Original Article

Frequency of EGFR Mutations among Thai Non-Small Cell Lung Cancer [NSCLC] Patients

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Background: Tyrosine kinase inhibitor is an important drug for lung adenocarcinoma patients with epidermal growth factor [EGFR] mutation. Asian patients with non-small cell lung cancer [NSCLC] have a high incidence of EGFR mutations.

Objective: To determine the frequency of EGFR mutations among Thai NSCLC patients using Sanger sequencing and a real-time polymerase chain reaction [PCR] assay.

Materials and Methods: DNA samples were extracted from formalin-fixed paraffin embedded [FFPE] tissues. EGFR mutations were analyzed by direct sequencing in 318 NSCLC cases and by a real-time PCR-based assay (therascreen® RGQ EGFR mutation kit) in 112 cases.

Results: With direct sequencing, EGFR mutations were detected in 156 of 318 cases (49.06%), including 101 exon 19 mutations (31.76%) and 89 exon 21 mutations (27.99%). Mutations in exons 18 through 21 were detected in 51.79% of the cases analyzed with the therascreen assay. Exon 19 deletion and L858R mutations were detected in 25.16% and 16.35% of cases, respectively, by direct sequencing and in 26.78% and 13.39% of cases, respectively, by the therascreen assay. T790M mutation, found in 7.14% of the cases, always occurred along with exon 19 deletion or L858R mutation. Patients aged >75 years had a significantly higher frequency of EGFR mutations than younger patients ($p<0.05$). In addition, female patients had a significantly higher frequency of EGFR mutation than male patients ($p<0.001$). EGFR mutations were detected at the same rate by direct sequencing and a real-time PCR assay.

Conclusion: This study supports that EGFR mutation is more prevalent in women and older (age >75) Asian patients with NSCLC. The two most common mutations are exon 19 deletion and L858R substitutions.

Keywords: Lung cancer, NSCLC, EGFR, Mutation, Tyrosine kinase inhibitor

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Lung cancer is the second most prevalent form of cancer among Thai men and the fourth most prevalent among Thai women[1]. Approximate 80% of Thai lung cancer cases are non-small cell lung cancers [NSCLC][2]. Tyrosine kinase inhibitors, such as erlotinib or gefitinib, are effective against NSCLCs with epidermal growth factor receptor [EGFR] gene mutations[3,4]. EGFR mutations in the tyrosine kinase domain are caused by single nucleotide substitutions, deletions, or insertions in exons18, 19, 20, or 21. In previous research, about 90% of EGFR mutations were deletions within exon

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19 or an amino acid substitution [L858R] in exon 21[5]. Several guidelines recommend EGFR mutation analysis before first-line therapy in NSCLC patients[6]. The mutations can be detected by many techniques and commercially available kits, such as direct sequencing, allele-specific polymerase chain reaction [PCR] (cobas EGFR mutation test kit, Roche™, USA), and amplified refractory mutation system (ARMS, therascreen® RGQ EGFR mutation kit, Qiagen™, UK)[7]. The detection techniques vary in sensitivity, specificity, limit of detection [LOD], convenience, and mutations detected. These factors are important for determining which technique benefits patients the most.

In this study, we determined the frequency of EGFR mutations among Thai patients with NSCLC using Sanger sequencing and a real-time PCR assay.

Materials and Methods

Patient samples

The protocol of this research was reviewed and approved by the Ethical Committee for Human Research, Chulabhorn Research Institute (EC No. 14/2554). Formalin-fixed paraffin embedded [FFPE] tissue or plural effusion cell block samples from 431 patients with lung adenocarcinoma were collected from March 2010 to May 2017. The percentage of malignant cells in the FFPE blocks was estimated by pathologists. DNA was extracted from FFPE tissue or plural effusion samples using a QIAamp DNA Micro Kit (Qiagen™, UK) following the manufacturer's instructions. The DNA concentration was determined by spectrophoto meter (Nano Drop, Thermo Fisher Scientific, USA).

PCR for EGFR exon 19 and exon 21

Exons 19 and 21 of the EGFR gene were amplified by PCR using the primers Ex19-F (5'GTAACATCCACCCAGATCACTG3'), Ex19R (5'GC CAGACATGAGAAAAGGTGG3'), Ex21F (5'GAG CCTGGCATGAACATGAC3'), and Ex21R (5'AACA ATACAGCTAGT GGGAAGG3'). The DNA was denatured for 4 minutes at 94°C, amplified for 35 cycles (94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds), and extended at 72°C for 5 minutes. The PCR products were detected by 2% agarose gel electrophoresis. Direct sequencing of PCR products was conducted by commercial sequencing companies (First BASE Laboratory, Malaysia and Macrogen Inc., Korea).

Sequencing analysis

DNA sequences were compared with the wild type EGFR sequence (LRG_304t1.1) from the GenBank database. Multiple sequence alignments were performed using Genalys Win3 (Metrowerks Code Warrior). The deduced amino acids (P00533.2) were archived using the National Center for Biotechnology Information website.

Therascreen® RGQ EGFR mutation kit

Therascreen® RGQ EGFR mutation kit (Qiagen, UK) was used for detection of mutations in exons 18, 19, 20, and 21. PCR reactions were conducted in a Rotor-Gene Q (Qiagen, UK) in accordance with the manual for the therascreen® RGQ EGFR mutation kit).

Statistical analysis

The association between mutations and demographic data for each method was analyzed using exact probability test. Logistic regression analysis was used to analyze the association between mutations and demographic data. The *p*-values <0.05 were considered significant.

Results

From March 2010 to May 2017, EGFR mutations were analyzed in samples from 430 patients with lung cancer. The first group of samples from 318 patients was analyzed with the direct sequencing technique, and the second group of samples from 112 patients was analyzed by real-time PCR using the therascreen. With direct sequencing, EGFR mutations were detected in 156 of 318 cases (49.06%), including 101 exon 19 mutations (31.76%) and 89 exon 21 mutations (27.99%) (Table 1). EGFR mutations were detected in 58 of 112 cases (51.79%) using the therascreen. The percentages of EGFR mutations in exons 18, 19, 20, and 21 were 1.79%, 32.14%, 8.93%, and 18.75%, respectively (Table 2).

Patients over 75 years old had a significantly higher frequency of EGFR mutations detected by the therascreen ($p<0.05$; Table 3). In addition, female patients had a significantly higher frequency of EGFR mutation detected by the therascreen than male patients ($p<0.001$). However, these age and sex differences were not detected by direct sequencing.

The two most common mutations were exon 19 deletion and L858R substitutions (Table 4). The frequencies of these two mutations were 25.16% and 16.35% by direct sequencing and 26.78% and 13.39% by therascreen. The T790M mutation was only detected along with an exon 19 deletion or L858R mutation. The rare mutations were detected data rate of 4.4% by direct

sequencing and included G729E, E736G, I740V, L747P, and P753L in exon 19 and M825V, V834M, P848L, M853V, R854A, D855E, and H870Y in exon 21.

Discussion

The frequency of EGFR mutations in this study was nearly 50% for both the direct sequencing and real-time PCR techniques. This result is consistent with recent reports showing that approximately 50% of Asian or Thai patients had EGFR mutations[8,9]. Patient populations in Europe, America, and India were found to have a lower rate of EGFR mutations (20 to 30%)[10]. We found that the frequency of EGFR mutations detected by the therascreen was significantly higher in women than in men, but we did not find the same difference by direct sequencing. In a previous study, EGFR mutations were more common in women, non-smokers, and patients with adenocarcinoma[11]. The therascreen can detect 29 mutations in exons 18, 19, 20, and 21. In this study, the direct sequencing assay analyzed only exons 19 and 20. With both detection methods, almost all the mutations were either deletions in exon 19 or L858R base substitution. These two types of mutations are known to sensitize tumors to tyrosine kinase inhibitor [TKI] treatment similar to other point mutations such as G719S, S768I, and L861Q[12]. In contrast, insertions in exon 20 and T790M base substitution have been classified as resistance markers for TKI therapies[13]. The majority of rare mutations in exons 19 and 21 that can be detected by direct sequencing had no effect on targeted drug sensitivity[14,15]. However, some rare mutations such as L747P increase resistance to TKI treatment[16]. In recent years, Osimertinib, a new drug effective against tumors with T790M mutations, has been used to treat patients with disease progression after first and second generation TKI treatment[17]. These data supported that EGFR mutation detection methods should analyze exons 18 to 21 because many mutations are important for selection of the optimal TKI therapy for each patient. Recently, several high sensitivity techniques have been developed for mutation detection, such as digital droplet PCR or next generation sequencing[18,19]. The circulating free tumor-derived DNA [ctDNA] in blood plasma is a source for tumor DNA diagnosis. By using ctDNA one can determine EGFR mutation in patient that had been treated with TKI therapy. Moreover ctDNA can be an alternative choice for patient show that tissue test results are not evaluable or tissue is not available[20,21]. These new technologies will be valuable tools for future TKI treatment selection.

Conclusion

Our data showed a high incidence of EGFR mutations by direct sequencing and real-time PCR in Thai NSCLC patients. These data are consistent with

Table 1. Demographics of patients with EGFR exon 19 or 21 mutations detected by direct sequencing

Variable	EGFR mutation		*p*-value*	Exon 19 mutation		*p*-value*	Exon 21 mutation		*p*-value*
	Yes n (%)	No n (%)		Yes n (%)	No n (%)		Yes n (%)	No n (%)	
Age group			0.510			0.739			0.428
<65	56	57		39	74		32	81	
	35.90	35.19		38.62	34.10		35.96	35.37	
65 to 75	54	65		36	83		29	90	
	34.61	40.12		35.64	38.25		32.58	39.30	
>75	46	40		26	60		28	58	
	29.49	24.69		25.74	27.65		31.46	25.33	
Sex						0.185			0.212
Male	76	96	0.072	49	123		43	129	
	48.72	59.26		48.51	56.68		48.31	56.33	
Female	80	66		52	94		46	100	
	51.28	40.74		51.49	43.32		51.69	43.67	
Total	156	162		101	217		89	229	
	49.06	50.94		31.76	68.24		27.99	72.01	

* Exact probability test

Table 2. Demographics of patients with EGFR exon 18, 19, 20, or 21 mutations detected by therascreen RGQ EGFR mutation kit

Variable	EGFR mutation		*p*-value*	Exon 18 mutation		*p*-value*	Exon 19 mutation		*p*-value*	Exon 20 mutation		*p*-value*	Exon 21 mutation		*p*-value*
	Yes n (%)	No n (%)		Yes n (%)	No n (%)		Yes n (%)	No n (%)		Yes n (%)	No n (%)		Yes n (%)	No n (%)	
Age			0.060			0.044			0.073			0.145			0.859
<65	33	19		0	52		22	30		77	45		11	41	
	56.90	35.18		0.00	47.27		61.11	39.47		0.00	44.12		52.38	45.05	
65 to 75	16	20		0	36		10	26		3	33		6	30	
27.58	37.04		0.00	32.73		27.78	34.21		30.00	32.35		28.57	32.97		
>75	9	15		2	22		4	20		0	24		4	20	
	15.52	27.78		100.00	20.00	11.11		26.32		0.00	23.53		19.05	31.98	
Sex			<0.001			0.159			<0.001			0.311			0.137
Male	12	33		2	43		5	40		2	43		5	40	
	20.69	61.11		100.00	39.09		13.89	52.63		20.00	42.16		23.81	43.96	
Female	46	21		0	67		31	36		8	59		16	51	
	79.31	38.89		0.00	60.91		86.11	47.37		80.00	57.84		76.19	56.04	
Total	58	54		2	110		36	76		10	102		21	91	
	51.79	48.21		1.79	98.21		32.14	67.86		8.93	91.07		18.75	81.25	

* Exact probability test

Table 3. Detection of EGFR mutations in exon 19 and exon 21 by the therascreen RGQ EGFR mutation kit and direct sequencing

Variable	Therascreen (all mutations)		Direct sequencing (exon 19, 21)		therascreen (exon 19, 21)	
	OR (95% CI)	*p*-value*	OR (95% CI)	*p*-value*	OR (95% CI)	*p*-value*
Age group						
<65	1	-	1	-	1	-
65 to 75	0.46 (0.19 to 1.10)	0.080	0.85 (0.50 to 1.42)	0.524	0.50 (0.21 to 1.18)	0.115
>75	0.35 (0.13 to 0.94)	0.037	1.17 (0.67 to 2.05)	0.583	0.26 (0.09 to 0.73)	0.011
Sex						
Male	1	-	1	-	1	-
Female	6.02 (2.60 to 13.93)	<0.001	1.53 (0.98 to 2.39)	0.060	7.16 (3.00 to 17.06)	<0.001

* Logistic regression analysis

Table 4. Summary of individual EGFR mutations detected by the therascreen RGQ EGFR mutation kit and direct sequencing

Mutation type	Direct sequencing (n = 318)		therascreen (n = 112)	
	n	%	n	%
Single mutation				
G719X	N/A	N/A	2	1.79
S768I	N/A	N/A	0	0
T790M	N/A	N/A	0	0
Exon 19 deletion	80	25.16	30	26.78
Exon 20 insertion	N/A	N/A	1	0.89
L858R	52	16.35	15	13.39
L861Q	1	0.31	0	0
Multiple mutation				
Exon 19 deletion + L858R	9	2.83	1	0.89
Exon 19 deletion + T790M	N/A	N/A	4	3.57
Exon 19 deletion + T790M + L861Q	N/A	N/A	1	0.89
L858R + T790M	N/A	N/A	3	2.68
L858R + S768I	N/A	N/A	1	0.89
Other mutation	14	4.4	N/A	N/A

previous reports demonstrating a high frequency of EGFR mutations in Asian populations, particularly women and elderly patients.

What is already known on this topic?

Lung cancer patients in Europe and America were found to have a lower rate of EGFR mutations (20 to 30%). Real-time PCR is more sensitive than direct sequencing for mutation analysis. Most rare mutations detected with direct sequencing may not influence TKI response.

What this study adds?

Our data shows that EGFR mutation is more prevalent in women and older Thai patients with NSCLC. EGFR mutations were detected at the same rate by direct sequencing and a real-time PCR assay. The T790M mutation was only detected together with exon 19 deletion or L858R mutation.

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Potential conflicts of interest

The authors declare no conflict of interest.

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