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Comparison of PANAMutyper and PNAClamp for Detecting KRAS Mutations from Patients With Malignant Pleural Effusion

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Abstract. *Background/Aim: KRAS is one of the frequently mutated genes in human cancers and often relates with drug resistance and poor prognosis. PANAMutyper™ is a novel technology that integrates PNAClamp™ and PANA S-Melting™. In the present study, PANAMutyper™ and PNAClamp™ were compared for the detection of KRAS mutations using different samples of patients with malignant pleural effusion. Patients and Methods: A total of 103 patients (including 56 lung adenocarcinoma, 10 lung squamous carcinoma, 17 small cell lung cancer, 3 large cell lung cancer, 3 stomach cancer, 2 ovarian cancer, and others) with malignant pleural effusion were investigated using matched tumor tissue, cell block, and pleural effusion samples. The diagnostic performance of these two methods was compared. Results: KRAS mutations were detected in 18 (17.5%) of 103 patients using tissue, cell block, and pleural effusion samples. All 18 patients with KRAS mutations were detected by PANAMutyper™ using any sample type, however, only 7 cases were detected by PNAClamp™. Among the subtypes of KRAS mutations, substitution in codon 12, 35G>T was the most frequent, followed by substitution in codon 12, 35G>A and codon 12, 34G>A. In pleural effusion specimens, PANAMutyper™ showed a better diagnostic performance compared to PNAClamp™. Conclusion: PANAMutyper™ had a diagnostic superiority for the detection of KRAS mutations in patients with malignant pleural effusion compared to PNAClamp™, although there was a concordance between PANAMutyper™ and PNAClamp™ results. Therefore, PANAMutyper™ can be used for a more sensitive and accurate detection of KRAS mutations.*

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KRAS is a well-known signaling molecule in the epidermal growth factor receptor pathway. For many years, KRAS has been recognized as one of the most frequently mutated oncogenes in multiple human cancers, including pancreatic, colorectal, lung, endometrial, gastric, biliary tract, and thyroid cancer (1, 2). The existence of KRAS mutations is commonly associated with poor prognosis and resistance to therapy (3-7).

Previously, we compared PNA clamping with direct sequencing for the detection of KRAS and EGFR mutations and found that the diagnostic performance and clinical outcome using PNA clamping are better compared to those of direct sequencing (8, 9). PNAClamp™ technology is based on the principle that PNA can inhibit amplification of wild-type DNA by hybridizing with wild-type sequences. Therefore, mutant DNA is preferentially amplified, and this is detected by an intercalating dye (10-13).

PANAMutyper™ R KRAS is a novel kit based on PANA C-Melting™ technology, combining PNAClamp™ and PANA S-Melting™, a multiplex detection system using specific PNA detection probes. Similar to PNAClamp™, a PNA clamp probe in PANAMutyper™ can only tightly bind to wild-type DNA sequences, and thus suppresses their amplification during PCR. Meanwhile, the PNA detection probe in PANAMutyper™ is conjugated with a fluorescent dye and a quencher and can specifically detect target mutant DNA. Each mutation can be genotyped by melting peak analysis (14, 15).

In the present study, we analyzed KRAS mutations in matched tumor tissues, cell blocks, and pleural effusion samples by PANAMutyper™ (both clamping-assisted fluorescence and melting curve analysis) and PNAClamp™ (clamping only), to compare their diagnostic performance and determine their usefulness in detecting KRAS mutations. To the best of our knowledge, this is the first study that uses PNA clamping-assisted fluorescence melting curve analysis to detect KRAS mutations in matched tissue, cell block, and pleural effusion samples to compare its performance with PNA clamping only.

Patients and Methods

Study subjects. We investigated 103 patients with malignant pleural effusion who underwent diagnostic thoracentesis at the Division of Pulmonology of Seoul St. Mary's Hospital (Seoul, Republic of Korea), between September 2008 and December 2016. We used samples of malignant pleural effusion diagnosed after confirming the presence of malignant cells using cytological examination, pleural biopsy or histology without other cause of pleural effusion. Pleural fluid samples from all patients were evaluated for KRAS mutations using both PANAMutyper™ and PNAClamp™. For patients whose tumor tissue and cell block samples were available, KRAS mutations were investigated from these as well.

All subjects provided a written informed consent for the procedure, and the study protocol was approved by the Institutional Review Board of Seoul St. Mary's Hospital, The Catholic University of Korea (IRB approval number: KC16TISI0672).

DNA extraction. 5-μm paraffin sections of tissues and cell blocks were used for DNA extraction. These sections were deparaffinized in xylene and were washed in ethanol. Five ml of pleural fluid specimens were centrifuged, and 1 ml of the supernatant was used for DNA analysis. DNA was extracted using a High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany). After eluting DNA in 50 μl of elution buffer, concentration and purity of extracted DNA were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The amount of DNA used was 35-70 ng (5-10 ng/reaction) for PNAClamp™ (version 4), and 40-80 ng (5-10 ng/reaction) for PANAMutyper™. A schematic representation of the workflow for PNAClamp™ *versus* PANAMutyper™ is presented in Figure 1.

PNAClamp™. PNAClamp™ analysis was performed using the PNAClamp™ KRAS Mutation Detection Kit (Panagene, Daejeon, Republic of Korea), following the manufacturer's instructions.

Table I. *Clinical characteristics of patients*.

Variables	n	%
Gender		
Male	65	63.1
Female	38	36.9
Age (years)		
Mean±standard deviation	72±12	
Median (range)	74 (31-93)	
Smoking status		
Never smoker	52	50.5
Ex-smoker	38	36.9
Current smoker	13	12.6
Pathology		
Adenocarcinoma	56	54.4
Squamous cell carcinoma	10	9.7
Small cell carcinoma	17	16.5
Large cell carcinoma	3	2.9
Sarcomatoid carcinoma	1	1.0
NSCLC NOS	2	1.9
Stomach cancer	3	2.9
Mesothelioma	2	1.9
Ovarian cancer	2	1.9
Thyroid cancer	2	1.9
Breast cancer	1	1.0
Endometrial cancer	1	1.0
Esophageal cancer	1	1.0
Head and neck cancer	1	1.0
Lymphoma	1	1.0
Total	103	

NSCLC NOS: Non-small cell lung cancer not otherwise specified.

Briefly, 7 μl of DNA template, 3 μl of each PNA mix, and 10 μl of 2X premix were mixed for a single amplification reaction. Amplification of the mixture (20 μl) was performed in a CFX96 real-time PCR instrument (Bio-Rad Laboratories, CA, USA) with the following thermal program: pre-incubation at 94˚C for 5 min, followed by 40 cycles of amplification at 94˚C for 30 sec (s), 70˚C for 30 s, 63˚C for 30 s, and 72˚C for 30 s.

Detection of signal from the intercalating dye was measured at every 63˚C step. Cycle threshold (Ct) value for the reaction (sample Ct value) was determined based on the fluorescence value measured at every 63˚C step. Results were assessed according to the delta-Ct value, calculated by subtracting the Ct value of the sample from the Ct value of the standard. Delta-Ct values larger than 2.0 were interpreted as corresponding to mutant DNA.

PANAMutyper™. PANAMutyper™ analysis was performed using the PANAMutyper™ R KRAS kit (Panagene, Deajeon, Republic of Korea), following the manual provided by the manufacturer. Briefly, 5 μl of DNA template, 19 μl of each master mix, and 1 μl of Taq polymerase were mixed for a single amplification reaction. Amplification of the mixture (25 μl) was performed in a CFX96 real-time PCR instrument (Bio-Rad) using the following thermal program: a UDG incubation at 50˚C for 2 min, a pre-incubation at 95˚C for 15 min, first amplification with 15 cycles of 95˚C for 30 s, 70˚C for 20 s, and 63˚C for 1 min, a second amplification with 35 cycles of 95˚C for 10 s, 53˚C for 20 s, and 73˚C for 20 s, a

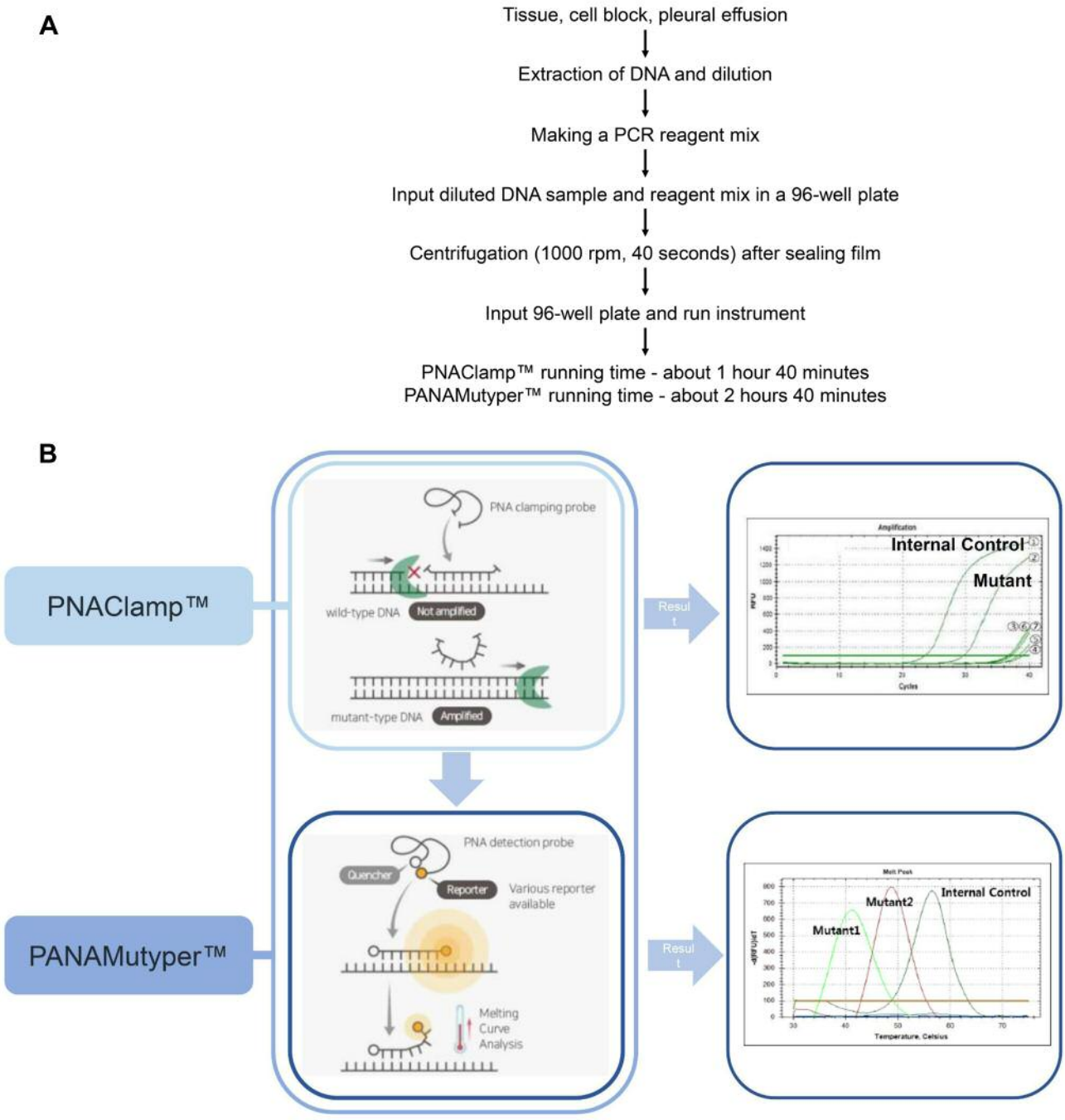

Figure 1. *Schematic representation of the workflow for PNAClamp™ vs PANAMutyper™. (A) Experimental methods for DNA extraction and the performance of PNAClamp™ and PANAMutyper™. (B) PANAMutyper™ is a fusion technology of PNAClamp™ for high sensitivity and PCR for multiplex genotyping. *Adapted from the PANAGENE INC. (http://www.panagene.com/_ENG/html/dh/tech2).*

product denaturation at 95˚C for 15 min, a detection probe binding at 35˚C for 5 min, and a melting curve analysis at 35˚C to 75˚C with an increment of 0.5˚C and detection for 3 s. Four color fluorescence signals (FAM, HEX, ROX, and Cy5) were measured during the melting curve analysis. Following amplification and melting analysis, the genotype of each sample was determined by the melting temperature (Tm) value obtained from the melting curve of each fluorescent dye. Each sample was assessed according to specific fluorescence and Tm ranges provided by the manual.

Table II. *Distribution of KRAS mutations in 103 patients with malignant effusion.*

No.	Gender	Age	Smoking status	Underlying malignancy	Samples and methods for the detection of KRAS mutations					
					Tissue		Cell block		Effusion	
					PANAMutyper™	PNAClamp™	PANAMutyper™	PNAClamp™	PANAMutyper™	PNAClamp™
1	M	69	Current	ADC	Ind.	Ind.	Codon 12 35G>T	WT	WT	WT
2	M	57	Current	ADC	NA	NA	NA	NA	Codon 12 34G>A	WT
3	F	59	Never	ADC	NA	NA	Codon 12 35G>A	WT	Codon 12 35G>A	WT
4	M	74	Never	ADC	NA	NA	Codon 61 182A>T	WT	WT	WT
5	M	74	Never	ADC	NA	NA	Codon 61 182A>T	WT	WT	WT
6	M	92	Ex	ADC	NA	NA	Codon 12 35G>C	WT	WT	WT
7	M	86	Ex	ADC	NA	NA	NA	NA	Codon 12 34G>A	WT
8	M	61	Current	ADC	Codon 13 38G>C	WT	WT	WT	WT	WT
9	F	71	Never	ADC	WT	WT	NA	NA	Codon 13 37G>A	WT
10	M	77	Ex	ADC	Codon 12 35G>T	WT	NA	NA	Codon 12 35G>T	Codon 12
11	F	31	Never	ADC	NA	NA	NA	NA	Codon 12 35G>A	WT
12	F	79	Never	ADC	NA	NA	NA	NA	Codon 12 35G>A	Codon 12
13	F	79	Never	ADC	Codon 13 37G>A	WT	WT	WT	WT	WT
14	F	62	Never	ADC	NA	NA	NA	NA	WT	WT
15	M	68	Ex	ADC	NA	NA	WT	WT	WT	WT
16	M	74	Never	ADC	NA	NA	NA	NA	WT	WT
17	M	68	Never	ADC	NA	NA	WT	WT	WT	WT
18	M	76	Never	ADC	WT	WT	NA	NA	WT	WT
19	M	65	Ex	ADC	WT	WT	NA	NA	WT	WT
20	M	74	Never	ADC	WT	WT	NA	NA	WT	WT
21	M	73	Never	ADC	NA	NA	WT	WT	WT	WT
22	M	47	Never	ADC	NA	NA	WT	WT	WT	WT
23	M	71	Never	ADC	NA	NA	NA	NA	WT	WT
24	M	52	Ex	ADC	NA	NA	WT	WT	WT	WT
25	F	76	Never	ADC	WT	WT	WT	WT	WT	WT
26	F	83	Never	ADC	WT	WT	WT	WT	WT	WT
27	F	48	Ex	ADC	WT	WT	WT	WT	WT	WT
28	M	60	Ex	ADC	NA	NA	WT	WT	WT	WT
29	F	60	Never	ADC	WT	WT	WT	WT	WT	WT
30	M	73	Ex	ADC	WT	WT	NA	NA	WT	WT
31	F	88	Never	ADC	WT	WT	WT	WT	WT	WT
32	M	77	Ex	ADC	NA	NA	NA	NA	WT	WT
33	F	86	Never	ADC	WT	WT	WT	WT	WT	Ind.
34	M	89	Ex	ADC	NA	NA	NA	NA	WT	WT
35	F	80	Never	ADC	NA	NA	NA	NA	WT	WT
36	F	64	Never	ADC	WT	WT	NA	NA	WT	WT
37	M	57	Ex	ADC	WT	WT	WT	WT	WT	WT
38	M	78	Ex	ADC	WT	WT	NA	NA	WT	WT
39	M	59	Ex	ADC	NA	NA	WT	WT	WT	WT
40	F	88	Never	ADC	NA	NA	WT	WT	WT	WT
41	F	62	Never	ADC	NA	NA	WT	WT	WT	WT
42	F	62	Never	ADC	NA	NA	WT	WT	WT	WT
43	F	84	Never	ADC	WT	WT	NA	NA	WT	WT
44	M	76	Never	ADC	WT	WT	NA	NA	WT	WT
45	M	72	Ex	ADC	NA	NA	WT	WT	WT	WT
46	F	69	Never	ADC	NA	NA	WT	WT	WT	WT
47	F	84	Never	ADC	NA	NA	WT	WT	WT	WT
48	M	78	Current	ADC	WT	WT	WT	WT	WT	WT
49	M	68	Never	ADC	NA	NA	WT	WT	WT	WT
50	M	72	Ex	ADC	NA	NA	WT	WT	WT	WT
51	M	86	Ex	ADC	NA	NA	WT	WT	WT	WT
52	M	61	Ex	ADC	NA	NA	WT	WT	WT	WT
53	F	49	Never	ADC	NA	NA	NA	NA	WT	WT
54	F	65	Never	ADC	NA	NA	NA	NA	WT	WT

Table II. *Continued*

Table II. *Continued*

No.	Gender	Age	Smoking status	Underlying malignancy	Samples and methods for the detection of KRAS mutations					
					Tissue		Cell block		Effusion	
					PANAMutyper™	PNAClamp™	PANAMutyper™	PNAClamp™	PANAMutyper™	PNAClamp™
55	F	81	Never	ADC	NA	NA	NA	NA	WT	WT
56	M	76	Current	ADC	NA	NA	NA	NA	WT	WT
57	M	71	Ex	SQCC	WT	WT	NA	NA	WT	WT
58	F	55	Never	SQCC	NA	NA	WT	WT	WT	WT
59	M	82	Never	SQCC	Ind.	Ind.	NA	NA	WT	WT
60	M	68	Never	SQCC	NA	NA	WT	WT	WT	WT
61	M	76	Current	SQCC	WT	WT	NA	NA	WT	WT
62	M	87	Ex	SQCC	WT	WT	NA	NA	WT	WT
63	M	62	Ex	SQCC	NA	NA	NA	NA	WT	WT
64	M	79	Ex	SQCC	WT	WT	WT	WT	WT	WT
65	M	81	Ex	SQCC	NA	NA	NA	NA	WT	WT
66	M	58	Ex	SQCC	NA	NA	NA	NA	WT	WT
67	M	72	Ex	SCLC	NA	NA	NA	NA	WT	WT
68	M	82	Never	SCLC	NA	NA	NA	NA	WT	WT
69	M	84	Ex	SCLC	WT	WT	NA	NA	WT	WT
70	M	77	Ex	SCLC	WT	WT	WT	WT	WT	WT
71	F	64	Never	SCLC	NA	NA	NA	NA	WT	WT
72	M	68	Ex	SCLC	WT	WT	WT	WT	WT	WT
73	M	70	Current	SCLC	NA	NA	WT	WT	WT	WT
74	M	79	Never	SCLC	WT	WT	NA	NA	WT	WT
75	F	79	Ex	SCLC	WT	WT	WT	WT	WT	WT
76	F	87	Never	SCLC	WT	WT	WT	WT	WT	WT
77	M	69	Ex	SCLC	WT	WT	NA	NA	WT	WT
78	M	93	Ex	SCLC	NA	NA	WT	WT	WT	WT
79	M	69	Current	SCLC	NA	NA	WT	WT	WT	WT
80	M	62	Current	SCLC	WT	WT	NA	NA	WT	WT
81	M	74	Current	SCLC	NA	NA	NA	NA	WT	WT
82	M	81	Ex	SCLC	WT	WT	NA	NA	WT	WT
83	F	74	Never	SCLC	WT	WT	WT	WT	WT	WT
84	M	78	Current	Large cell	WT	WT	NA	NA	Codon 12 35G>T	Codon 12
85	M	80	Ex	Large cell	WT	WT	NA	NA	WT	WT
86	M	64	Ex	Large cell	WT	WT	NA	NA	WT	WT
87	M	79	Ex	Sarcomatoid carcinoma	WT	WT	NA	NA	WT	WT
88	M	79	Current	NSCLC NOS	NA	NA	Codon 12 35G>T	Codon 12	Codon 12 35G>T	WT
89	M	90	Ex	NSCLC NOS	NA	NA	WT	WT	WT	WT
90	M	54	Current	Stomach cancer	NA	NA	Codon 13 38G>A	WT	Codon 13 38G>A	Codon 13
91	M	87	Never	Stomach cancer	WT	WT	NA	NA	WT	WT
92	F	85	Never	Stomach cancer	NA	NA	NA	NA	WT	WT
93	F	62	Never	Mesothelioma	NA	NA	NA	NA	WT	WT
94	F	64	Never	Mesothelioma	NA	NA	NA	NA	WT	WT
95	F	84	Never	Ovarian cancer	NA	NA	Codon 12 34G>C	Codon 12	Codon 12 34G>C	Codon 12
96	F	49	Never	Ovarian cancer	WT	WT	Codon 13 38G>A	Codon 13	WT	WT
97	F	74	Never	Thyroid cancer	NA	NA	WT	WT	WT	WT
98	M	83	Ex	Thyroid cancer	NA	NA	WT	WT	WT	WT
99	F	62	Never	Breast cancer	NA	NA	WT	WT	WT	WT
100	F	54	Never	Endometrial cancer	NA	NA	NA	NA	WT	WT
101	M	75	Ex	Esophageal cancer	NA	NA	NA	NA	WT	WT
102	F	70	Never	Head & neck cancer	WT	WT	NA	NA	WT	WT
103	F	84	Never	Lymphoma	WT	WT	NA	NA	WT	WT

ADC: Adenocarcinoma; Current: current smoker; Ex: ex-smoker; Ind: indeterminate; NA: not available; Never: never smoker; NSCLC NOS: Non-small cell lung cancer not otherwise specified; SQCC: squamous cell carcinoma; SCLC: small cell lung cancer; WT: wild type.

Table III. *Distribution of KRAS mutations detected by PANAMutyper™ and PNAClamp™.*

	Tissue		Cell block		Effusion	
	PANAMutyper™	PNAClamp™	PANAMutyper™	PNAClamp™	PANAMutyper™	PNAClamp™
Mutation	3 (6.8)	-	9 (17.6)	3 (5.9)	11 (10.7)	5 (4.9)
Codon 12	-	-	-	2 (3.9)	-	4 (3.9)
Codon 12 34G>C	-	-	1 (2.0)	-	1 (1.0)	-
Codon 12 34G>A	-	-	-	-	2 (1.9)	-
Codon 12 35G>T	1 (2.3)	-	2 (3.9)	-	3 (2.9)	-
Codon 12 35G>A	-	-	1 (2.0)	-	3 (2.9)	-
Codon 12 35G>C			1 (2.0)	-	-	-
Codon 13	-	-	-	1 (2.0)	-	1 (1.0)
Codon 13 37G>A	1 (2.3)	-	-	-	1 (1.0)	-
Codon 13 38G>C	1 (2.3)	-	-	-	-	-
Codon 13 38G>A	-	-	2 (3.9)	-	1 (1.0)	-
Codon 61	-	-	-	-	-	-
Codon 61 182A>T	-	-	2 (3.9)	-	-	-
Wild type	39 (88.6)	42 (95.5)	42 (82.4)	48 (94.1)	92 (89.3)	97 (94.2)
Indeterminate	2 (4.5)	2 (4.5)	-	-	-	1 (1.0)
Total	44	44	51	51	103	103
Not Measured	59	59	52	52	0	0

Data are presented as n (%).

Statistical analyses. Demographic data are presented as a mean±SD or n (%). Agreement between PANAMutyper™ and PNAClamp™ for tissues, cell blocks, or effusion samples was determined based on overall agreement and Cohen's kappa value. McNemar's test was used to identify any discordance between the two methods for tissues, cell blocks, and effusion samples, after categorizing all patients into: i) wild and ii) mutant type.

Diagnostic performance of the two methods for detecting KRAS mutations in pleural fluids was presented with sensitivity, specificity, positive predictive value, and negative predictive value, with the mutation status in all samples combined as a reference standard. A "wild type" in the reference standard was defined as a failure to detect any mutation in all samples combined, identified using either PANAMutyper™ or PNAClamp™. In addition, Cohen's kappa statistic was calculated to compare the agreement of each method between the result of pleural fluid and the reference standard.

A two-sided *p*-Value of equal or less than 0.05 was considered statistically significant. All statistical analyses were performed using the SAS 9.4 software (SAS Institute, Inc., Cary, NC, USA).

Results

Patient characteristics. Baseline demographic characteristics of enrolled patients are summarized in Table I. Of 103 patients, 65 (63.1%) were males. The mean age of all patients was 72 ± 12 years. Fifty-one (49.5%) patients had a history of smoking. Eighty-nine patients were diagnosed as primary lung cancer (72 patients with NSCLC and 17 patients with SCLC). Fourteen patients had other malignancies (3 with stomach cancer, 2 with mesothelioma, 2 with ovarian cancer, 2 with thyroid cancer, 1 with breast cancer, 1 with endometrial cancer, 1 with esophageal cancer, 1 with head and neck cancer, and 1 with lymphoma).

Among these 103 subjects, primary malignancies were diagnosed based on pathologic confirmation of the primary tumor (n=81) or by examination of pleural specimens only (pleural tissue or cell block of the pleural fluid) without confirmation of the primary tumor site (n=22). Pleural malignancy was diagnosed *via* pleural biopsy (n=10), cell block or cytology of the pleural fluid (n=62), or exclusion of other causes of pleural effusion (n=31). The major histological type of lung cancer was adenocarcinoma (54.4%).

Comparison of KRAS mutations detected by PANAMutyper™ and PNAClamp™. KRAS mutations were detected in 18 (17.5%) of 103 patients (Table II). Among these 18 patients with a KRAS mutation, 11 were males and 9 had a history of smoking. There was no KRAS mutation in patients with squamous cell carcinoma or small cell carcinoma of the lung. All 18 patients with KRAS mutations were detected by PANAMutyper™ using any sample type, but only 7 cases were detected by PNAClamp™.

Codon 12 had the highest incidence of KRAS mutations, present in 11 patients (4 with codon 12 35G>T, 3 with codon 12 35G>A, 2 with codon 12 34G>A, 1 with codon 12 34G>C, and 1 with codon 12 35G>C), followed by codon 13 present in 5 patients (2 with codon 13 37G>A, 2 with codon 13 38G>A, and 1 with codon 13 38G>C) and codon 61 in 2 patients (182A>T).

Concordance of PANAMutyper™ and PNAClamp™. Detailed comparisons of KRAS mutations according to sample type and detection method are shown in Tables II and

Table IV. *Concordance between PANAMutyper™ and PNAClamp™ for the detection of KRAS mutations.*

	PNAClamp™						
	Mutation		Wild type/ indeterminate	Total	K coefficient (95%CI)*	Overall agreement* (95%CI)	McNemar's test *p*-Value*
	Codon 12	Codon 13					
PANAMutyper™							
Tissue							
Mutation							
Codon 12 35G>T	0	0	1	1	0	0.93 (0.81-0.99)	0.0833
Codon 13 37G>A	0	0	1	1			
Codon 13 38G>C	0	0	1	1			
Wild type/Indeterminate	0	0	41	41			
Total	0	0	44	44			
Cell block							
Mutation							
Codon 12 34G>C	1	0	0	1	0.45 (0.11-0.80)	0.88 (0.76-0.96)	0.0143
Codon 12 35G>T	1	0	1	2			
Codon 12 35G>A	0	0	1	1			
Codon 12 35G>C	0	0	1	1			
Codon 13 38G>A	0	1	1	2			
Codon 61 182A>T	0	0	2	2			
Wild type/indeterminate	0	0	42	42			
Total	2	1	48	51			
Effusion							
Mutation							
Codon 12 34G>C	1	0	0	1	0.60 (0.31-0.88)	0.94 (0.88-0.98)	0.0143
Codon 12 34G>A	0	0	2	2			
Codon 12 35G>T	2	0	1	3			
Codon 12 35G>A	1	0	2	3			
Codon 13 37G>A	0	0	1	1			
Codon 13 38G>A	0	1	0	1			
Wild type/indeterminate	0	0	92	92			
Total	4	1	98	103			

Data are presented as n. *All patients were categorized into two groups: mutant and wild type.

III. In tumor tissues, KRAS mutations were only identified in 3 (6.8%) out of 44 patients by using PANAMutyper™ (Table III). In cell blocks,9 (17.6%) and 3 (5.9%) out of 51 patients were found to have KRAS mutations using PANAMutyper™ and PNAClamp™, respectively. The three mutations identified using PNAClamp™ were concordant with those detected using PANAMutyper™. The six other KRAS mutations detected by PANAMutyper™ were identified as wild type by PNAClamp™.

Regarding pleural effusion samples, PANAMutyper™ identified KRAS mutations in 11 (10.7%) samples, whereas PNAClamp™ identified KRAS mutations in 5 (4.9%) samples. The 5 mutations identified by PNAClamp™ were consistent with those detected by PANAMutyper™. The 6 other KRAS mutations detected by PANAMutyper™ were identified as wild type by PNAClamp™.

The diagnostic concordance between PANAMutyper™ and PNAClamp™ in tissues, cell blocks, and effusion samples is shown in Table IV. K coefficients between the two methods were 0.45 and 0.60 for cell blocks and effusion samples, respectively, indicating a moderate agreement between PANAMutyper™ and PNAClamp™. However, McNemar's test showed a significant superiority of PANAMutyper™ over PNAClamp™ in both cell blocks and pleural effusion samples.

Diagnostic performance of pleural effusion to detect KRAS mutations. The diagnostic performance of pleural effusion compared to results obtained from all samples combined, showed a sensitivity of 61%, a specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 92% for PANAMutyper™. For PNAClamp™, the diagnostic performance of pleural effusion showed a sensitivity of 28%, a specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 87% (Table V).

Table V. *Diagnostic performance of two mutation detection methods using pleural effusion samples compared to results obtained from all samples combined.*

Effusion	Results from all samples combined		Total	Sensitivity (95%CI)*	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)	K coefficient (95%CI)
	Mutation	Wild type						
PANAMutyper™								
Mutation	11	0	11	0.61 (0.36-0.83)	1.00 (0.96-1.00)	1.00 (0.72-1.00)	0.92 (0.85-0.97)	0.72 (0.53-0.91)
Wild type	7	85	92					
PNAClamp™ †								
Mutation	5	0	5	0.28 (0.10-0.53)	1.00 (0.96-1.00)	1.00 (0.48-1.00)	0.87 (0.78-0.93)	0.39 (0.14-0.63)
Wild type	13	84	97					

*p-Value of 0.025 in McNemar's test for comparing sensitivities. †A total of 102 out of 103 samples were evaluated due to one indeterminate result. 95%Cl: 95%Confidence Interval.

Discussion

The aim of the present study was to compare the performance of PANAMutyper™ and PNAClamp™ for the detection of KRAS mutations in matched tumor tissues, cell blocks, and pleural effusion samples. In addition, the diagnostic performance of both methods using pleural fluid samples for the detection of KRAS mutations was investigated.

The detection rate of KRAS mutations varied from 0% to 17.6%, depending on specimen and detection method. There was an acceptable concordance between PANAMutyper™ and PNAClamp™ for cell blocks and pleural effusion samples. However, tissue specimens did not show any agreement due to their low sensitivities for detecting KRAS mutations. PANAMutyper™ had a superior diagnostic performance over PNAClamp™ for cell blocks and pleural effusion specimens. Compared to results obtained for all samples combined, a good diagnostic performance regarding the detection of KRAS mutations was achieved when pleural effusion samples were used. In the current study, rates of KRAS mutations detected by PANAMutyper™ in tissue, cell block, and pleural effusion samples were 6.8%, 17.6%, and 10.7%, respectively. These rates detected by PNAClamp™ were 0%, 5.9%, and 4.9%, respectively.

The frequency of KRAS mutations is known to vary among different ethnic groups. In Caucasian patients, the prevalence of KRAS mutations in lung cancer is 17% in the COSMIC database (16) and 33% in lung adenocarcinoma in TCGA (17). In Eastern Asians, the prevalence of KRAS mutations is 2.3-9.4% (18, 19). In the present study, the frequency of KRAS mutations caused by lung cancer from all specimens combined was 16.9% (15 out of 89) and 4.5% (4 out of 89) detected by PANAMutyper™ and PNAClamp™, respectively.

Mutation of the RAS gene usually occurs in adenocarcinoma (20) and rarely in squamous cell carcinoma, while it never occurs in small cell lung cancer (21). Wu *et al.* have studied the prevalence of KRAS mutations in Taiwanese and found that the prevalence is 3.8% (9 out of 237 patients) in lung cancer (18). Among these 9 patients with KRAS mutations, 8 (88.9%) had adenocarcinoma while one (11.1%) had squamous cell carcinoma (18). The results of the present study correspond well with the previous study on the Taiwanese. Thirteen (86.7%) of 15 patients with KRAS mutations exhibited adenocarcinoma histology in the present study. However, squamous cell carcinoma or small cell carcinoma identified no KRAS mutations.

Approximately 97% of KRAS mutations in non-small cell lung cancer occur in exons 2 and 3. They are commonly found in codon 12, occasionally in codon 13 and rarely in codon 61 (20, 22, 23). In the current study, among the 18 detected KRAS mutations, most of them were detected in codon 12 (11 patients), followed by those in codon 13 (5 patients) and codon 61 (2 patients).

Specific subtypes of KRAS mutations could be associated with different clinical implications, such as drug sensitivity or prognosis. Regarding the distinct subtype of KRAS mutations, the most common mutation is a codon 12 34G>T point mutation, followed by codon 12 35G>T and codon 12 35G>A mutations (24). The present study also demonstrated that four KRAS mutations had a codon 12 35G>T point mutation, while three KRAS mutations had a codon 12 35G>A point mutation. The Taiwanese study has indicated that the most commonly identified KRAS mutation is codon 12 35G>T, followed by codon 12 35G>A, consistent with our results. These findings suggest that specific subtype of KRAS mutations could be different depending on ethnic group. Further large-scale studies are needed to verify the clinical role of the distinct subtypes of KRAS mutations.

Direct sequencing of DNA is traditionally a reasonable approach to identify the KRAS mutation status. We have previously reported that the diagnostic performance of PNA clamping is better than that of direct sequencing (9),

however, more sensitive methods are being developed these days. Superior results of the PANAMutyper™ technology for detecting EGFR mutation using specimens of plasma and bronchoalveolar lavage fluid have been reported recently (15, 25). To the best of our knowledge, this is the first study concerning the diagnostic performance of PNA clamping-assisted fluorescence melting curve analysis, by comparing matched tissue and cell block specimens with pleural effusion samples for the detection of KRAS mutations.

Our study has several limitations. First, the number of patients was not large. Second, the number of tissue and cell block specimens was not matched to that of pleural effusion samples because some specimens were of insufficient volume following routine pathological examination.

Taken together, we found that PANAMutyper™ had a superior diagnostic performance over PNAClamp™ for the detection of KRAS mutations, although there was concordance between the PANAMutyper™ and PNAClamp™ results. Furthermore, the good diagnostic accuracy of using pleural fluid sample can provide useful clinical information offering better prediction and personalized therapy.

Clinical Practice Points

- PNAClamp™ is currently used to detect KRAS mutations because of its superior diagnostic performance over conventional Sanger sequencing.
- PANAMutyper™ is a novel technology that integrates PNAClamp™ and PANA S-Melting™ .
- PANAMutyper™ and PNAClamp™ were compared for the detection of KRAS mutations in patients with malignant pleural effusion.
- PANAMutyper™ had a diagnostic superiority for the detection of KRAS mutations compared to PNAClamp™ .
- Frequency and specific subtypes of KRAS mutations in the current study correspond well with those in a previous study on Taiwanese.
- PANAMutyper™ can be used for more sensitive and accurate detection of KRAS mutations.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conception and design: Su Yeon Choi, Seung Joon Kim; Development of methodology: Hyung Woo Kim, Sang Hoon Jeon, Bit Na Kim, Nahyeon Kang; Acquisition of data: Chang Dong Yeo, Chan Kwon Park; Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Mi Sun Park, Hyeon Woo Yim; Writing, review, and/or revision of the manuscript: Su Yeon Choi, Jong Y. Park, Seung Joon Kim; Administrative, technical, or material support: Yoon Ho Lee, Kyo Young Lee, Young Kyoon Kim, Sug Hyung Lee; Study supervision: Seung Joon Kim.

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