

ORIGINAL PAPER

# Comparison of EGFR mutation detection between the tissue and cytology using direct sequencing, pyrosequencing and peptide nucleic acid clamping in lung adenocarcinoma: Korean multicentre study

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## Summary

**Background:** The importance of sensitive methods for the detection of epidermal growth factor receptor (EGFR) mutation is emphasized. The aim of this study is to perform comparative and concordance analyses of direct sequencing, pyrosequencing and peptide nucleic acid (PNA) clamping for detecting EGFR gene mutations using archived tissue and cytology specimens.

**Methods:** Samples from a total of 112 cases, which were diagnosed with adenocarcinoma of the lung at nine hospitals in Korea were collected. Using the above three methods, the concordance rates of EGFR mutations in exons 18, 19, 20 and 21 were analysed and validated in comparative tissue and cytology specimens.

**Results:** Comparison of EGFR mutation detection between the tissue and cytology had a high concordance rate. The diagnostic performance of pyrosequencing and PNA clamping in tissue was higher than that of direct sequencing as well as cytology. Additionally, among some of the patients who had EGFR wild type by single method, EGFR mutations were detected by other methods. Cytology specimens had a diagnostic performance for the detection of EGFR mutations.

**Conclusions:** Cytology specimens had a diagnostic performance for the detection of EGFR mutations that was comparable to that of tissues. For detecting EGFR mutations, pyrosequencing or PNA clamping was more sensitive than direct

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sequencing. In EGFR mutation negative patients who are difficult to obtain tissue, repeating test using pyrosequencing or PNA clamping is recommended to improve the detection rate of EGFR mutation than only one, especially in cytology.

## Introduction

The detection of activating mutations of the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) is nowadays the best predictive marker to treat adenocarcinoma with EGFR-tyrosine kinase inhibitors (TKIs) as the therapeutic target.<sup>1</sup> For selecting the patients who show an appropriate therapeutic response, molecular testing for EGFR mutations is one of the clinically important tools in routine practice.

Direct sequencing (DS) has historically been the standard method for EGFR mutation testing, and it is still widely used for discovering cancer-related mutations in previously unidentified genes that may respond to targeted therapies. However, DS involves complex steps, including DNA extraction, PCR-based amplification, DNA sequencing and sequence interpretation. This complexity is confounded by the fact that clinical samples often contain a small subpopulation of mutant cells mixed with a greater level of normal tissue, thus sometimes resulting in non-detection of mutations by sequencing technologies.<sup>2</sup> Therefore, the sensitivity of DS is suboptimal in representative clinical tumour samples and it can be meaningfully used only when there are sufficient levels of mutant DNA.<sup>3</sup>

Pyrosequencing is a DNA sequencing technology based on the sequencing-by-synthesis principle.<sup>4,5</sup> It is a non-electrophoretic real-time luminescence technique, in which the phosphate released during the incorporation of a nucleotide into a growing DNA chain is converted into the light signal through a series of enzymatic reactions. It is a simple, robust, fast and sensitive method as well as a cost-effective alternative.<sup>6-8</sup>

The recently Korean Food and Drug Administration (FDA)-approved peptide nucleic acid (PNA) clamping (PNAc) technology (Panagene Inc. Daejeon, Korea) uses PNA-mediated real-time PCR using benzothiazole-2-sulfonyl as the amine-protecting groups (PNA Clamp TM EGFR Mutation Detection kit). PNA is an artificially synthesized polymer that has the properties of both nucleic acids and proteins.<sup>9</sup> PNA can bind to a complementary sequence in the DNA, and the binding capacity is stronger than that with DNA because of the lack of electrostatic repulsion. Real-time PCR using a PNA oligomer designed to bind to the bottom strand of the wild-type EGFR gene for the detection of EGFR mutations is a highly sensitive, rapid and simple protocol, but it cannot discover new mutations. PNAc enables the detection of mutations in specimens containing as few as approximately 1 % mutant alleles.<sup>10</sup>

The obtainment of a sufficient tumour biopsy sample for diagnosis in patients with lung cancer may not always be possible due to comorbidities or other reasons. Cytological samples also frequently permit the initial, rapid and effective diagnosis of cancer.<sup>11</sup> There are several published studies that have used various methods to analyse EGFR mutations in the cytology samples.<sup>12-17</sup> A large-scale, retrospective, multicenter study demonstrated a correlation between clinical outcomes and the results of EGFR mutational analyses through various methods.<sup>18</sup>

We attempted to analyse the samples of lung adenocarcinoma using DS, pyrosequencing and PNAc. The aim of this study was to compare the efficacy of the three methods for EGFR

testing, and to assess variable results of EGFR mutation testing in tissue or cytology samples. This study also evaluated the clinical response to EGFR-TKIs in patients with EGFR mutations defined by these different detection methods.

## Materials and methods

### Patient selection

A total of 127 cases, which were diagnosed with lung adenocarcinoma at nine hospitals in Korea between February 2006 and March 2012 were collected. Fifteen cases were excluded because of lack of availability of tissue and cytology samples for making the diagnosis and inadequate clinical history. One hundred twelve formalin-fixed paraffine-embedded (FFPE) tissues and cytology samples were reviewed. For tissue, hematoxylin and eosin (H-E) stained tissue slides from each corresponding FFPE block including 76 biopsies and 36 surgical resections were reviewed. For cytology, papanicolaou (Pap) or hematoxylin and eosin (H-E) stained cytology slides, including 98 conventional smears (samples smeared directly onto a slide after collection) and 14 cell blocks (paraffin-embedded specimen derived from cytology samples) were reviewed (Figure 1) (Supplementary Tables S1 and S2). One hundred and twelve cases were diagnosed with lung adenocarcinoma based on the histological and/or immunohistochemical stains, such as TTF-1 (1:200, Lab vision, Fremont, CA), napsin A (1:100, Cell Marque, Rocklin, CA), Cytokeratin 5/6 (predilution, Ventana, Tucson, AZ), p63 (1:100, BioCare, Concord, CA). Clinicopathological information about the patients such as age, sex, smoking history, tumour location, American Joint Committee on Cancer (AJCC) tumour stage, recurrence with metastasis after treatment, EGFR TKI treatment modality and follow up was obtained from the pathology and hospital records.

### EGFR mutation analysis

On microscopic view, the tumour contents revealed various morphological features such as necrotic materials, normal bronchial epithelium, inflammatory cells, vessel and mucous materials, etc. For detection of EGFR mutation, we obtained the most glandular or tubular formation from tissue and harvested at least one portion including the highest number of tumour cells from cytology (proportion of tumour cells  $\geq 70\%$ , tumour cell count 100-300). Tissue and cytology samples were analysed using three different EGFR mutation tests performed by three different testing laboratories: DS was performed by Asan Medical Center (Seoul, Korea); pyrosequencing was performed by Konkuk University Medical Center (Seoul, Korea); and PNAc was performed by PANAGENE Inc (Daejeon, Korea). The three methods were performed using the experimental set up of the laboratories, with complete data analysis and quality control according to their own specific protocols (further details in the Supplementary Methods). The EGFR mutations detected by pyrosequencing and PNAc are shown in Supplementary Table S3.

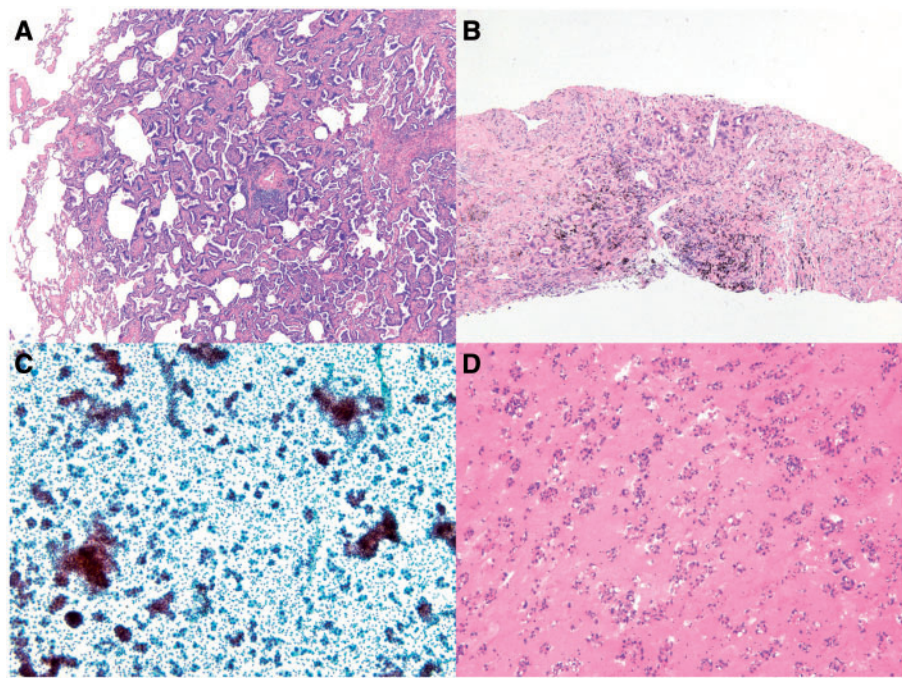


Figure 1. Representative microphotographs showing tissue slide from resection (A) and biopsy (B) and cytology slide from conventional Pap smear (C) and cell block (D) in lung adenocarcinoma (original magnification  $\times 100$ ).

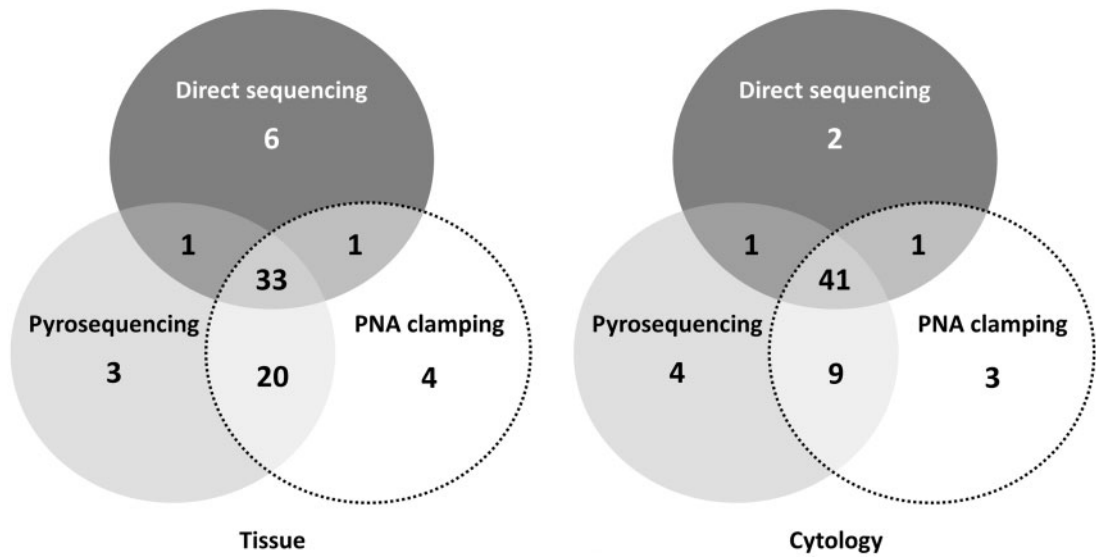


Figure 2. Venn diagram showing the distribution of EGFR mutation detection using direct DNA sequencing, pyrosequencing and PNA clamping methods in the tissue and cytology.

Statistical analyses

Comparisons of EGFR mutation detection between tissue and cytology samples using DS, pyrosequencing and PNAc were performed using  $\kappa$  statistics. Comparisons between EGFR mutational status and clinicopathological parameters were analysed by the  $\chi^2$  test. Continuous variables were analysed using the independent samples t test. For analysis of diagnostic performance, receiver operating characteristic (ROC) curves plotting sensitivity versus 1—specificity were used. A two-tailed  $P < 0.05$  was considered statistically significant. All data were analysed by using SPSS software for Windows 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics and tumour EGFR mutation status detected by direct sequencing, pyrosequencing and PNA clamping in tissue and cytology samples

In tissue samples, EGFR mutations were identified in 41 (37%), 57 (51%) and 58 (52%) of the 112 patients by DS, pyrosequencing and PNAc, respectively. Unlike DS, pyrosequencing detected 57 patients who were positive for EGFR mutations; 16 cases more than those detected by DS. PNAc detected 58 patients who were positive for EGFR mutations; 17 cases more than those detected

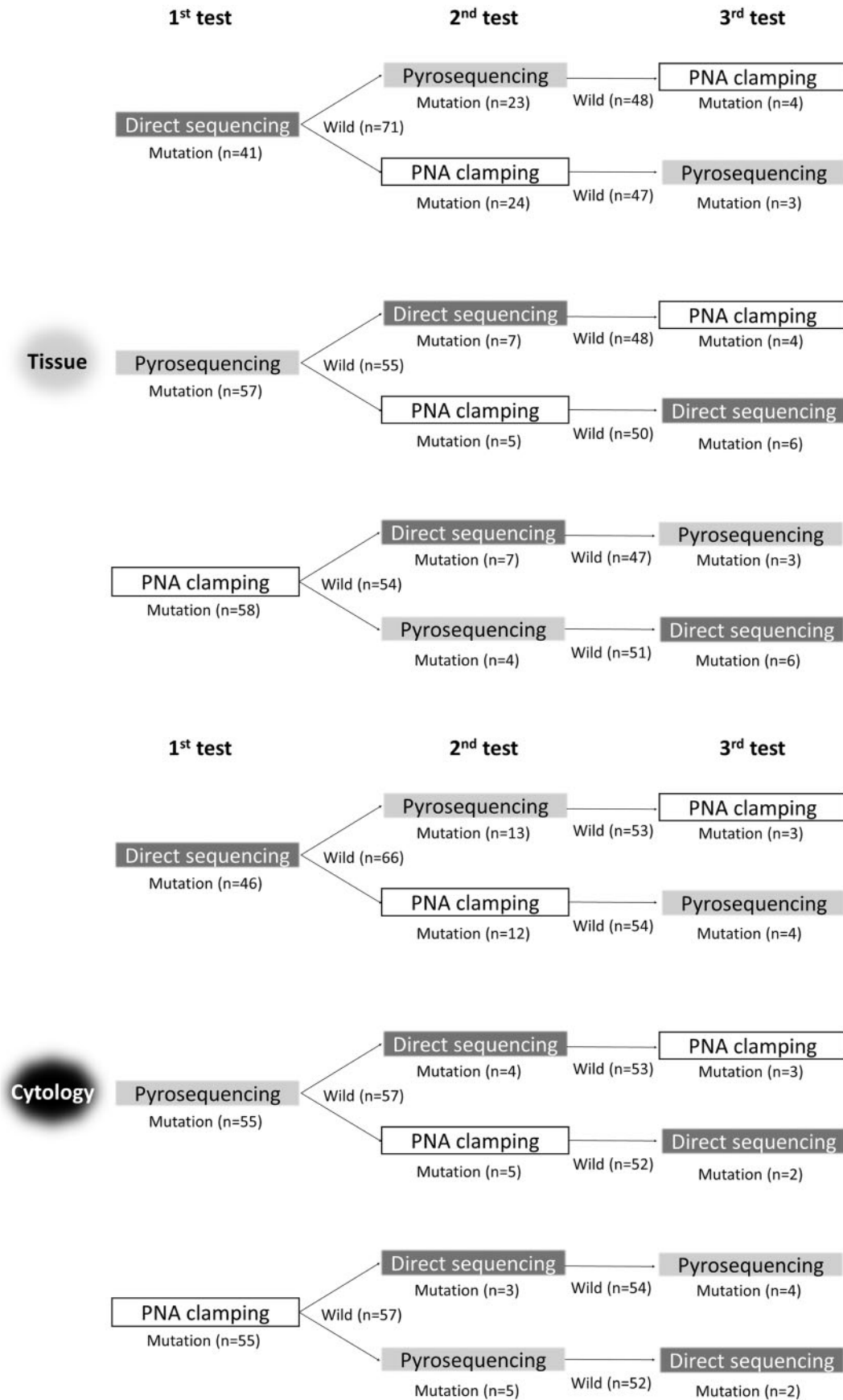


Figure 3. EGFR mutation status detected by direct sequencing, pyrosequencing and PNA clamping methods in the tissue and cytology.



**Table 1.** Concordance among the direct DNA sequencing, pyrosequencing and PNA clamping methods for the detection of EGFR mutations in the tissue and cytology

Tissue				CR
		Pyro		
		Wild	Mutation	
DS	Wild	48 (87.3%)	23 (40.4%)	46.7%
	Mutation	7 (12.7%)	34 (59.6%)	
		DS		
		Wild	Mutation	
PNAc	Wild	46 (66.2%)	7 (17.1%)	45.2%
	Mutation	24 (33.8%)	34 (82.9%)	
		PNAc		
		Wild	Mutation	
Pyro	Wild	50 (92.6%)	5 (8.6%)	83.9%
	Mutation	4 (7.4%)	53 (91.4%)	
Cytology				CR
		Pyro		
		Wild	Mutation	
DS	Wild	53 (93%)	13 (23.6%)	69.5%
	Mutation	4 (7%)	42 (76.4%)	
		DS		
		Wild	Mutation	
PNAc	Wild	54 (81.8%)	3 (6.5%)	73.1%
	Mutation	12 (18.2%)	43 (93.5%)	
		PNAc		
		Wild	Mutation	
Pyro	Wild	52 (91.2%)	5 (9.1%)	82.1%
	Mutation	5 (8.8%)	50 (90.9%)	

CR, concordance rate; DS, direct sequencing; Pyro, pyrosequencing; PNAc, PNA clamping.

by DS. Using pyrosequencing and PNAc, two mutations were found in six and three cases, respectively, but double mutations could not be detected by DS. Exon 18 mutation was detected only by DS. In cytology samples, EGFR mutation detection rate was different according to each method. In cytology samples, EGFR mutations were identified in 46 (41%), 55 (49%) and 55 (49%) of the 112 patients by DS, pyrosequencing and PNAc, respectively (Figure 2). Compared with DS of tissue samples, two mutations were found in two cases, but exon 18 mutation was not observed in cytology samples. Using pyrosequencing and PNAc, two mutations were found in five and three cases, respectively (Supplementary Table S4).

Twelve patients harbouring EGFR double mutations were detected. Ten cases harbouring EGFR double mutations were detected by single method, but two cases were identified by combination of at least more than two methods (Supplementary Table S5).

Among the patients who had EGFR wild type by single method, EGFR mutations were identified by other methods in tissue samples (Figure 3). Among the 71 patients who had EGFR wild type by DS, 23 (32.3%) and 24 (33.8%) mutations were detected by pyrosequencing and PNAc, respectively. Among the 55 patients who had EGFR wild type by pyrosequencing, 7 (12.7%) and 5 (9%) mutations were detected by DS and PNAc, respectively. Among the 54 patients who had EGFR wild type by PNAc, 7 (12.9%) and 4 (7.4%) mutations were detected by DS and pyrosequencing, respectively. Among the 66 patients who had EGFR wild type by DS in cytology samples, 13 (19.6%) and 12 (18.1%) mutations were detected by pyrosequencing and PNAc, respectively. Among the 57 patients who had EGFR wild type by pyrosequencing in cytology samples, 4 (7%) and 5 (8.7%) mutations were detected by DS and PNAc, respectively. Among the 57 patients who had EGFR wild type by PNAc in cytology samples, 3 (5.3%) and 5 (8.8%) mutations were detected by DS and pyrosequencing, respectively.

### Concordance rates of direct sequencing, pyrosequencing and PNA clamping

In tissue samples, the concordance rates between two diagnostic tests were as follows: DS and pyrosequencing, 46.7%; DS and PNAc, 45.2%; and pyrosequencing and PNAc, 83.9%. In cytology samples, the concordance rates between two diagnostic tests were as follows: DS and pyrosequencing, 69.5%; DS and PNAc, 73.1%; and pyrosequencing and PNAc, 82.1% (Table 1).

The concordance rates between the tissue using DS and cytology using DS, pyrosequencing and PNAc were 45.6, 39 and 39%, respectively. The concordance rates between the tissue using pyrosequencing and cytology using DS, pyrosequencing and PNAc were 66.2, 82.1 and 75%, respectively. The concordance rates between the tissue using PNAc and cytology using DS, pyrosequencing and PNAc were 68.1, 80.4 and 91.1%, respectively. Intriguingly, the concordance rates between pyrosequencing and PNAc in both tissue and cytology samples were significantly higher than those between DS and the above methods. On comparing the concordance rates between the tissue and cytology, the combination showed a high concordance rate of 81.7% (Table 2).

### Diagnostic performance of the tissue and cytology according to the EGFR mutation detected by direct sequencing, pyrosequencing and PNA clamping

The diagnostic performance of the tissue was as follows: DS (58% sensitivity, 0.793 area under ROC), pyrosequencing (81% sensitivity, 0.907 area under ROC), PNAc (83% sensitivity, 0.914 area under ROC). The diagnostic performance of cytology was as follows: DS (66% sensitivity, 0.829 area under ROC), pyrosequencing (79% sensitivity, 0.893 area under ROC), PNAc (79% sensitivity, 0.893 area under ROC). In tissue and cytology samples, the EGFR mutation detection rate of pyrosequencing and PNAc was higher than that of DS.

With respect to the diagnostic performance of the tissue and cytology, there was high sensitivity in both the tissue and cytology (97 and 88% sensitivity, 0.986 and 0.943 area under ROC, respectively) (Supplementary Table S6).

### Correlation between clinicopathological characteristics and EGFR mutation

In the 112 paired tissue and cytology samples, a total of 70 (62.5%) mutations were found by at least one of the three

**Table 2.** Comparison of EGFR mutation detection between the tissue and cytology according to the direct DNA sequencing, pyrosequencing and PNA clamping methods

Tissue		Cytology											
		DS			Pyro			PNAc			Combination <sup>a</sup>		
		W	M	CR	W	M	CR	W	M	CR	W	M	CR
DS	W	54	17	45.6%	47	24	39%	47	24	39%	44	27	42.7%
	M	12	29		10	31		10	31		6	35	
Pyro	W	51	4	66.2%	51	4	82.1%	49	6	75%	45	10	73.2%
	M	15	42		6	51		8	49		5	52	
PNAc	W	51	3	68.1%	50	4	80.4%	53	1	91.1%	48	6	85.7%
	M	15	43		7	51		4	54		2	56	
Combination <sup>b</sup>	W	43	1	58.7%	42	2	69.8%	43	1	73.3%	42	2	81.7%
	M	23	45		15	53		14	54		8	60	

CR, concordance rate; DS, direct sequencing; Pyro, pyrosequencing; PNAc, PNA clamping; M, mutation; W, wild.

<sup>a</sup>EGFR mutations detected by at least one of the three methods in cytology samples.

<sup>b</sup>EGFR mutations detected by at least one of the three methods in tissue samples.

methods such as DS, pyrosequencing and PNAc. Mutations were more frequently observed in females than in males and in non-smokers than in ex/current smokers. Furthermore, tumour involvement of multiple lobes was significantly correlated with mutations (all  $P < 0.05$ ) (Supplementary Table S7).

## Discussion

A variety of methods are available for identifying EGFR mutations, but there is no consensus on which method is the most effective or accurate. DS is labor-intensive, time consuming and has a relatively lower sensitivity. Pyrosequencing is a simple, robust, fast and sensitive method as well as a cost-effective alternative. PNAc is also a highly sensitive, rapid and simple method. However, PNAc with pyrosequencing cannot identify novel EGFR mutations.<sup>10</sup> In this study, one of discrepant cases was detected as having EGFR exon 18 mutation using DS in the tissue. Furthermore, two cases with E19 p.E747\_A751del mutation were identified using DS, but these were not detected using pyrosequencing and PNAc. It was an underlying EGFR mutation, and hence it was not identified by pyrosequencing and PNAc. The weakness of pyrosequencing and PNAc is that they can only be used to detect mutations for which primers have been previously designed. Therefore, the EGFR mutation detection could be incomplete with the use of pyrosequencing and PNAc. For discovery of novel gene mutations, DS is still considered as a useful tool.

Previous studies demonstrated that pyrosequencing and PNAc have higher sensitivity than DS for detecting EGFR mutations.<sup>6,10,19</sup> In our study, EGFR mutations were frequently detected by pyrosequencing and PNAc in tissue and cytology samples, compared with those detected by DS. However, a study by Lee et al. reported that sensitivity of pyrosequencing and PNAc is slightly lower than that of DS.<sup>20</sup> Thus, controversy still exists. This discrepancy can be explained by differences in DNA extraction procedures between laboratories as well as in study designs, patient characteristics and the number of study subjects.

Biopsy sampling is an invasive procedure and it is often difficult to obtain tumour tissue from patients with advanced tumour stage. Therefore, the cytology specimen is considered to be an attainable source of molecular analysis, which requires a relatively simple, minimally invasive and repeatable procedure. Previous studies have demonstrated that cytology samples

were successfully assessed for EGFR mutations using various methods.<sup>13,21,22</sup> In our results, EGFR mutations detection rate was highly concordant between the tissue and cytology, especially using pyrosequencing and PNAc, which is consistent with the results of other studies.<sup>23,24</sup> The concordance rate between the tissue and cytology was high, and the diagnostic performance for both samples was high. Therefore, it is thought that the cytology sample is an easily available sample, which is successfully assessed for EGFR mutations similar to the tissue sample.

With respect to the detection of EGFR mutations, various factors may have contributed to the discordances between the tissue and cytology and these may include tumour cell percentage in the specimen, tumour heterogeneity or sampling of different sites.<sup>25</sup> Generally, sufficient tissue samples could have a high diagnostic yield, but the tissue may have variations in the tumour cell content within and across the samples, compared with cytology. In our results, the detection rate of EGFR mutations in cytology was higher than that in the tissue using DS, which especially requires sufficient levels of mutant DNA.

In at least one of the tissue and cytology samples, double mutations within the EGFR exons 19, 20 and 21 by pyrosequencing and PNAc were used to identify 10 and 3 cases, respectively. However, double mutations by DS in the tissue were not found, although two cases were detected by DS in cytology. The clinical significance of double mutations is still unclear, but it could be due to tumour heterogeneity. A study by Sakurada et al. suggested that some tumours may demonstrate intratumoural heterogeneity for the occurrence of EGFR mutation.<sup>25</sup>

The presence of EGFR mutations is an important molecular biologic factor for therapeutic planning. A more sensitive screening method would allow for better prediction of the response to EGFR-TKIs and enable personalized therapy. This study demonstrated that EGFR mutation detection rate in cytology is similar to that in the tissue and hence it is feasible. The diagnostic performance of pyrosequencing and PNAc was superior to that of DS in both tissue and cytology samples. In our study, among the 71 patients who had EGFR wild type by DS, EGFR mutations were often detected by pyrosequencing and PNAc.

In summary, high diagnostic performance of cytology corresponding to that of tissue is a useful screening tool for the detection of EGFR mutations in patients who cannot tolerate the invasive diagnostic procedure. The concordance among DS,

pyrosequencing and PNAc was variable according to tissue or cytology specimens. Thus, the second test in reference to the other sensitive methods should be performed to improve diagnostic accuracy, when the first test could not detect the EGFR mutation.

## Supplementary material

Supplementary material is available at QJMED online.

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Conflict of interest: None declared.

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