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Research Article

Histologic Grade with Thyroid Transcription Factor 1 and Sample Type Serve as Independent Factors for the Incidence of EGFR Mutations in Non-small Cell Lung Cancer

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Abstract

Objectives: This study aims to describe the characteristics of epidermal growth factor receptor (*EGFR*) mutations in correlation with clinical features of Vietnamese non-small cell lung cancer (NSCLC), and identify the predictive factor for the incidence of *EGFR* mutations.

Methods: A total of 1.548 newly diagnosed NSCLC patients were selected for this retrospective study. *EGFR* mutations were detected in the tumor, lymph node tissue and pleural fluid by the pyrosequencing method.

Results: *EGFR* mutations were detected in 623 patients (40.2%). Mutations were more frequent in the female patient ($p<0.001$), in adenocarcinoma ($p=0.004$), in pleural effusion sample ($p=0.002$), in the low-intermediate grade of the tumor ($p<0.001$), and in those with CK7-positive ($p=0.001$) and TTF1-positive result ($p<0.001$). Notably, the low grade of the tumor ($p<0.001$), TTF1-positive marker ($p=0.001$) and pleural fluid ($p=0.002$) were detected as independent factors for the higher incidence of *EGFR* mutations in multivariable analysis. In addition, CK7 marker played the role as an independent factor when TTF1 marker was not applied ($p=0.011$).

Conclusion: *EGFR* mutations occur with high frequency in Vietnamese NSCLC patients. Histologic grade, TTF1 marker, and sample type are independent factors for the incidence of *EGFR* mutations.

Keywords: *EGFR* mutations; NSCLC; histologic grade; pleural fluid; TTF1; CK7 markers

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Lung cancer is the most common cause of cancer death in over the world.[1] The disease develops silently with no symptoms while almost all patients are diagnosed in late stages (III-IV). Approximately 85-90% lung cancer cases are non-small cell lung cancer which includes adenocarcinoma (ADC), squamous cell carcinoma (SCC), large cell carcinoma, and a few of sarcomatoid carcinoma.[2, 3] Before the 2000s, chemotherapy with radiotherapy and surgery were the principal treatment methods for NSCLC. Erlotinib (Tarceva, OSI/Genentech) and Gefitinib (Iressa/AstraZeneca), the tyrosine kinase inhibitors (TKI), were first approved in 2004 by the Food and Drug Administration, and later as the first-line agents in treatment for NSCLC patients who have the activating *EGFR* mutations in exon 19 (E19del) or exon 21 (L858R) substitution mutation.[3, 4] This is the breakthrough in treatment for NSCLC patients. These drugs act to inhibit the tyrosine kinase activity of mutated *EGFR* molecules, therefore inhibit the transduction of intracellular signals, leading to inhibition of malignant cell proliferation.

Regarding TKI treatment, *EGFR* diagnostic test plays an important role in selecting the suitable patients (with activating *EGFR* mutations) for the treatment regimen. The mutation testing on the tumor tissue was considered as the gold standard method in clinical practice. Many studies have demonstrated that *EGFR* mutations occur in 15-27% of European and American NSCLC patients, but higher in

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Eat-Asian patients (40-64%).[5-10] In addition, the mutation occurs more frequently in female and non-smoker patients compared to others.[5-8, 10] In spite of that, a little information about *EGFR* mutations in Vietnamese lung cancer patients was reported.[7, 9] Our study aims to describe the characteristics of *EGFR* mutations in correlation with clinical features of Vietnamese NSCLC population, and afterward identify the predictive factor for the incidence of *EGFR* mutations.

Methods

Patient and Parameters

A total of 1.548 newly diagnosed NSCLC patients from February 2014 to April 2018 were selected for this retrospective study at Cho Ray hospital. Patient selection based on the criteria of the primary lung tumor and the available result of *EGFR* mutations. The clinical and paraclinical characteristics such as histological type, grade of tumor differentiation, immunohistochemical results, and disease stage were recalled from the laboratory database and clinical records. The study was reviewed and approved by the Ethics Committee of Cho Ray Hospital (Reference number: 258/BVCR-HDDD/2016). Authors were allowed to access the clinical records and collect the requested data with the responsibility of information security. Patients were not requested to write the consent form.

Genomic DNA Extraction

Genomic DNA was extracted from 3-5 slices of the formalin fixed paraffin embedded (FFPE) tumor tissue, lymph node tissue or pleural effusion sample. Extraction protocol was performed automatically on QIAcube machine, using kit QIAamp DNA FFPE Tissue (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, paraffin in FFPE tissue samples was removed by xylene solution, and afterward by ethanol 100%, then evaporating in room temperature for 3 minutes. Tumor tissue was destructed by 180µl ATL solution and 20µl proteinase K in 56°C for 60 minutes, then in 90°C for 60 minutes. Tumor cells were lysed completely by 200µl AL solution, then precipitated by 400µl ethanol 100%. The entire lysate was transferred to the QIAamp MinElute column where DNA was captured on the silica membrane and eluted after washing twice with AW1 and AW2 solutions. Concentration and purity of DNA were checked by the BioDrop µLITE machine (BioDrop Ltd, UK), then stored in -40°C until uses.

Detecting *EGFR* Mutations

The mutations in FFPE sample were detected by the pyrosequencing method using kit Therascreen *EGFR* Pyro (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The technical protocol consists of three steps: polymerase chain reaction (PCR) for amplification of *EGFR* gene from exon 18 to exon 21; immobilization of PCR products to Streptavidin-Sepharose high-performance beads, then collecting the target gene by filter probe; and pyrosequencing step. The required load of DNA per action was 2-10ng. Sequencing results were reported by the PyroMark Q24 v.2.0.6 software.

Statistical Analysis

The chi-square test was used to compare the frequency between groups of clinical characteristics. The logistic regression model with multivariable analysis was used to identify the useful factors for the incidence of *EGFR* mutations. All data analyses were performed on the R statistical software v.3.5.1 (R foundation, 1020 Vienna, Austria). $P<0.05$ was considered as significant difference.

Results

Patient Characteristics

The median age of all study subjects was 59 years (ranging from 18 to 92). The majority of the cases were male patients (978, 63.2%). Most of them (1.354, 87.5%) were classified as clinical stage IV according to the TNM classification system (Table 1).

The initial diagnosis and *EGFR* mutation testing were performed on the tumor tissue, lymph node tissue, and pleural effusion sample for 1.333 (86.1%), 116 (7.5%), and 99 (6.4%) patients, respectively. Among the confirmed patients with histological type (1.330), adenocarcinoma was accounted for most of the cases (93.3%), and higher in female (96.4%) compared to male patients (91.5%) ($p=0.015$). The poorly differentiated tumor (grade III) was recorded in 232 of 431 (53.8%) patients, whereas the moderately (grade II) to the well-differentiated tumor (grade I) were recorded in 199 (46.2%) cases.

From 894 available data, 94.5% of patients were positive with the CK7 marker. Similarly, the TTF1 marker was highly positive in NSCLC (80.4%). We also found that the expression of CK7 was coherent with TTF1 marker (odds ratio, OR=13.38, $p<0.001$), and these booth markers were closely associated with adenocarcinoma (OR were 20.94 and 5.84, respectively) ($p<0.001$). Conversely, Cytokeratin 20 (CK20) marker was negative in most of all patients (94.8%). The smoking history was not fully collected from the medical records.

EGFR Mutations in Different Characteristics

Totally, *EGFR* mutations were detected in 623 of 1.548 NSCLC patients equivalent to the mutation rate of 40.3% (Table 1). The mutations were found more frequently in female patients (59.3%, $p<0.001$), in adenocarcinoma (42.1%,

Table 1. Patient characteristics and *EGFR* mutation status

Characteristics	Frequency	*EGFR* status, n (%)		P
		(+)	(-)	
Total	1.548	623 (40.3)	925 (59.7)	-
Age: median: 59 (18-92) yrs				0.695
<59	741	302 (40.8)	439 (59.2)	
≥59	807	321 (39.8)	486 (60.2)	
Gender				<0.001
Female	570	338 (59.3)	232 (40.7)	
Male	978	285 (29.1)	693 (70.9)	
Clinical stage		0.523		
I-III	194	74 (38.1)	120 (61.9)	
IV	1.354	549 (40.5)	805 (59.5)	
Sample type				0.002
Tumor tissue	1.333	527 (39.5)	806 (60.5)	
Lymph node tissue	116	40 (34.5)	76 (65.5)	
Pleural fluid	99	56 (56.6)	43 (43.4)	
Histological type				0.004
ADC	1.241	523 (42.1)	718 (57.9)	
SCC	72	17 (23.6)	55 (76.4)	
Large cell carcinoma	17	4 (23.5)	13 (76.5)	
NSCLC: non-specific	218	79 (36.2)	139 (63.8)	
Histologic grade				<0.001
High (III)	232	53 (22.8)	179 (77.2)	
Low-Intermediate (I-II)	199	100 (50.2)	99 (49.8)	
Not assessed	1.117	470 (42.1)	647 (57.9)	
Cytokeratin 7				0.001
Negative	49	8 (16.3)	41 (83.7)	
Positive: 1+	158	53 (33.5)	105 (66.5)	
Positive: 2+	170	61 (35.9)	109 (64.1)	
Positive: 3+	517	219 (42.4)	298 (57.6)	
Cytokeratin 20				0.150
Negative	704	280 (39.8)	424 (60.2)	
Positive	39	11 (28.2)	28 (71.8)	
Thyroid transcription factor 1				<0.001
Negative	170	39 (22.9)	131 (77.1)	
Positive: 1+	273	107 (39.2)	166 (60.8)	
Positive: 2+	204	84 (41.2)	120 (58.8)	
Positive: 3+	219	103 (47.0)	116 (53.0)	

ADC: adenocarcinoma; SCC: squamous cell carcinoma.

p=0.004), in low to the intermediate grade of the tumor (50.2%, p<0.001), and in pleural fluid (56.6%, p=0.002). We also noted that *EGFR* mutation rate was higher in CK7-positive patients (333/845, 39.4%) compared to those of negative group (8/49, 16.3%), and correlated with the positive levels of CK7 marker (p=0.001) (Table 1). The similar results were also observed in sub-groups of TTF1 marker (p<0.001). Mutation rate of TTF1-negative, TTF1-1+, TTF1-2+ and TTF1-3+ groups were 22.9%, 39.2%, 41.2% and 47.0%, respectively. No significant difference of mutation rate between groups of the age, CK20 marker status, and clinical stage was observed.

In multivariable analysis, four factors including gender (p<0.001), sample type (p=0.002), grade of tumor differentiation (p<0.001) and TTF1 marker (p=0.001) were identified as independent predictive factors for the incidence of *EGFR* mutations (Table 2). When TTF1 marker was excluded from the multivariable model, CK7 marker played the role as an independent factor for *EGFR* mutations (adjusted OR=2.86, 95%CI: 1.24-6.58, p=0.011).

Table 2. Independent factors for the incidence of *EGFR* mutations in multivariable analysis

Variable	Adjusted OR (95%CI)	P
Gender		
Female vs male	3.45 (2.75-4.32)	<0.001
Sample type		
Fluid vs tissue	2.03 (1.31-3.16)	0.002
Histologic grade		
Low-intermediate vs high	2.82 (1.81-4.38)	<0.001
TTF1 marker		
Positive vs negative	2.01 (1.31-3.06)	0.001
CK7 marker		
Positive vs negative	2.86 (1.24-6.58)*	0.011*

*: when TTF1 was excluded from the multivariable model; TTF1: thyroid transcription factor 1; CK7: cytokeratin 7; OR: odds ratio; Vs: versus.

Table 3. The proportion of mutations in four exons

Exon, mutation type	Frequency	Proportion, %
Exon 18	71	11.4
G719S	41	6.6
G719A	22	3.5
G719C	8	1.3
Exon 19	350	56.2
E746_A750	277	44.5
E746_S752	22	3.5
E746_T751	6	1.0
L747_A750	11	1.8
L747_P753	9	1.4
L747_T751	7	1.1
Other	18	2.9
Exon 20	34	5.5
S768I	14	2.2
T790M	10	1.6
Insertion	10	1.6
Exon 21	195	31.3
L858R	169	27.1
L861Q	26	4.2
Multiple: 2 mutations	26	4.2
Multiple: 3 mutations	1	0.2
Total	623	100

The Proportion of Mutation in Four Exons

Among 623 *EGFR* mutated patients, mutations at exon 18, exon 19, exon 20 and exon 21 were recorded in 71 (11.4%), 350 (56.2%), 34 (5.5%) and 195 (31.3%) cases, respectively (Table 3). Multiple mutations (2 or 3 mutations) were observed in 27 cases (4.4%). The most common type of mutation in exon 18, 19 and 21 was G719S, E746_A750, and L858R, respectively.

Discussion

The *EGFR* mutations in NSCLC patients were characterized well in other countries, but limited in Vietnam. We investigated and showed a higher rate of *EGFR* mutations in Vietnamese NSCLC population (40.3%) compared to those of the western (15-25%).[5, 6, 8] The mutations were found in all major types of NSCLC including ADC, SCC and the large cell carcinoma which is consistent with previous findings.[5,11-15] Besides, the mutations we observed more frequently in exon 19 and 21 (total of 87.5%) with the common type of mutation were E746_A750 and L858R, respectively.

The patient's gender, ethnicity, smoking status, and histological type have been demonstrated in numerous studies as the predictive factors for *EGFR* mutations in NSCLC.[5-8, 10, 11, 16-20] Moreover, the high level of serum biomarker such as carcinoembryonic antigen, cancer antigen 125, cancer antigen 199 and cancer antigen 242 have been proved to be associated with the higher rate of *EGFR* mutations.[21-25] The results of this study indicated that the grade of tumor differentiation, TTF1 marker, and sample type are additionally independent factors for the incidence of *EGFR* mutations. Furthermore, the CK7 marker can be used as the independent factor when TTF1 marker is absent.

In recent five years, many studies have mentioned the association of histologic grade and TTF1 marker with *EGFR* mutations in NSCLC.[11, 17-20, 26-34] The frequency of mutation was documented to be higher in patients with the low grade of histologic tumor and TTF1 positive results than in others. Therefore, these two factors might help clinicians to distinguish the potential patients of *EGFR* mutations once they have the initially pathological results. However, the independent role of these factors has just only been established recently in the studies of Shiau et al.[17] and Levy et al.,[20] which is in accordance with our results. In the other way, patients with well-differentiated tumor and TTF1 positive results have a better survival prognosis in *EGFR* TKI treatment.[19, 20, 30-32, 34] This indicates that the histologic grade and TTF1 marker are very useful in clinical practice, and should be examined for all NSCLC patients as the routine diagnostic tests. For the patients with the high grade of histologic tumor and the TTF1 negative result, especially accompanying with severe symptoms, the clinical decisions with the first-line chemotherapy, radiotherapy or immunotherapy (PD-L1/PD1 inhibitors) should be given quickly while awaiting the *EGFR* mutation testing. After that, the TKI treatment might be reintroduced if applicable. This is because of the low rate of *EGFR* mutations in these situations. We investigated and showed a mutation rate of 7.1% (1/14 cases) which is comparable with the report of Levy et al.[20] (1.8%).

Pleural effusion is a usual complication associated with lung cancer which might contain the tumor cells from the primary site of lung tumor with high percentage. Hence, this fluid can be used in the pathological assessments and genetic testings. The equally successful rate of testing compared to the testing on tumor tissue confirmed that pleural fluid is suitable for *EGFR* mutation analysis.[35, 36] Whereas, the mutation rate in the fluid sample was even reported to be higher than in the tumor tissue and lymph node tissue,[17, 37-40] which is compatible with our observation. This might be explained by the effect of *EGFR* mutations on the development of effusion.[41-44] To our knowledge, this is the first time the pleural fluid is identified as an independent predictor for the higher incidence of *EGFR* mutations. In the clinical aspect, survival outcomes of patients with *EGFR* mutations in pleural fluid seem to be better than those without mutations.[45, 46]

This study reported the prevalence of *EGFR* mutations in correlation with demographic features in a large cohort of Vietnamese NSCLC patients, however, has limitations of the single center and retrospective study. The smoking history was not sufficiently collected from the medical records, so that was not a cofactor in the multivariable model for analysis.

In summary, *EGFR* mutations were detected with high frequency in Vietnamese NSCLC patients; higher in female patients, adenocarcinoma, the well-differentiated tumor, and in pleural effusion sample. Besides the previously confirmed factors such as ethnicity, gender, and smoking status, results of this study show that the histologic grade, TTF1 marker, and sample type are additionally independent factors for the incidence of *EGFR* mutations which help to guide the clinical decisions.

Disclosures

Ethics Committee Approval: The study was approved by the Ethics Committee of Cho Ray Hospital (Number: 258/BVCR-HDDD/2016).

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

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