Routine genetic testing of lung cancer specimens derived from surgery, bronchoscopy and fluid aspiration by next generation sequencing

GOU YAMAMOTO[1], MARI KIKUCHI[1*], SHIHO KOBAYASHI[1*], YOSHIKO ARAI[1], KENJI FUJIYOSHI[1,4], TOMOKAZU WAKATSUKI[1], MIHO KAKUTA[1], YUKI YAMANE[2], YOSHIHITO IIJIMA[3], HIDEAKI MIZUTANI[2], YUKI NAKAJIMA[3], JUNKO SUDO[2], HIROYASU KINOSHITA[3], FUTOSHI KURIMOTO[2], HIROHIKO AKIYAMA[3], HIDETAKA URAMOTO[5], HIROSHI SAKAI[2], YOSHITO AKAGI[4] and KIWAMU AKAGI[1]

Departments of [1]Molecular Diagnosis and Cancer Prevention, [2]Thoracic Oncology and [3]Thoracic Surgery, Saitama Cancer Center, Ina, Kitaadachi, Saitama 362-0806; [4]Department of Surgery, Kurume University, Kurume, Fukuoka 830-0011; [5]Department of Thoracic Surgery, School of Medicine, Kanazawa Medical University, Uchinaka, Kahoku, Ishikawa 920-0293, Japan

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Abstract. After the development of EGFR tyrosine kinase inhibitors (TKIs), genetic testing of *EGFR* became required for effective treatment of lung cancer. Initially, the testing was conducted separately for each mutated region. However, many *EGFR* mutations have since been identified that determine the efficacy of EGFR-TKIs. Therefore, genetic testing of *EGFR* by next generation sequencing (NGS) may be a suitable strategy for lung cancer. Here we examined the applicability of the NGS method in regard to sensitivity, time and cost. A total of 939 specimens were obtained from 686 lung cancer patients at our hospital. DNA and RNA were simultaneously extracted from specimens derived from surgery, bronchoscopy, and fluid aspiration. Specimens included cerebrospinal fluid, pleural effusion, abdominal fluid, and pericardial effusion. From RNA, target regions (*EGFR, KRAS, ALK* fusion and *RET* fusion) were enriched by RT-PCR and sequenced with MiSeq. From DNA, PCR or PCR-RFLP conventional methods were performed. NGS and conventional methods were carried out routinely per week. Among the total 939 specimens, 38 specimens could not be examined with NGS. Among these, 34 specimens were analyzed by conventional testing with simultaneously extracted DNA. The remaining four specimens could not be tested with either method. Compared with the conventional method, the concordance rate of mutations was 99% (892/901), excluding specimens with NGS failure. The time period required from processing of specimens to results was 4 days, and the cost per sample was sufficiently low. In conclusion, the genetic testing with NGS method was useful for lung cancer treatment. The cost, sensitivity and time were able to tolerate routine examinations.

Correspondence to: Dr Kiwamu Akagi, Department of Molecular Diagnosis and Cancer Prevention, Saitama Cancer Center, 780 Oaza-Komuro, Ina, Kitaadachi, Saitama 362-0806, Japan
E-mail: akagi@cancer-c.pref.saitama.jp

*Contributed equally

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Introduction

Lung cancer is one of the most common cancers and the leading cause of cancer-related death worldwide (1). Non-small cell lung cancer (NSCLC) accounts for 80% of lung cancer and is classified broadly in adenocarcinoma (AdCa) and squamous cell carcinoma (SCC). Approximately half of NSCLC cases are AdCa (2). A decade ago, therapeutic strategies for lung cancer were histologically classified only as small-cell carcinoma (SCLC) or NSCLC. However, in recent years, somatic mutations within the epidermal growth factor receptor (*EGFR*) gene were discovered as driver mutations in lung cancer, thus dramatically changing the therapeutic strategy for unresectable lung cancer (3). Driver mutations are a predictive factor of the effect of EGFR tyrosine kinase inhibitors (TKI), which are molecular-targeted agents (such as gefitinib, erlotinib, and afatinib). The response rate of these drugs against cancers harboring driver mutations is >70% in many studies (4,5). Moreover, rearrangement of the anaplastic lymphoma receptor tyrosine kinase (*ALK*) gene (6) was also found to predict the response to ALK inhibitors (crizotinib and alectinib) (7). Thereafter, rearrangements of ret proto-oncogene (*RET*) and ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*) genes (8) were revealed as predictors for NSCLC. The molecular-targeted therapies crizotinib and vandetanib were used to target cancers harboring these two rearrangements (9,10). An ALK TKI regimen, targeted against cancer

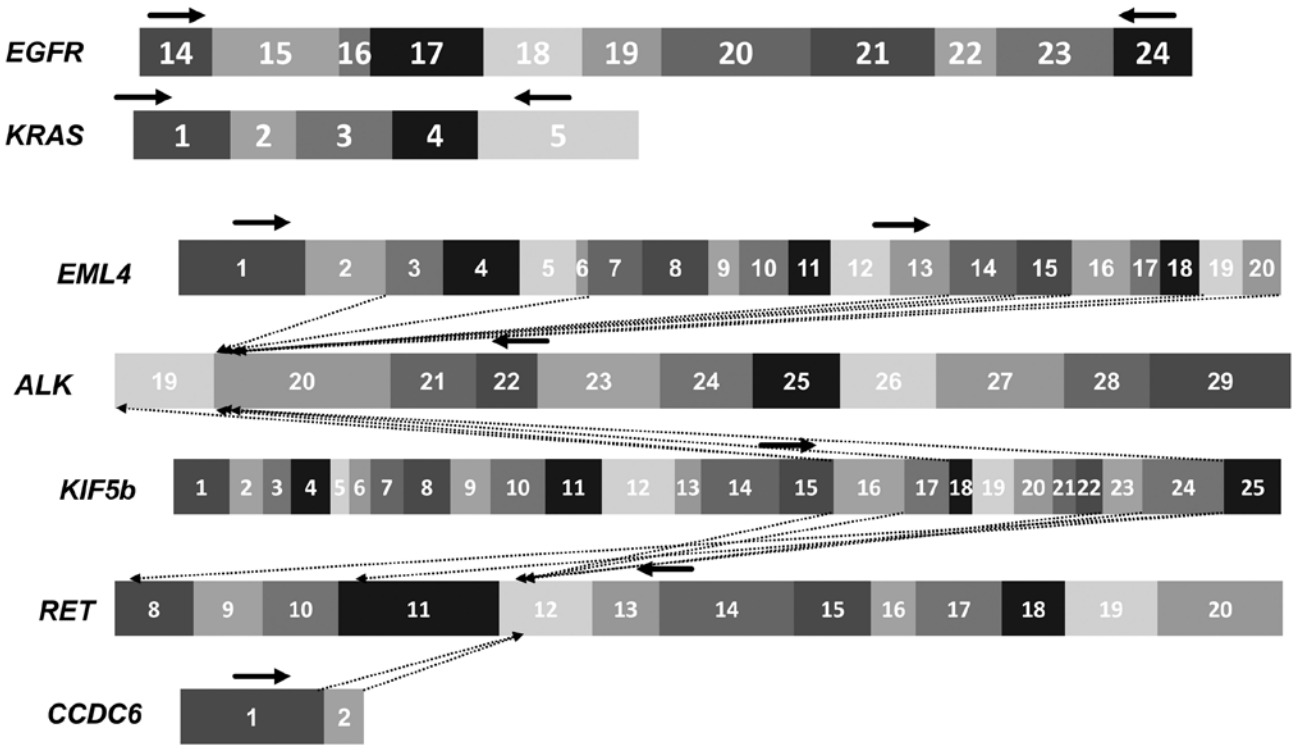


Figure 1. Primer design in target genes. Black arrows indicate positions of designed primers. White numbers indicate exon numbers of each gene. Dotted lines indicate regions of gene fusion.

harboring *ALK* rearrangement, was approved shortly after the discovery of *ALK* rearrangement. This compares with the standard approval time of therapeutic drugs of >10 years. This early approval suggests that an agent targeted to gene alterations (molecular-targeted agent) is also likely to be approved quickly in expectation of a high antitumor effect.

Precise patient classification is necessary to determine the most appropriate therapeutic strategy and obtain the maximum effect of molecular target drugs. Several *EGFR* mutations were initially found to influence the efficacy and administration of TKIs, but currently over 10 types of mutations have been shown impact the efficacy of TKIs. In addition, many mutations other than those in the *EGFR* gene are used to select a method of treatment (11). Previously, companion diagnostics for gene mutations that impacted the success of anticancer drugs were conducted separately for each mutation, followed by other diagnostics. However, choosing the appropriate molecular targeted drug now requires an extensive analysis of gene regions. Many institutions are using next generation sequencing (NGS) methods to detect comprehensive gene mutations associated with lung cancer treatment (12-14). However, genetic testing performed by NGS is fraught with problems of sensitivity, complexity and cost. Comparisons of the sensitivity of conventional methods using NGS and analysis from non-surgical specimens such as bronchoscope lavage and pleural effusion have not been reported.

In this study, we obtained 939 surgical, biopsy, bronchoscope, and fluid aspiration specimens from 686 lung cancer patients and examined the sensitivity and efficiency of NGS analyses using RNA (15) compared with conventional analytical methods (PCR-RFLP and RT-PCR) using DNA (16).

Materials and methods

Samples. This study included a total of 686 patients suspected with lung cancer at Saitama Cancer Center. All patients provided informed consent. A total of 939 specimens were obtained by surgery, bronchoscopy and fluid aspiration, and submitted for genetic testing at a specialized department in our hospital. Genetic tests were carried out only once for each patient, which is typical in Japan. Control DNA containing *EGFR* T790M (Riken Genesis, Japan) was used for a comparison of sensitivity between NGS and conventional methods.

Testing workflows. DNA and RNA were extracted simultaneously from all specimens using the AllPrep DNA/RNA Micro kit (Qiagen, Hilden, Germany). From extracted RNA, cDNA was synthesized with both oligo-dT and random primers using the SuperScript First-Strand Synthesis system for RT-PCR (Thermo Fisher Scientific, Waltham, MA, USA). Conventional examination of the *EML4-ALK* fusion gene (RT-PCR method) was performed with part of the cDNA. The remaining cDNA sample was used for multiplex PCR for targeted *EGFR* (exons 15-23), *KRAS* (all coding regions), *EML4*/*KIF5b*-*ALK* and *KIF5b*/*CCDC6*-*RET* (Fig. 1) using the Takara Taq™ Hot Start Version (Takara Bio, Japan). The PCR conditions were as follows: *EGFR* and *KRAS*, 95˚C for 3 min, followed by 40 cycles of 95˚C for 30 sec, 57˚C for 30 sec and 72˚C for 90 sec, and finally 72˚C for 5 min; for multiplex PCR, 95˚C for 3 min, followed by 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 105 sec, and finally 72˚C for 5 min. Primer sequences are shown in Table I. PCR products were purified with the QIAquick PCR Purification kit (Qiagen) and quan-

Table I. Primer sequence.

Primers	Sequence (5'-3')	Product length (bp)
EGFR forward	GAACATCACCTGCACAGGACG	1,148
EGFR reverse	ATCTGCGTCTATCATCCAGC	
KRAS forward	CATTTCGGACTGGGAGCGAG	836
KRAS reverse	CTGGGAATACTGGCACTTAGAGG	
EML4 forward-1	CCGGCAGTCTCGATGATAG	Variable
EML4 forward-2	TGGAGTAGGATGCCTGGATT	344-2,305
KIF5B forward	AAATGACCAACCACCAGAAA	
CCDC6 forward	TGCAGCAAGAGAACAAGGTG	
ALK reverse	ATCCAGTTCGTCCTGTTCAGAGC	
RET reverse	CAGGCCCCATACAATTTGAT	

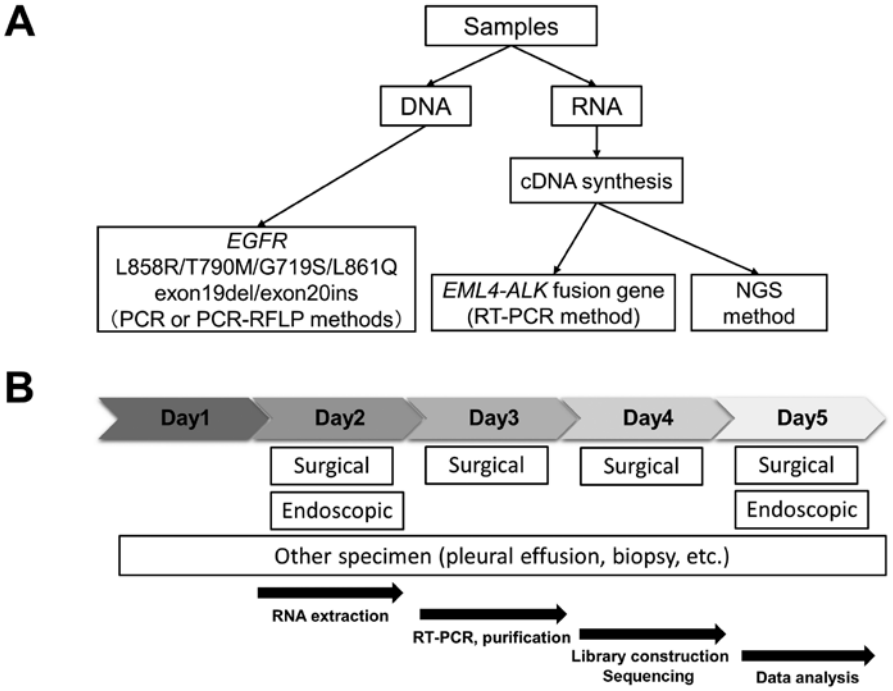


Figure 2. Overview of genetic testing. (A) DNA was used for the conventional method. RNA was used for both conventional and NGS methods. (B) Surgical indicates surgical materials. Endoscopic indicates bronchoscope lavage.

tified with the Qubit ds DNA HS assay kit (Thermo Fisher Scientific). Purified and quantified PCR products were mixed at equal molar ratios. Because multiplex PCR products were not amplified in most cases for fusion genes, reflecting their rarity, they could not be quantified. However, NGS is very sensitive and sometimes detects fusion genes. Therefore, in unquantifiable cases, 10 μl fusion gene PCR product was mixed. Mixed PCR products were quantified again, and libraries for NGS were prepared using the Nextera XT DNA Sample Prep kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Assessments of quality and quantification of libraries were performed with the D1000 Screen Tape system (Agilent, Santa Clara, CA, USA) and Qubit ds DNA HS assay kit (Thermo Fisher Scientific), respectively. Constructed libraries were adjusted to 4 nM and were sequenced using MiSeq (Illumina) with the MiSeq Reagent Kit Nano v2 (300 cycles, Illumina). Extracted DNA was used for conventional testing (Fig. 2A) (16). The overview of the genetic testing strategy performed each week by NGS method is shown in Fig. 2B.

Verification of discrepancy by NGS panel. Samples showing different results between the conventional method and NGS method were verified using the TruSight Tumor 15 (TST15, Illumina) with DNA according to the manufacturer's protocol. Several samples containing lower concentrations of DNA specified by the protocol (2 ng/μl) were only used to prepare libraries.

Table II. Patient characteristics.

	N=686	(%)
Median age		
Years	68.4	
(range)	(28-91)	
Gender		
Male	447	(65)
Female	239	(35)
Smoking status (Brinkman index)		
≥800	236	(34)
<800	51	(7)
<600	44	(6)
<400	24	(3)
<200	12	(2)
Index unknown (smoker)	57	(8)
Never	203	(30)
Smoking status unknown	59	(9)
Histology available (N=321)		
Adenocarcinoma	169	(25)
SCC	62	(9)
Other NSCLC	5	(1)
Small	8	(1)
Atypical lesion	4	(1)
Benign tumor	2	(1)
Carcinoma (undifined)	34	(5)
Metastatic lung cancer		
Colorectal cancer	15	(2)
Other site	17	(2)
No tumor cells (inflammatory tissue etc.)	5	(1)
Cytology only (N=365)		
Class I	0	
Class II	92	(13)
Class III	40	(6)
Class IV	16	(2)
Class V	208	(30)
Not classified	9	(1)

Sequence data analysis. Data output in the FASTQ format by MiSeq were analyzed with CLC Genomics Workbench (Qiagen). FASTQ files were trimmed by quality value, mapped and realigned to reference sequence (hg19), called variants. Variants with allele frequency of ≥1%, allele count of ≥3 and that covered ≥300 reads were listed. Target ratios exceeded 95% and minimum coverage of analyzing regions was >500. Detected variants were annotated from databases such as dbSNP, HapMap, ThousandGenome, HGVD (17), ToMMo (18), Clinver, and COSMIC. Candidates for fusion genes were acquired by a list of broken pairs and were validated by mapping directly to sequences of fusion genes and RT-PCR method. FASTQ files obtained from the NGS panel for verification were also analyzed with CLC Genomics Workbench. Variant data were visualized with OncoPrimter (cBioPortal; http://www.cbioportal.org/) (19,20).

Table III. Summary of obtained samples from lung cancer patients.

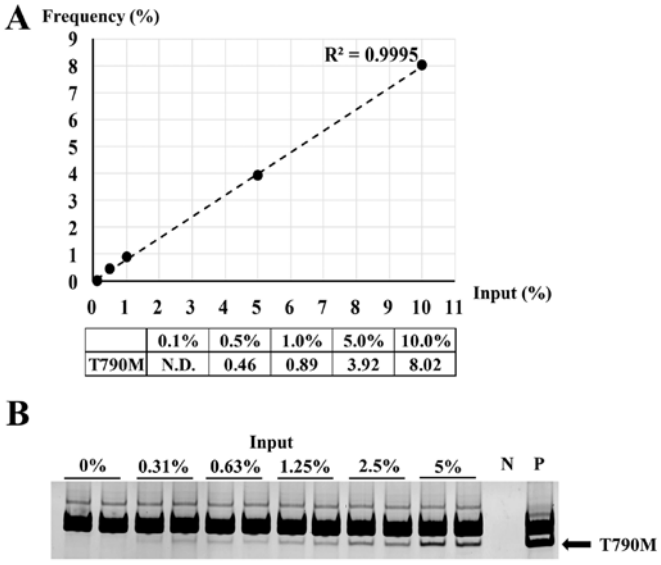
Sample type	RNA concentration		
	No. of samples	Average (ng/μl)	No. of samples <20
Bronchoscope lavage	413 (18)	4.5	403
Surgery	311 (2)	18.3	46
Biopsy	77 (2)	13.9	33
Pleural effusion	83 (7)	16.1	29
Cerebrospinal fluid	18 (3)		
Ascites fluid	5		
Sputum	3 (1)		
Pericardial effusion	2		
Others	27 (5)	8.2	19
Total	939 (38)		

The numbers in parentheses indicate the numbers of specimens with NGS failure.

Results

Patient characteristics. A total of 686 patients were included in this study and 939 specimens were obtained. Patient characteristics are presented in Table II. Of the total patients, 321 were categorized according to histopathological type. AdCa accounted for 53% of pathologically defined cases, followed by SCC (19%). Thirty-two cases showed lung metastasis from cancer of other organs. In 365 patients, only cytological specimens were examined and more than half the specimens were diagnosed as class 4 or 5. The cytology cases included patients that previously underwent surgery before this study and were pathologically diagnosed.

Summary of specimens. Among the total 939 specimens, ~44% were obtained by bronchoscopic examination and these specimens showed low RNA concentrations compared with other specimens (Table III). Thirty-eight specimens (4%) could not be examined with NGS because of PCR failure, although 34 of these specimens could be examined by conventional testing with simultaneously extracted DNA. The remaining four specimens could not be tested with either method. Unanalyzable specimens included bronchoscope lavage with few cells, cerebrospinal fluid, hemorrhagic pleural effusion and surgical material containing mostly necrotic debris. The time period required for reporting of results from processing of specimens was 4 days.



	0.1%	0.5%	1.0%	5.0%	10.0%
T790M	N.D.	0.46	0.89	3.92	8.02

Figure 3. Sensitivity of NGS and conventional methods. (A) NGS method. y-axis indicates input amount of T790M mutation. x-axis indicates detected frequency of the mutation. Table shows the actual number. (B) Conventional method. N, negative control (H_2O), P, positive control (H1975 cells).

Comparison of sensitivity between conventional and NGS methods using T790M harboring H1975 cells. Sensitivity of mutation detection was compared between NGS and conventional methods (PCR-RFLP method). First, a dilution series of control DNA was examined for detection of *EGFR* T790M using each method. The NGS method could detect up to 0.5% of T790M mutation, while the conventional method could detect explicitly up to 0.63% (Fig. 3).

Distribution of EGFR and KRAS mutations, and fusion genes in lung cancer. Among the total 686 patients, 411 were diagnosed pathologically as AdCa or strongly suspected as AdCa. These cases included cytological specimens diagnosed as class 4 or 5 from patients previously decided as AdCa. Typical EGFR TKI-sensitizing mutations (ex19del, L858R, G719X, L861Q) were detected in 32% of these cases, and fusion genes (*ALK, RET*) were detected in 2.4% of cases (Fig. 4A). The most common mutations in the *KRAS* gene were found in exon 2 (exon 2; G12X or G13X, ~13% of cases), followed by mutations in exon 3 (exon 3; Q61L or Q61H, 2.4%). Only one case harbored mutation in exon 4 (A146T). *EGFR* mutations were detected in 40% of cases pathologically diagnosed as AdCa or as strongly suspected AdCa by surgical materials alone (Fig. 4B). Fusion genes were detected in 11 cases among all patients (Table IV).

Among the *EGFR* exon 19 deletions, a 15-bp deletion from p.K745 to p.A750 was most frequently observed (Table V). Exon 20 insertion mutations were different in all cases. One exon 19 deletion was found in squamous papilloma. No *EGFR* and *KRAS* mutations or fusion genes were detected in SCC cases.

Discordance of results between conventional and NGS methods. Among the 901 specimens with results obtained by both conventional and NGS methods, 9 cases showed varied results between the two methods (Table VI). Almost all cases

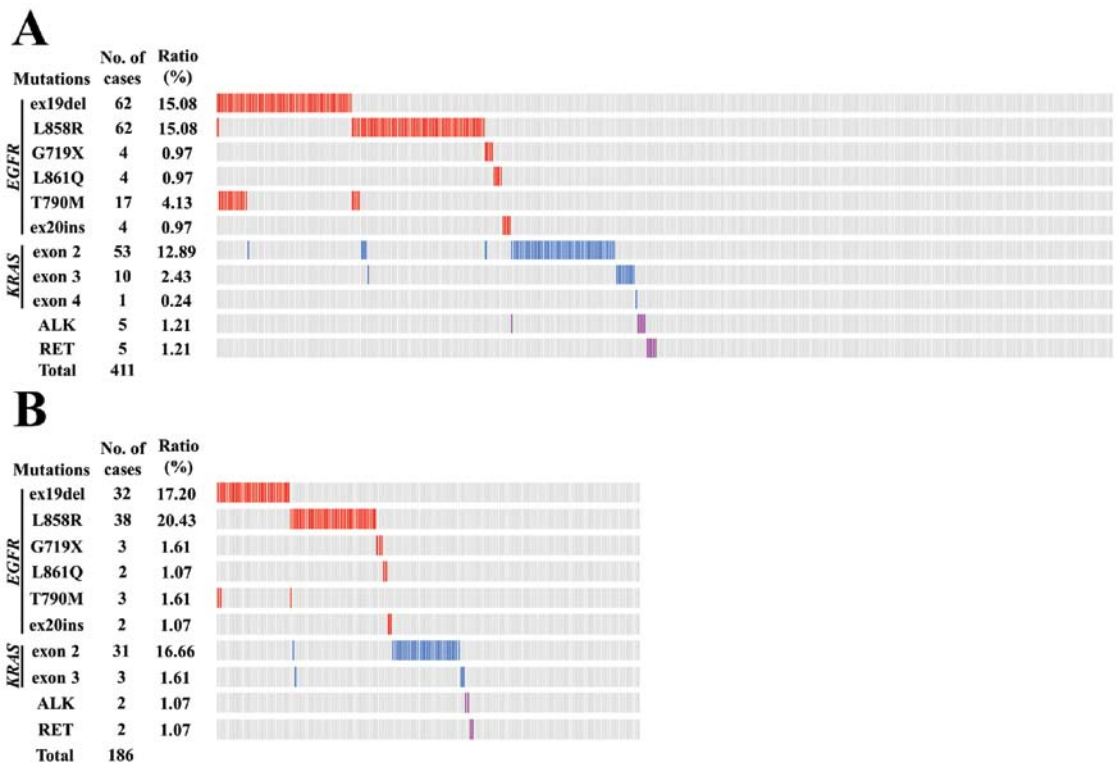


Figure 4. Frequency and distribution of mutations. Plot of mutations of (A) pathologically diagnosed AdCa or strongly suspected AdCa according to all materials (n=411), and (B) by surgical material alone (n=186). ex19del, L858R, G719X, L861Q, T790M and ex20ins; mutations of *EGFR*, exons 2-4; mutation of *KRAS, ALK* and *RET* fusion genes.

Table IV. Fusion genes.

Gender	Sample type	RNA concentration (ng/μl)	Cytology	Histology	Fusion genes
M	BL	33.9	V		*CCDC6-RET*
M	AF	2.5	V		*EML4-ALK*
M	PE	20	V		*EML4-ALK*
F	Surgery	20		AdCa	*EML4-ALK*
M	Surgery	20		AdCa	*EML4-ALK*
F	BL	7.4	V		*EML4-ALK*
F	Surgery	20		AdCa	*KIF5b-RET*
F	PE	20	V		*CCDC6-RET*
M	Biopsy	20	V		*CCDC6-RET*
M	Biopsy	1.6	Unknown		*EML4-ALK*
M	BL	2	V		*KIF5b-RET*

BL, bronchoscope lavage; AF, ascites fluid; PE, pleural effusion.

Table V. *EGFR* insertions/deletions.

Types of insertion/deletion	Sequence of insertion/deletion	No. of cases	Length
Exon 19 deletion			
p.Lys745_Ala750delinsLys	GGAATTAAGAGAAGC	29	15
p.Glu746_Ala750del	GAATTAAGAGAAGCA	11	15
p.Leu747_Pro753delinsSer	TAAGAGAAGCAACATCTC	5	18
p.Glu746_Ser752delinsVal	delAATTAAGAGAAGCAACATCinsT	5	18
p.Glu746_Thr751delinsGlu	ATTAAGAGAAGCAAC	5	15
p.Glu746_Arg748del	GAATTAAGA	3	9
p.Leu747_Ser752del	TTAAGAGAAGCAACATCT	1	18
p.Lys745_Ala750delinsLys	delCAAGGAATTAAGAGAAGCinsTAA	1	15
p.Glu746_Thr751delinsAla	AATTAAGAGAAGCAA	1	15
p.Glu746_Ala750delinsGlnPro	delGAATTAAGAGAAGinsCAAC	1	9
p.Glu746_Ser752delinsLeu	delGAATTAAGAGAAGCAACATCinsCT	1[a]	18
Exon 20 insertion			
His773_Val774insHisProHis	ACCCCCACC	1	9
p.Ala767delinsAlaSerValAsp	CAGCGTGGA	1	9
p.Val769_Asp770insAlaSerVal	TGGCCAGCG	1	9
p.Asp770_Asn771insLysAsp	GGACAA	1	6

[a]Deletion detected in squamous papilloma.

involved bronchoscope lavage specimens and had low RNA concentration. Cytological results were diverse. In all 9 cases, the conventional method could detect mutations while the NGS method was unable to detect mutations at >1% frequency, except for one exon 19 deletion. For the specimens with remaining DNA sample available, sequencing was performed with TST15 by MiSeq. Although concentrations were low, all mutations detected with the conventional method were confirmed using TST15. In some cases, the variant allele frequency analyzed from RNA was greatly different than that obtained from DNA.

Minor mutations. Many minor mutations were detected from the NGS results. *EGFR* mutations >10% of allele frequency are listed in Table VII. Although minor mutations were mostly accompanied by major mutations, such as L858R and exon 19 deletion (21,22), gefitinib sensitizing mutations (L833V+H835L) and a resistance mutation (L747S) were also found (23,24).

Mutations found in specimens with cytological class 3b or lower. Cytological samples were frequently obtained from

Table VI. Cases that showed discordance between NGS and conventional methods.

Sample type	RNA concentration (ng/μl)	Cytology	Conventional	NGS RNA (%)	NGS DNA (%)
Bronchoscope	2.5	III	ex19del	ND	-
Bronchoscope	2.2	III	ex19del	E709K (1.4)	ex19del (1.2)
Bronchoscope	5.3	II	ex19del	ND	-
Bronchoscope	3.4	II	ex19del	ND	-
Bronchoscope	5.6	III	ex19del	ex19del (0.36)	ex19del (1.7)
Bronchoscope	2.6	IV	ex19del, T790M	ex19del (0.5), T790M (0.6)	ex19del (8.2), T790M 1.7)
Bronchoscope	0.7	V	ex19del, T790M	ex19del (42.4)	ex19del (17.5), T790M (1.3)
Bronchoscope	1.4	IV	L858R	L858R (0.9)	L858R (1.3)
Pleural effusion	1.9	V	G719S, L861Q	ND	G719S (26.8), L861Q (26.6)

ND, not detectable.

Table VII. Minor mutations.

Gender	Sample type	RNA concentration (ng/μl)	Cytology	Histology	Sensitive (%)	Resistant (%)	Minor mutations (%)
M	FBS	3.2	V		ex19del (47.4)	T790M (25.4)	A750P (52.2)
F	FBS	5.5	V		L858R (55.3)		S720F (56.5)
M	FBS	3.4	V		L858R (23.3)		R776C (23.6)
F	Surgery	100		AdCa	ex19del (45.0)		A755G (48.2)
M	FBS	5.9	II				A743T (55.3), H584R (13.5)
M	FBS	3.6	V				L777P (16.0
M	Surgery	100		AdCa	L858R (35.4)		R776C (38.0)
F	Biopsy	20		AdCa	ex19del (52.2)	T790M (27.5)	T605N (43.4)
M	FBS	20	V				I586V (16.2)
M	Surgery	20		Ca	G719C (86.7)		S768I (85.4)
F	FBS	5.7	II				M825V (18.0), G901R (17.8)
M	PE	20	II				N816S (12.6), Q849L (11)
M	Surgery	20		AdCa			ex15del (43.8)
M	FBS	1.2	V				Y827C (32.9)
M	FBS	1.2	II				K593R (10.2)
F	PE	20	-	-	ex19del (37.4)		A750P (38.9), G873E (16.2)
M	FBS	4.9	V				G721S (11.3), L883fs (10.1)
M	FBS	3.4	V				G863D (17.5)
M	PE	20	V				A871S (14.9)
M	FBS	4.5	II				D800G (12.1)
M	FBS	1.6	V				K754E (11.4)
M	PE	20	V		L858R (32.8)		R776C (33.4)
F	Surgery	20		AdCa			L833V (72.3), H835L (69.7)
M	FBS	1.6	II				P631R (27.3)
M	Surgery	100		AdCa	ex19del (45.8)		L747S (13.4)
M	Surgery	24.5		Ca			R705K (10.1)
M	FBS	3.9	V				K642R (11.3)
M	Surgery	45.1		AdCa	G719A (32.6)		R776H (29.5)
F	FBS	1.1	V				I923T (33.9), K676R (27.6)
M	FBS	2.9	V	AdCa	L858R (28.9)		E709G (30.5)
F	FBS	3	V				E922K (12.0)
F	PE	20	V		ex19del (49.4)		K754E (50.0), K757R (50.0)
M	Other	3.8	II				N771S (10.8)
F	Surgery	20		AdCa			D916H (10.4)

FBS, fiberoptic bronchoscope; PE, pleural effusion.

Table VIII. Mutations detected in cytological specimens under class 3b.

Sample type	RNA concentration (ng/μl)	Cytology	*EGFR* mutation	Freqency (%)	*KRAS* mutation	Freqency (%)
Bronchoscope	2.9	II	L858R	3		
Bronchoscope	4.0	II			G13C	10.6
Pleural effusion	20	II			G12C	13.3
Bronchoscope	2.2	II			G12S	41.1
Bronchoscope	5.6	IIIb	ex19del	0.36		
Pleural effusion	20	IIIb	ex19del	75.1		
Pleural effusion	20	IIIb	ex19del	10.7		
Cerebrospinal fluid	2.9	IIIb	ex19del	90.5		
Bronchoscope	0.8	IIIb			G12C	42.5

Table IX. Total cost of conventional and NGS methods.

Procedure	Conventional method (US$)	NGS method (US$)
No. of samples	15	15
Target region	*EGFR*: G719S, exon19del, T790M, ex20ins, L858R, L861Q, *EML4-ALK*	*EGFR*: exons 15-23 *KRAS*: all CDS region *EML4/KIF5b-ALK* *KIF5b/CCDC6-RET*
DNA/RNA extraction	234.6	234.6
Reverse transcription	209.3	209.3
PCR	49.8	25.0
Enzymatic treatment	25.3	-
Electrophoresis	2.8	-
Library construction	-	911.2
Sequencing	-	483.9
Total	521.8	1,864.0

patients with suspected lung cancer, and in rare cases *EGFR* mutations were detected in samples diagnosed as cytological class 3b or lower. This sample was diagnosed as cancerous by genetic testing but 'suggestive of but not conclusive for malignancy' or 'no evidence of malignancy' by cytology. Extracting *EGFR* mutations confirmed by conventional methods and *KRAS* mutations with an allele frequency >10%, resulted in 9 cases harboring *EGFR* or *KRAS* mutations below cytological class 3b (Table VIII).

Discussion

In this study, we examined whether variant analysis by the NGS method using RNA was suitable for clinical use as genetic testing for lung cancer. Over 900 specimens were examined by the conventional method and NGS method in parallel over the course of one year. Moreover, we used solid samples, such as surgical and biopsy materials, as well as liquid samples, such as bronchoscope lavage and pleural effusion. Our results showed that the RNA-based method was practical for clinical examination as demonstrated by high sensitivity, short duration (4 days), and low expense (approximately JP\15,000 or US$144, as of June 2016) for each sample. The more detailed data are shown in Table IX. Supposing the number of the specimens per one week were 15, total cost of NGS method was approximately 3.5-fold. However, conventional method could only analyze six kinds of *EGFR* mutations and one kind of *EML4-ALK* fusion gene. On the other hand, NGS was able to analyze *EGFR* (exons 15-23), *KRAS* (all CDS region) and five kinds of fusion genes. Especially, because mutations within exon 2 of *KRAS* are various, the analysis by NGS was efficient. Moreover, in conventional method, operation time increased in proportion to the increase of the target region, while almost unchanged in NGS method.

The positive rate of EGFR-TKI sensitizing mutations was 40% in surgical materials diagnosed as AdCa. The ratio was lower than one previous report (49%) (25), but was similar to that of a recent study (26). The frequencies of *EML4/KIF5b-ALK* and *KIF5b/CCDC6-RET* were in total 2.2% among these patients, which was lower than that of a previous study (3.8%) (13). The ratios of EGFR-TKI sensitizing mutations among smokers (58%) and non-smokers (22%) were

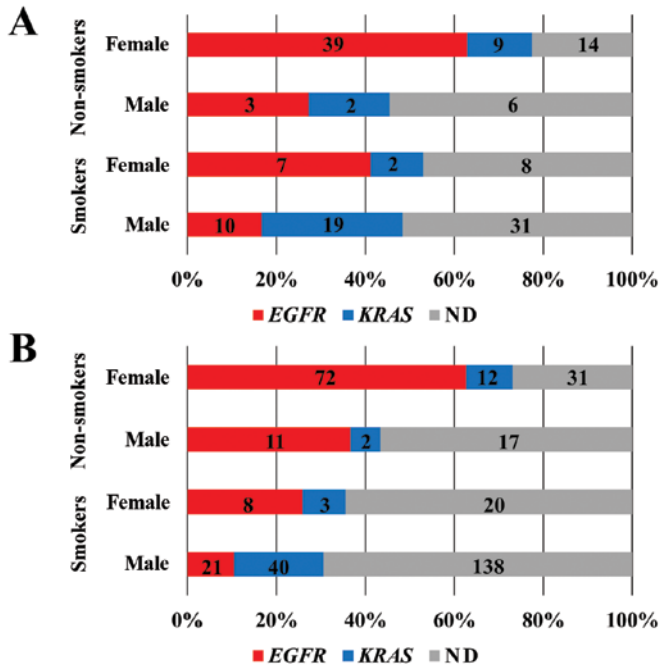


Figure 5. Distribution of *EGFR* and *KRAS* mutations by smoking status. (A) Pathologically diagnosed AdCa or strongly suspected AdCa according to all materials. (B) AdCa diagnosed pathologically from surgical materials only. Numbers represent case numbers.

also less than that of a previous report (68% and 31%, respectively) (25). The positive rate of *KRAS* mutations (18%) was higher than that from previous findings in Japan (7%) and lower than that in the west (20%) (27). Both *EGFR* and *KRAS* mutation frequencies were markedly different according to gender and smoking status (Fig. 5). EGFR-TKI sensitizing mutations were high in female non-smokers (63%) and *KRAS* mutations were high in male smokers (32%) (25).

Compared with the conventional method, the concordance rate of mutations was 99% (892/901), excluding specimens with NGS failure. Almost all discordant cases were confirmed by DNA sequencing. Moreover, regarding the cases of NGS failure, 34 of these 38 specimens could still be analyzed by DNA sequencing. In a previous study, the frequency of the mutation allele in DNA was similar to the ratio of cancer cells. However, in RNA, the frequency is much higher because genes with driver mutations have high transcriptional activity (15). However, our results suggest that DNA sequencing could detect mutations with higher sensitivity than analyses using RNA. In some cases, the frequency of the mutant allele was higher in RNA analyses, thus the sensitivity of analyses using RNA might depend on experimental conditions. We propose the following three explanations for the increased sensitivity with DNA analyses: i) the small number of specimens may have been affected by experimental conditions; ii) even in specimens containing very few viable cells, necrotic tissue and dead cells still contained sufficient DNA; and iii) degradation of RNA may have occurred between sample collection to RNA extraction.

In the present study, >30 kinds of minor mutations were detected in the examined regions of *EGFR*. Although most of the minor mutations were accompanied by the presence of major mutations, some clinically relevant mutations were found, such as L833V+H835L (23) and L747S (24). With the advance of sequencing technology, a correlation between various mutations within the regions of *EGFR* from exon 18 to exon 21 and sensitivity (28,29) or resistance (24,30) of EGFR-TKIs has been reported. Regarding the major mutations, a previous study indicated that EGFR-TKI sensitivity decreased when the frequency of T790M-harboring cells exceeded 25% (31), thus information on the allele frequency might be important for treatment. Moreover, the emergence of T790M after the administration of EGFR-TKIs was considered to contribute toward drug resistance (32), and *EGFR* C797S mutation indicated resistance against EGFR-TKIs targeting T790M (33). Therefore, genetic testing targeting a widespread region of *EGFR* could provide clinically useful information and could help develop a system to repeatedly analyze many genes and regions with efficiency and the ability to quantify. In addition, by examining various kind of specimens such as bronchoscope lavage and pleural effusion, the existence of cancer cells that did not emerge from cytodiagnosis was possibly revealed.

Several clinical trials of lung cancer with targeted *KRAS* mutations are ongoing (34). Recently, a small molecule with an effect on cancer cells harboring only the G12C mutation of *KRAS* was discovered (35), and testing on individual *KRAS* mutations has become important for developing other therapeutic strategies (36).

Many candidate driver and resistance mutations were discovered in lung cancer (37-39) and genome-based diagnosis was attempted (40). Treatment selection with molecular profiling seems to bring a huge clinical benefit (41). So, some trials applying NGS to clinic are ongoing, for example SCRUM-Japan and NCI-MATCH (USA) (42). About the detection of fusion genes, superior sensitivity of NGS method was reported (43). Recently, a trial to detect mutation derived from a cancer cell was performed noninvasively by liquid biopsy such as plasma DNA (44). However, sensitivity of the diagnostic agents which FDA authorized was 5%, and there is much false-negative. There were some reports about the usefulness of NGS in the liquid biopsy (45), and clinical application for the cancer treatment of NGS was expected in Japan.

The method used in this study was able to easily enrich broad protein-coding regions from RNA using PCR. In addition, this method could perform simultaneous detections of mutations and fusion genes and required fewer primer pairs than DNA sequence analyses. Sensitivity levels were approximately equal to those of conventional methods. Moreover, the NGS method with RNA sequence is cost-effective, as the sequencing cost per sample using this method is within the cost of 'Malignant tumor genetic testing' in Japan.

In conclusion, the strategy of analyzing mutations of target genes by the NGS method with RNA extracted from cancer cells for the purpose of developing treatment strategies compares favorably with the conventional method.

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