-three underwent diagnostic procedures, i.e., needle aspirates under bronchoscopic or computed tomographic/fluoroscopic guidance, or forceps biopsies via bronchoscopy. Separate samples for research purposes were obtained from these 33 patients during the diagnostic procedure. All samples were analyzed for mutations in *EGFR* exons 18 to 21, *p53* exons 4 to 9, and *Kras* exon 2.

Results: No deaths occurred in this study. Success rates in obtaining sequence data from surgical samples versus low-volume samples for *EGFR*, *p53*, and *Kras* were 100% versus 85%, 100% versus 82%, and 100% versus 85%, respectively. Sequencing nine polymerase chain reaction products from each low-volume sample resulted in the exhaustion of all extracted DNA from three samples.

Conclusions: Acquiring a separate low-volume lung biopsy sample for mutational analysis in lung cancer patients during the diagnostic procedure is feasible and may be a valuable complement to the usual diagnostic workflow in future.

Key Words: Non-small cell, Mutational analysis, Diagnosis, Asian, *EGFR*, *p53*, *Kras*.

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Lung cancer remains the leading cause of cancer mortality, comprising 17.6% of cancer deaths worldwide, with an average 5-year survival rate of 9% to 15%.[1] The need for improved strategies in the management of non-small cell lung cancer (NSCLC) is made more urgent as it constitutes the majority of cases compared with small cell lung cancer (SCLC): 85% versus 15%.

As a clinical entity, East Asian nonsmoking women with lung adenocarcinoma have historically intrigued clinicians in Asia. Epidemiological studies have shown lower smoking rates of 16% to 52% in Chinese female lung cancers in a scattered geographic distribution, in contrast with 77% to 90% in white women.[2] In Chinese-dominated Singapore, records of lung cancer patients showed that one third were never smokers, of whom two thirds were female; females comprised one tenth of smokers/ex-smokers. This suggests that in Chinese women, the majority of NSCLC cannot be attributed to smoking.[3]

Since the discovery of mutations in the tyrosine kinase (TK) domain of EGFR and their prediction for clinical response to TK inhibitors (TKIs),[4,5] and the correlation with female gender, nonsmoker status, East Asian ethnicity, and adenocarcinoma subtype,[6–10] there have been efforts focusing on optimizing the diagnostic process. In so doing, rational

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treatment decisions can be made with maximal impact on patient benefit and minimal wastage in misallocated resources. This is especially pertinent in Asia where the most populous nations reside, where the target and resident populations coincide, and where health care resources vary regionally.

The majority of NSCLC cases present in the advanced stages, when surgery is not recommended. For these late-stage NSCLCs, tissue specimens for analysis are typically limited to low-volume samples acquired during the diagnostic procedure, e.g., forceps biopsies via bronchoscopy and needle aspirates guided by bronchoscopy or computed tomography (CT)/fluoroscopy. Treatment decisions based on *EGFR* mutational status rely wholly on the accurate detection of mutations in these tissue samples.

Mutational analysis of diagnostic samples is possible using paraffin-embedded tissue.[11–13] However, results from DNA sequencing performed on paraffin-embedded materials should be interpreted with caution and may require independent repeat polymerase chain reaction (PCR) for confirmation[14] (see Discussion).

Patients with advanced-stage disease do not undergo surgery, receiving chemotherapy and/or radiotherapy instead. As standard first-line treatment of late-stage NSCLC is similar for all histological subtypes, there had been no clinical need for subtype specification beyond distinguishing NSCLC from SCLC in the diagnostic samples. Cell numbers and available morphological features of the diagnostic biopsy sample also limit the level of confidence in assigning histological subtypes. With the advent of *EGFR* mutations, however, perhaps the paradigm for lung cancer diagnosis requires modification, such that treatment decisions regarding TKIs are not based on phenotype, but on genotype (mutational analysis), to derive optimal clinical benefit.

This was less of a biological study and more of an exploration of an alternative approach to mutational analysis in NSCLC patients, from the perspective of clinical feasibility. First, we adopted a sequencing strategy and evaluated the frequency of mutations in *EGFR*, *p53*, and *Kras* in surgical samples to demonstrate the reliability of our sequencing technique and validity of the sequence quality. Second, we showed that within the daily constraints of busy hospitals, we were able to extract data of similar quality from low-volume lung biopsy samples obtained in parallel with diagnostic biopsy samples, bypassing paraffin embedding. We also estimated the likely maximum amount of sequencing information that can be acquired from these low-volume tissue samples to set the preliminary stage for possible incorporation of other gene mutations of clinical significance into the routine diagnostic process in the future. As the main objective was to discover the maximal yield of mutations from low-volume samples, this study did not assess the accuracy of mutation results by comparing mutations between matched large-volume (surgical) and low-volume samples from the same patients.

In summary, this study explored the feasibility of performing mutational analysis on low-volume tissue samples that are procured separately from those for histological examination during the diagnostic biopsy procedure in the context of routine clinical workflow.

PATIENTS AND METHODS

Patients and Sample Collection

Approval for this study was obtained from the institutional review boards of the National University Hospital and Tan Tock Seng Hospital, Singapore. Samples were obtained from patients with informed consent. For patients undergoing surgery, tumor tissue (1 cm^3) was obtained from each patient ex vivo. For patients undergoing nonsurgical diagnostic procedures, study samples were obtained if the preceding sample was assessed by a cytologist to contain sufficient cells for diagnosis. Therefore, study samples were acquired at the same time as the diagnostic procedure, but were separate from the diagnostic biopsy samples, thereby not compromising the usual diagnostic process. Bronchoscopic biopsy samples were obtained with a 22-gauge Wang cytology needle (Bard Endoscopic Technologies, Billerica, MA) or forceps (Olympus America Inc., Center Valley, PA). Percutaneous needle aspirates were obtained with CT/fluoroscopic guidance using an 18- or 20-gauge Quick-Core needle (Cook Inc., Bloomington, IN), or 20-gauge Chiba or Westcott needles (Medical Device Technologies Inc., Gainesville, FL). As these were diagnostic procedures, a range of pathologies, both malignant and benign, was included (Table 1). NSCLCs of various histological subtypes were covered, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Each aspirate and surgical sample was collected in 80 μl and 1 ml of RNAlater (Ambion, Austin, TX), respectively.

Preparation of Genomic DNA

Genomic DNA was extracted from fresh surgical tissues with Puregene DNA purification kits (Gentra Systems Inc., Minneapolis, MN), using isopropanol for DNA precipitation. To maximize yield for the low-volume samples, DNA precipitation was achieved with oyster glycogen (Sigma-Aldrich, St. Louis, MO) added to the last step to a final concentration of 0.02 μg/μl and then incubated overnight at -20°C before centrifugation.[15] DNA extracted from each sample was resuspended in 15 μl of Tris-ethylenediaminetetraacetic acid (pH 7.5) buffer. Quantification of extracted DNA was performed using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

PCR and Purification of PCR Products

Exons 18 to 21 of *EGFR*, exons 4 to 9 of *p53*, and exon 2 of *Kras* were amplified by PCR. Primer sequences (forward and reverse, respectively) and PCR conditions are as follows: *EGFR* exon 18: 5′-CAAATGAGCTGGCAAGTGCCGTGTC-3′ and 5′-GAGTTTCCCAAACACTCAGTGAAAC-3′; *EGFR* exon 19: 5′-GCAATATCAGCCTTAGGTGCGGCTC-3′ and 5′-CATAGAAAGTGAACATTTAGGATGTG-3′; *EGFR* exon 20: 5′-CCATGAGTACGTATTTTGAAACTC-3′ and 5′-CATATCCCCATGGCAAACTCTTGC-3′; *EGFR* exon 21: 5′-CTAACGTTCGCCAGCCATAAGTCC-3′ and 5′-GCTGCGAGCTCACCCAGAATGTCTGG-3′; *p53* exon 4: 5′-GTAAGGACAAGGGTTGGGCT-3′ and 5′-ACTGACAGGAAGC

TABLE 1. Characteristics of Patients and Tumors

		Low-Volume Samples	
	Surgical Samples	Total	Excluding 'Others'
No. of patients	35	33	24
Age, yr			$p = 0.023$[a]
Median	64	70	70
Range	39–81	33–83	52–83
Gender			$p = 0.67$
Male	26 (74%)	25 (76%)	19 (79%)
Female	9 (26%)	8 (24%)	5 (21%)
Smoking status			$p = 0.006$
Never smokers	12 (34%)	4 (12%)	1 (4%)
Smokers and ex-smokers	23 (66%)	29 (88%)	23 (96%)
No. of samples	35	34	24
Histology/cytology			$p = 0.0004$
Adenocarcinoma	16 (46%)	6 (17%)	6 (25%), $p = 0.1$
Squamous cell carcinoma	15 (43%)	8 (23%)	8 (33%), $p = 0.46$
Large cell carcinoma	1 (3%)	5 (15%)	5 (21%), $p = 0.02$
Adenosquamous carcinoma	1 (3%)	0	0, $p = 0.4$
Undifferentiated	2 (6%)	1 (3%)	1 (4%), $p = 0.79$
NSCLC, unspecified	0	4 (12%)	4 (17%), $p = 0.01$
Others			
Malignant, non-NSCLC primary	0	3 (9%)	
Malignant, metastases	0	2 (6%)	
Nonmalignant	0	5 (15%) (2 samples were from the same patient)	

For comparisons in histology/cytology, the p value for overall distribution of subtypes is shown at the top; individual subtype comparisons are also given. The two groups being compared are surgical samples and low-volume samples, excluding others.
[a] Student's t test was used to compare the distribution of age between both groups.

CAAAGGGT-3′; *p53* exons 5-6 5′-CTAGCTCGCTAGTGGGTT G-3′ and 5′-AGGAGAAAGCCCCCCTACTG-3′; *p53* exon 7: 5′-TGCTTGCCACAGGTCTCC-3′ and 5′- AAGCTCCAGCT CCAGGTAGG-3′; *p53* exons 8 to 9: 5′-TTCCTTACTGCCTC TTGCTT-3′ and 5′-AGAAAACGGCATTTTGAGTG-3′; *Kras* exon 2: 5′-TTCTTAAGCGTCGATGGAGG-3′ and 5′-ACA GAGAGTGAACATCATGGAC-3′.

All PCR assays were carried out in 25 μl that contained 5 to 100 ng of genomic DNA, 1 unit of platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) and 0.3 mM of deoxyribonucleoside triphosphate (Bioline Ltd., London, UK). For DNA samples of concentration greater than 100 ng/μl, 100 ng was used as template; for concentration less than 100 ng/μl, 1 μl was used. Each PCR reaction was subjected to 95°C for 5 minutes before DNA amplification for 35 cycles at 95°C for 30 seconds, and 56°C for 30 seconds, 72°C for 1 minute, with final extension at 72°C for 10 minutes. QiaQuick PCR purification kits (Qiagen, Hilden, Germany) were used on PCR products amplified from surgical tissue. To avoid DNA loss through purification kit columns, exonuclease I (New England Biolabs, Ipswich, MA) and shrimp alkaline phosphatase (Promega, Madison, WI) in a 1:10 concentration ratio (ExoSAP) was added to the PCR products amplified from low-volume samples to a final volume ratio of 1.1:10, incubated at 37°C for 1 hour and then at 72°C for 15 minutes, according to manufacturer's protocols. This step removes excess primers and deoxyribonucleoside triphosphate.

DNA Sequencing

Purified PCR products were sequenced in forward and reverse directions using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Version 3) and ABI PRISM 3700 Genetic Analyzer (Applied Biosystems). Chromatograms were analyzed by manual review.

Statistical Analysis

The hypergeometric distribution was used to assess the correlation between gene mutations and patient characteristics (Tables 2 and 3). Fisher's exact test yielded similar results. The majority of p values were calculated using the χ^2 test, which was used in comparisons of patient characteristics (Table 1) and frequencies of gene mutations (Table 4); the t test was used to compare ages (Table 1).

RESULTS

Alternative Approach to Obtaining Low-Volume Lung Tissue For Mutational Analysis is Feasible

There was no mortality associated with the acquisition of patient samples in this study. The pneumothoraces induced

TABLE 2. Mutations Detected in Surgical Samples

Sample Identity	Gender	Age, yr	Histology	Smoking Status	Mutations		
					EGFR	*p53*	*Kras*
CT3	F	41	Adenocarcinoma	NS	—	—	—
CT7	M	79	Squamous cell carcinoma	S	—	—	—
CT9	M	81	Adenocarcinoma	S	—	—	—
CT11	M	61	Large cell carcinoma	S	—	—	—
CT13	F	57	Adenocarcinoma	NS	Exon 18:E709A (G>A)G719S (A>G)	—	—
CT15	M	44	Squamous cell carcinoma	S	—	—	—
CT17	M	57	Adenocarcinoma	S	—	—	—
CT19	M	73	Squamous cell carcinoma	NS	Exon 21:L858R (G>T)	—	—
CT21	M	62	Squamous cell carcinoma	S	—	—	—
CT23	M	69	Squamous cell carcinoma	S	—	—	—
CT25	M	47	Adenocarcinoma	S	—	—	—
CT27	F	72	Adenocarcinoma	NS	—	—	—
CT29	M	69	Squamous cell carcinoma	S	—	—	—
CT31	F	52	Squamous cell carcinoma	NS	Exon 21:L833V (G>T)L858R (G>T)	—	—
CT33	M	72	Adenocarcinoma	S	Exon 20:Q787Q (G>A)	—	—
CT35	F	75	Adenocarcinoma	NS	Exon 18:G719S (G>A)	—	—
CT37	M	71	Adenocarcinoma	S	—	Exon7:S241A(T>G)	—
CT39	M	74	Squamous cell carcinoma	S	—	—	Exon2:G12D(G>A)
CT41	M	66	Squamous cell carcinoma	S	—	—	—
CT43	F	76	Adenocarcinoma	NS	—	—	—
CT45	M	78	Squamous cell carcinoma	S	—	—	—
CT53	M	42	Adenocarcinoma	S	—	—	—
CT59	M	53	Undifferentiated	S	—	—	—
CT63	M	48	Squamous cell carcinoma	NS	—	—	—
CT65	M	61	Adenocarcinoma	S	—	—	—
CT71	M	64	Squamous cell carcinoma	S	—	—	—
CT73	M	64	Squamous cell carcinoma	S	—	—	—
CT75	F	65	Adenocarcinoma	NS	Exon 21:L858R (G>T)	—	—
CT77	M	69	Squamous cell carcinoma	S	—	—	—
CT81	F	39	Adenocarcinoma	NS	Econ 21:L858R (G>T)	—	—
CT105	M	67	Adenocarcinoma	S	—	—	—
S21	M	63	Undifferentiated	S	—	—	—
S25	M	73	Adenosquamous	NS	Exon 21:L858R (G>T)	—	—
S27	M	58	Squamous cell carcinoma	S	—	Exon5:R158L(G>T)	—
S29	F	63	Adenocarcinoma	NS	Exon 21:L858R (G>T)	—	—

NS, never smoker; S, smoker or ex-smoker.

iatrogenically were small and did not require draining, occurring at a rate of 14% to 15%, comparable with previous internal audit data of the participating institutions. There was no other procedure-related morbidity, e.g., uncontrollable hemorrhage, hypoxia.

The time taken to acquire a separate research sample in the same sitting as the diagnostic procedure did not significantly affect the work flow of the interventional radiologists or respiratory physicians. Their usual clinical load was adequately managed within the usual working hours. The samples for this study circumvented the usual flow to the Pathology Department and were directly processed for mutational analysis.

Patient and Tissue Sample Characteristics

A total of 68 ethnic Chinese patients were included in this study; 35 patients provided 35 surgical samples and 33 patients provided 34 low-volume samples (one patient provided two samples).

All surgical samples had confirmed histological diagnoses of various NSCLC subtypes (i.e., adenocarcinoma, squamous cell carcinoma). The 34 low-volume samples were not microscopically examined, but their corresponding diagnostic biopsy reports showed that five were nonmalignant with inflammatory and/or necrotic changes, two were metastases of other adenocarcinoma primaries (breast, salivary gland), one was a mesothelioma, one was a sarcoma, and one was a malignancy of neuroendocrine origin. Of the remaining 24 low-volume samples, all were biopsies of lung primaries, and their corresponding diagnostic biopsy samples were cytologically assessed as NSCLC, although four could not be further subtyped as adenocarcinoma, squamous cell carcinoma, etc., owing to the limitations of cell numbers and

TABLE 3. Low-Volume Samples: Mutations Detected, Biopsy Procedures, DNA Yield

Sample Identity	Gender	Age, yr	Cytology	Smoking Status	Mutations			Biopsy Procedure	DNA Yield (ng)
					EGFR	*p53*	*Kras*		
G1	M	65	Non-small cell lung cancer	S	Exon 21: L833V (G>T)L858R (G>T)	—	—	Needle aspirate	76
G2	M	78	Adenocarcinoma	S	—	—	—	Core biopsy	2941
G3	M	58	Squamous cell carcinoma	S	—	Exon 5:R158L (G>T)	—	Core biopsy	2904
G4	M	73	Squamous cell carcinoma	S	—	—	—	Needle aspirate	287
G6	M	84	Squamous cell carcinoma	S	Exon 21:L833V (G>T)L858R (G>T)	—	—	Needle aspirate	67
G7	M	54	Squamous cell carcinoma	S	—	Exon 7:M237I (G>A)	—	Needle aspirate	403
G9	M	63	Squamous cell carcinoma	S	Exon 21:L833V (G>T)L858R (G>T)	—	—	Needle aspirate	106
G10	M	70	Large cell carcinoma	S	—	—	—	Needle aspirate	198
G21	F	33	Adenocarcinoma (metastasis from breast)	NS	—	—	—	Core biopsy	1773
G22	M	75	Non-small cell lung cancer	S	Exon 21:L833V (G>T)L858R (G>T)	—	—	Core biopsy	3000
G23	F	58	Adenocarcinoma	NS	—	—	—	Needle aspirate	226
G24	M	68	Non-small cell lung cancer	S	—	—	—	Core biopsy	3214
G25	M	33	Nonmalignant, necroinflammatory	NS	—	—	—	Core biopsy	1864
G26	M	63	Undifferentiated	S	—	—	—	Needle aspirate	144
G27	M	75	Mesothelioma	S	—	—	—	Needle aspirate	657
G28	M	74	Adenocarcinoma	S	—	Exon 5:A138V (G>T)	—	Forceps	256
G29	M	59	Large cell carcinoma	S	—	—	—	Needle aspirate	196
G32	F	83	Squamous cell carcinoma	S	—	—	—	Forceps	2378
G36	M	73	Squamous cell carcinoma	S	Exon 21:L833V (G>T)L858R (G>T)	—	—	Needle aspirate	76
G37	M	70	Adenocarcinoma	S	Exon 21:L833V (G>T)	—	—	Needle aspirate	98
G38	M	59	Large cell carcinoma	S	—	Exon 6:Q192stop (C>T)	—	Needle aspirate	410
G39	M	52	Adenocarcinoma	S	—	—	—	Core biopsy	1726
G40	M	60	Adenocarcinoma	S	—	Exon 7:Y236stop (G>A)	—	Needle aspirate	321
G41	M	81	Nonmalignant	S	—	—	—	Forceps	1779
G46	M	81	Nonmalignant	S	—	—	—	Forceps	812
G67	F	82	Adenocarcinoma (metastasis from salivary gland)	S	—	—	—	Core biopsy	5993
H1	M	65	Nonmalignant, necrotic	S	—	—	—	Needle aspirate	1378

(*Continued*)

TABLE 3. (*Continued*)

Sample Identity	Gender	Age, yr	Cytology	Smoking Status	Mutations			Biopsy Procedure	DNA Yield (ng)
					EGFR	*p53*	*Kras*		
H16	M	73	Carcinoma, neuroendocrine	S	—	—	Exon 2:K5N (A>T)	Needle aspirate	2496
H18	F	58	Nonmalignant, necrotic	NS	—	—	—	Core biopsy	2856
T23	M	81	Large cell carcinoma	S	—	Exon 8:R273L (T>G)	—	Needle aspirate	2031
T24	F	80	Large cell carcinoma	S	—	—	—	Needle aspirate	707
T25	M	75	Sarcoma	S	—	—	—	Core biopsy	6901
T26	F	81	Squamous cell carcinoma	S	—	—	—	Forceps	1692
T27	M	83	Non-small cell lung cancer	S	—	—	—	Forceps	3523

G41 and G46 were obtained from the same patient. NS, never smoker; S, smoker or ex-smoker.

available morphological features. All 24 patients were in the advanced stage of disease; therefore, surgery was not part of their clinical management. Table 1 lists the patient characteristics and tumor histology/cytology. The distribution of smoking status and histological subtypes are significantly different (see Discussion).

High Success Rate in Obtaining Sequence Data from Low-Volume Lung Tissue

Genomic DNA was successfully extracted from all surgical and low-volume samples. For the low-volume samples, the range of DNA quantities extracted varied between 67 and 6900 ng (median, 812 ng; see Table 3). Typical values for the Quick-Core needle biopsies and forceps biopsies were greater than 1000 ng, whereas those for the Wang, Chiba, and Westcott needle biopsies were less than 300 ng. The size and location of the lesions helped determine which needles were used: the larger and more peripheral the lesion, the more likely the Quick-Core needle was used. The choice of needle was left to the operator's discretion.

We were able to obtain sequence data from all the surgical samples. The success rates of obtaining sequence data from the low-volume samples for *EGFR*, *p53*, and *Kras* were 85%, 82%, and 85%, respectively. The *EGFR* (exons 18, 19, 20, 21) and *p53* (exons 4, 5 + 6, 7, 8 + 9) analysis included sequencing four PCR products each, whereas only one PCR product was sequenced for *Kras* (exon 2). For *EGFR* and *p53*, success was scored as being able to obtain sequence data from at least three of four PCR products (Table 2). Additionally, sequence data from both *EGFR* exons 19 and 21 (which contained the most common mutations) had to be obtained to be considered a success (Table 5).

Figure 1 illustrates the quality of the mutation detection. All PCR products were sequenced bidirectionally, which served to confirm mutations.

Comparing Detection Rates of *EGFR*, *p53*, and *Kras* Mutations in Low-Volume Lung Tissue Samples and Surgically Resected Lung Tissue

Tables 2 and 3 illustrate the *EGFR* and *p53* mutations detected in both surgical and low-volume lung samples.

TABLE 4. Mutation Frequencies

		Surgical Group	Low-Volume Group
EGFR		9/35 (26%)	6/24 (25%), $p = 0.95$
p53		2/35 (6%)	6/24 (25%), $p = 0.034$
Kras		1/35 (3%)	0, $p = 0.4$
EGFR mutations			
	Shigematsu et al.[8]		
Females	55% (110)	67% (6/9), $p = 0.48$	0% (0/5), $p = 0.2$
Males	19% (251)	12% (3/26), $p = 0.33$	32% (6/19), $p = 0.017$
Never smokers	56% (135)	73% (8/11), $p = 0.29$	0% (0/1), $p = 0.13$
Smokers or ex-smokers	14% (226)	4% (1/24), $p = 0.17$	26% (6/23), $p = 0.26$
Adenocarcinoma	48% (214)	44% (7/16), $p = 0.74$	17% (1/6), $p = 0.13$
Nonadenocarcinoma	3% (147)	11% (2/19), $p = 0.086$	21% (3/14), $p = 0.001$; 'unspecified NSCLC' is disregarded

Comparing *EGFR*, *p53*, and *Kras* mutations between surgical and low-volume lung samples. Comparing *EGFR* mutations between Asian men and women, smokers/ex-smokers and never smokers, adenocarcinoma and nonadenocarcinoma histology, with Shigematsu et al.[8] as reference. NSCLC, non-small cell lung cancer.

TABLE 5. Success in Obtaining Sequence Data from PCR Products Amplified from Low-Volume Lung Samples

EGFR		*p53*		*Kras*	
***n* of 4 PCR Products**	**No. of Samples**	***n* of 4 PCR Products**	**No. of Samples**	***n* of 1 PCR Product**	**No. of Samples**
4	29	4	14	1	29
3	2 (exon 19 sequence not obtained)	3	14	0	5
2	0	2	1		
1	0	1	4		
0	3	0	1		
Success rate					
29/34 = 85%		28/34 = 82%		29/34 = 85%	

PCR, polymerase chain reaction.

EGFR mutations are positively correlated with female gender ($p = 0.003$), nonsmoking status ($p = 1.6e-04$) and adenocarcinoma subtype ($p = 0.03$) in the surgical group. This correlation was, however, not observed in the low-volume samples. All *EGFR* mutations detected in the low-volume group occurred in male smokers (see Discussion). Similarly, all *p53* mutations occurred in male smokers (see Discussion).

Table 4 compares the frequency of mutations detected in the surgical specimens and low-volume samples. For *EGFR*, the mutation rates were not significantly different ($p = 0.95$) at 26% and 25% for surgical and low-volume samples, respectively. No novel mutations were detected. The most frequently detected were the commonly reported L858R missense mutation, occurring in 11 of 15 samples with *EGFR* mutations, followed by the L833V mutation, occurring in seven cases. In fact, L833V was present in combination with L858R in six cases (one surgical, five low-volume samples). For *p53* mutations, the detection rates were significantly different ($p = 0.034$) at 6% and 25% for surgical and low-volume samples, respectively. Only one of 35 (3%) surgically resected tumors had a *Kras* mutation (G12D, G>A), and one *Kras* mutation (K5N, A>T) was detected in a low-volume sample that corresponded to a malignancy of neuroendocrine origin (categorized as malignant, non-NSCLC primary in Table 1). None of the mutations detected coexisted with mutations of another gene.

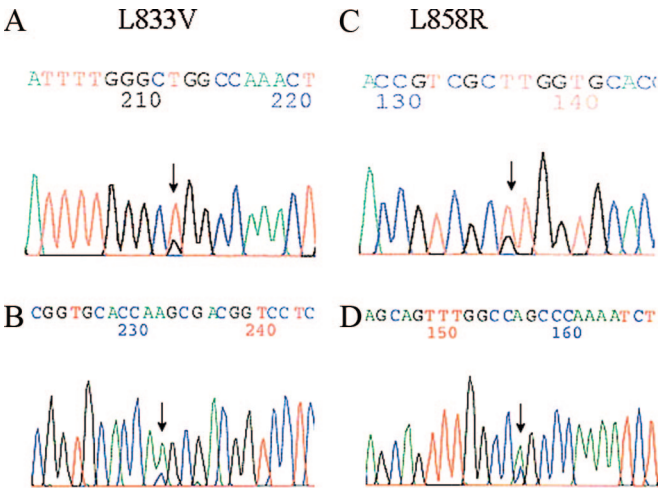

FIGURE 1. Quality of sequence data from low-volume samples. *A* and *B*: *EGFR* L833V mutation, in forward and reverse directions. *C* and *D*: *EGFR* L858R mutation, in forward and reverse directions.

Apart from the single *Kras* mutation found in the malignancy of neuroendocrine origin, there were no other mutations found in the remaining nine low-volume samples that were nonmalignant or from non-NSCLC primary malignancies and metastases.

DISCUSSION

This report examines an alternative approach to mutational analysis of low-volume lung tissue samples, with a view to clinical application. It also offers further insight into the mutational status of NSCLC in an ethnic Chinese population.

The dilemma of obtaining histological validation and maximal sequence information from a low-volume sample is not easily resolved. It would be ideal for every sample to be visually examined before the relevant cells are selected (e.g., using laser capture microdissection) for sequencing. Less than ideal would be to split the sample contents for histological validation and direct sequencing. Both scenarios are subject to acquiring enough cells per sample. Increasing the cell yield per patient can be achieved by additional sampling. The issue of sampling error then arises where the site of sampling may not be duplicated, although this may be minimized (see below). Nonetheless, it may be reasonable to regard directly sequencing a separate sample as being equivalent to directly sequencing one part of a sample that has been split (one part for direct sequencing, one part for histological validation), in the sense that in both cases, the cells for sequencing were not first directly validated histologically. On this basis, we designed the study such that a diagnostic sample is obtained for histological validation, and a research sample is acquired for direct sequencing.

With each additional sample acquisition, the impact on clinical safety and work flow also needs to be considered. Unlike other sites where sample acquisition poses low clinical risk, e.g., breast, skin, obtaining samples of lung tissue involves greater morbidity. Clinical risk notwithstanding, as sequencing expertise is getting more widely available and increasingly affordable, it seemed sensible to explore its

inclusion in hospital work flows, especially in Asia where the expected clinical impact is great. On balance, we considered it a worthwhile effort to obtain more cells per patient for direct sequencing, not only to increase the chances of capturing sequence data from as many patients as possible, but also to see whether the reliance on paraffin-embedded cells could be lessened. Our emphasis was on striking a balance between the practical feasibility within usual hospital constraints (e.g., financial, clinical safety, hospital work flow, timeliness of results), and the delineation of the limits of DNA sequence information obtainable. We would not lay claim that we have obtained results comparable with the ideal situation outlined earlier. Instead, this is an exploration of alternative measures to circumvent the inherent limitations and difficulties in analyzing low-volume samples.

Essentially, the existing issues in current practice that impede the broad application of mutational analysis to all newly diagnosed lung cancer patients are as follows: (1) insufficient cells in the diagnostic samples; (2) paraffin embedding may cause deamination, masking true mutational status; (3) limited availability of precision equipment for cell selection, e.g., laser capture microdissection.

Not every case has sufficient cells left over from the diagnostic biopsy with which to construct paraffin-embedded cell blocks. In the Shih et al.[16] retrospective study of lung cancer patients receiving gefitinib treatment, in 75 of 139 (54%) patients, there were no paraffin-embedded cells from their diagnostic biopsy samples, and thus they were excluded from analysis. Scraping cells off the diagnostic glass slide is an alternative that we have not evaluated. However, the physical manipulation and staining/destaining processes may be expected to compromise DNA quality and quantity.

Analyzing mutations in paraffin-embedded cells has been criticized, owing to the likely deamination of cytosine/adenine residues.[14] This change is reflected in subsequent PCR amplifications, giving rise to inaccurate mutation results. Taking a separate sample for immediate DNA sequencing offers an alternative route to acquiring similar information from paraffin-embedded tissue. Additional sampling also ensures increased cell numbers, but introduces another foreseeable limitation, namely, the possible nonconcordance of both samples due to the lack of morphological confirmation of the additional sample. Attempting to biopsy twice at the same site may minimize this, although this is dependent on the skill of the operators and the size and location of the lesion. For core biopsy samples, they are obtained coaxially through a sheath. By keeping the sheath in the same position, the likelihood of being able to obtain a biopsy sample from the same site again is increased. Bronchoscopists are guided visually by the preceding puncture site.

Laser capture microdissection can enrich for tumor cells, increasing the sensitivity of analysis. However, this equipment is not widely available and requires additional processing time. Constructing paraffin-embedded cell blocks is also time-consuming. Recently developed methods of detecting mutations, e.g., denaturing high-performance liquid chromatography,[17,18] may be used as screening measures before sequencing, thereby increasing overall efficiency of analysis. Again, availability of instruments is limited.

For any new measure to be successfully incorporated into clinical practice requires consideration of the degree of clinical benefit that can be promptly derived and the ease of integration into hospital workflows. Obtaining an additional sample at diagnosis appears to satisfy both criteria.

The ongoing IRESSA pan-Asian study trial (AstraZeneca) directly compares first-line gefitinib with standard intravenous platinum-based chemotherapy for advanced NSCLC in an Asian population. If the outcome clarifies and supports the role of *EGFR* mutational analysis at diagnosis, then devising hospital workflows to provide timely mutation results to the clinic will benefit patients.

By investigating the presence or absence of *EGFR*, *p53*, and *Kras* mutations, we had simultaneously explored the limits of low-volume samples for mutational analysis by sequencing. We conclude that the majority of low-volume samples had sufficient DNA to accommodate the sequencing of nine PCR products, even allowing for failed attempts. There were three samples where the extracted DNA was entirely used for this study. This may be taken into consideration in the future, when synthesizing a panel of the most clinically useful mutations to be analyzed in the diagnosis of lung cancer.

The lack of correlation between *EGFR* mutations and female gender, histology, and smoking status in the low-volume samples may be related to the different distributions of smokers and histological subtypes between the surgical and low-volume sample groups (Table 1). Whether this was affected by the patients' perception of risk and consent giving is unclear. Although all patients who consented to surgery also consented to providing research samples (no risk), only 40% of patients approached for low-volume samples gave consent (risk of morbidity).

Table 4 compares the rates of Shigematsu et al.[8] of *EGFR* mutations in surgical tumors from East Asians with our corresponding rates in the surgical and low-volume sample groups. Although there is good concordance of results between Shigematsu et al. and our surgical group, validating the quality of our sequence analysis, there are apparent differences as compared with the low-volume sample group. The greater proportion of males, smokers, and nonadenocarcinomas with *EGFR* mutations in the low-volume sample group may represent a true phenomenon or may be partly attributed to a hidden selection bias from the consent-giving process.

We compared results from our low-volume sample group with those reported by Shih et al.[16] They had examined *EGFR* mutations in paraffin-embedded cells from low-volume lung samples, which occurred in 49% of adenocarcinomas, in contrast with our result of 17%. However, unlike our unselected series, their retrospective series was selected for NSCLC and comprised mainly adenocarcinomas (92%). Our lower-than-expected figure could also be attributed to the exclusion from our calculation of NSCLC cases that could not be further subtyped but harbored *EGFR* mutations. If we had assumed they were adenocarcinomas, the proportion

would be 50%. Although we did not report exon 19 deletions here, we found them in subsequent analysis of tumors not included in this study, using the same sequencing method.

Nevertheless, the observed discrepancies need not detract from the main focus of this study, i.e., the feasibility of acquiring additional low-volume lung samples for mutational analysis. We note that two patients with *EGFR* mutations from the low-volume sample group had cytological reports of NSCLC without further subtyping. According to phenotype, the likelihood of their being offered TKI would have been diminished, as they are male smokers. In so doing, the potential clinical benefit for these two patients would not have been realized.

Both *p53* and *Kras* mutations have been implicated in smokers with NSCLC.[8,19–21] With the greater preponderance of smokers in the low-volume sample group than the surgical group, we would expect a higher frequency of *p53* and *Kras* mutations in the former than the latter. This is the case for *p53*, where the frequency of mutations is significantly higher (25% versus 6%). However, there were no *Kras* mutations in the low-volume sample group, and only 3% of the surgical samples had a *Kras* mutation. This could be partly due to the smaller proportion of adenocarcinomas (25%) in the low-volume group compared with the surgical group (46%), as *Kras* mutations are positively correlated with adenocarcinomas.[8,22,23]

Stage of disease is an important determinant of prognosis and treatment decisions in NSCLC. The role of p53 as a prognostic factor in lung cancer has been studied in relation to disease stage and included in several meta-analyses.[24–26] We could hypothesize that the increased frequency of *p53* mutations in the low-volume sample group, as compared with the surgical group, is a reflection of the different stages of disease represented by both groups. Whereas the surgical group consisted of patients with early-stage disease (stages I/II), the low-volume group comprised patients with late-stage disease (stages III/IV). In this context, the difference in disease stage between the surgical group and the low-volume group has allowed a preliminary comparison of mutation patterns between early- and late-stage disease.

There are several other aspects to consider regarding the limitations of our study. The expected low cell numbers per low-volume sample was a compelling factor in our decision to sacrifice histological confirmation of the low-volume research samples and use the histology reports of the corresponding diagnostic biopsies as proxy to achieve the overall objective of gaining the maximal amount of sequencing information from the maximal number of exons. This led to the preclusion of mutation confirmation by separate independent PCR, as recommended by Marchetti et al.,[14] in evaluating mutations from paraffin-embedded cells, although the PCR products in this study were all sequenced bidirectionally (Figure 1). The higher-than-expected frequency of *L833V* mutation occurring synchronously with the *L858R* mutation in our series could not be further confirmed with separate independent PCR as the DNA was interrogated for mutations in other exons. The *L833V* mutation has only been previously reported by Huang et al.[7] and occurred independent of the *L858R* mutation. This limitation has highlighted the importance of prioritization of exons to sequence and mutation confirmation.

In conclusion, acquiring an additional sample of lung tissue during the diagnostic procedure for lung cancer patients is feasible. This is easily incorporated into clinical practice and does not pose unacceptable risks to patients. Although there are technical limitations, it allows for mutational analysis in more patients, influencing treatment decisions in a timely fashion, thereby serving as a possible useful complement to the usual diagnostic work flow in hospitals. With ongoing advances in technology, specifically issues on its availability and affordability, this step may be further refined in future.

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