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Original Article

Relationship between *EGFR* gene copy number variation and *EGFR* mutations, protein expression, and clinicopathologic parameters in lung adenocarcinoma

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Abstract: Mutations of the *epidermal growth factor receptor (EGFR)* gene represent one type of genetic change in lung adenocarcinoma. Tyrosine kinase inhibitors are first line therapy for lung adenocarcinoma patients with *EGFR* mutations. *EGFR* gene copy number (GCN) variation is one screening tool for detecting *EGFR* mutations. However, the usefulness of *EGFR* GCNs is controversial. The aims of this study were to compare *EGFR* GCN, detected by single colored silver-enhanced in situ hybridization (SISH), with *EGFR* mutations, protein expression, and clinicopathologic parameters. Samples from 88 surgically resected lung adenocarcinoma patients were analyzed. EGFR protein expression was assessed by immunohistochemistry. *EGFR* GCN was examined by SISH. Scores of 5 (high polysomy) and 6 (amplification) were classified as *EGFR* SISH positive according to the Colorado scoring system. *EGFR* mutations were analyzed by direct deoxyribonucleic acid sequencing. The results showed that EGFR protein expression was present in 18.2% (16/88), *EGFR* SISH positivity in 20.5% (18/88), and *EGFR* mutations in 31.8% (28/88) of cases. *EGFR* SISH positivity was significantly correlated with EGFR protein expression ($P<0.001$). Results also showed that an increase in *EGFR* copy number (detected by SISH) was not significantly related to *EGFR* mutations. Therefore, *EGFR* SISH positivity cannot be used as a substitute for *EGFR* mutation analysis.

Keywords: Lung, adenocarcinoma, EGFR, SISH, mutation

Introduction

Lung cancer is one of the leading causes of cancer-related death in the world [1]. Adenocarcinoma is the most common type of primary lung cancer [2]. Multiple genetic changes are involved in the development and progression of this disease [3]. Several driver gene alterations have been identified in lung adenocarcinomas, including those of *EGFR, KRAS, BRAF, ALK, ROS1, RET, NTRK1, and NRG1* [2].

Epidermal growth factor receptor (EGFR) is a proto-oncogene located on chromosome band 7p12 [4]. EGFR belongs to ErbB receptor family, which mediates cell proliferation and signal transduction [5, 6]. EGFR overexpression has been reported and is implicated in the pathogenesis of human malignancies including adenocarcinoma [7-9]. EGFR can be deregulated, which drives uncontrolled proliferation in tumor cells, resulting in the ability to evade programmed cell death, thereby enhancing migratory ability and facilitating metastasis [10].

EGFR mutations in lung adenocarcinoma are different based on patient ethnicity, with a prevalence of 10-15% in Caucasians and 30-40% in Asians [2]. *EGFR* mutationsare clinically relevant, because EGFR tyrosine kinase inhibitors (TKIs) represent a class of targeted drugs available for lung adenocarcinoma patients with *EGFR* mutations [7, 11, 12]. *EGFR* mutations are prognostic factors and are predictive of the response to EGFR TKI treatment [2].

Several *EGFR* mutation detection methods including polymerase chain reaction (PCR)-based methods, immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), and chromogenic in situ hybridization (CISH) have been developed [13]. FISH and CISH are not recommended as a replacement for standard *EGFR* mutation analysis. *EGFR* gene copy num-

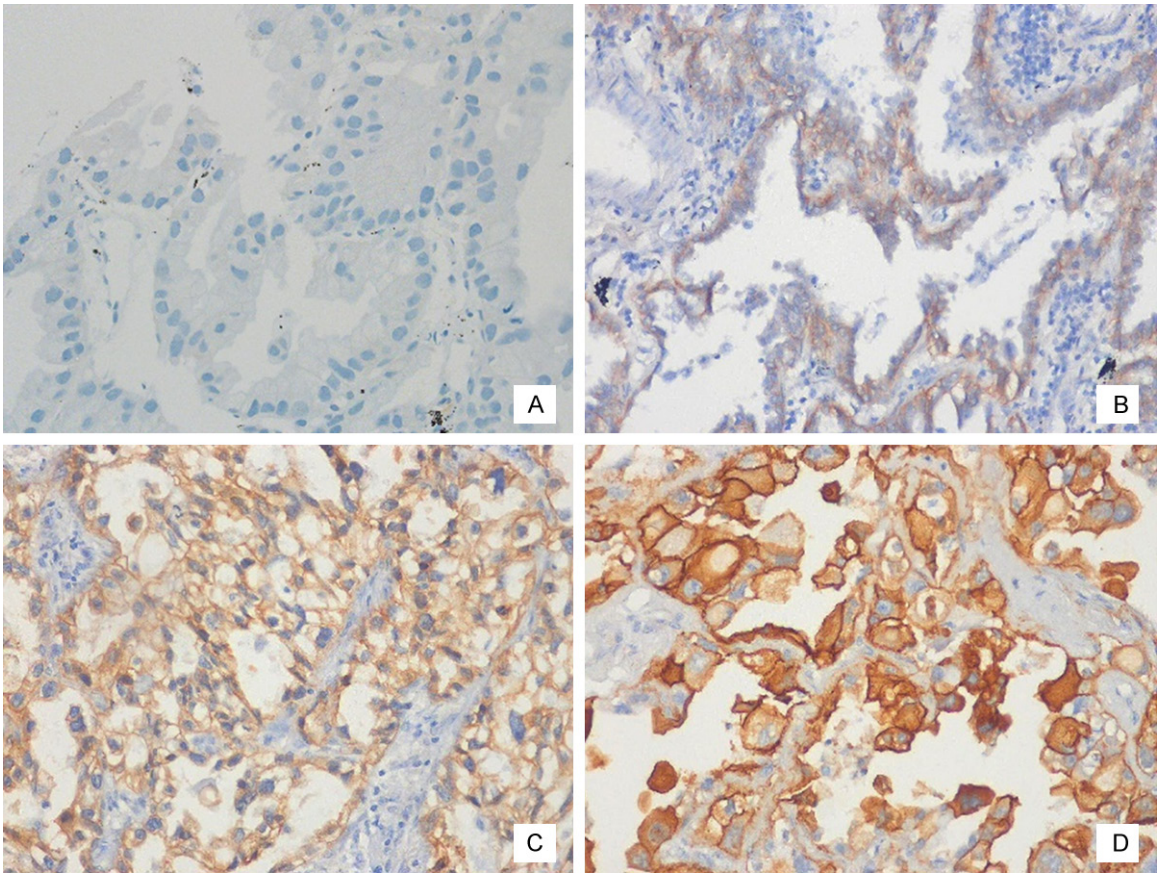

Figure 1. Immunohistochemistry for EGFR. A. 0, no staining. B. 1+, faint cytoplasmic staining in >10% of tumor cells. C. 2+, moderate membranous staining. D. 3+, strong membranous staining.

ber (GCN) variation is one method for assessing *EGFR* mutations. Silver-enhanced in situ hybridization (SISH) is a recently introduced, bright-field in situ hybridization technique for the detection of deoxyribonucleic acid (DNA) signals. In recent studies, SISH proved to be useful and accurate for the evaluation of the *Her-2/neu* gene amplification status in breast cancer [14]. High polysomy and amplification of *EGFR* (according to criteria of Cappuzzo et al. [15]) were observed in 20.7-42.1% of lung adenocarcinomas [16-18]. Liang et al. [17] found that *EGFR* GCN, detected by FISH, was significantly associated with *EGFR* mutations in lung adenocarcinoma. However, *EGFR* GCN detected by SISH was not suitable to select lung adenocarcinoma patients for *EGFR* mutation testing [18]. *EGFR* GCN evaluated by SISH is controversial in regards to its ability to predict *EGFR* mutation status and predict response to anti-EGFR therapy.

The aims of this study were to examine the relationship between *EGFR* GCN detected by SISH and *EGFR* mutations, EGFR protein expression, and clinicopathologic parameters.

Materials and methods

Selection of cases and construction of tissue microarray (TMA) blocks

We collected 88 primary lung adenocarcinomas from patients that were treated surgically at Yeungnam University Hospital, Daegu, Korea between December 2001 and October 2011. We reviewed the slides of all collected cases and selected a representative tumor block for each case for the construction of TMAs. A pair of tissue cores of 2 mm in diameter were retrieved from each tumor block and transferred to the recipient block (Quick-Ray™ array; UNITMA, Seoul, Korea).

Table 1. Colorado scoring system

Colorado score	Definition
1	1 or 2 signals in ≥90% of the counted nuclei (disomy)
2	3 signals in 10% to 40% and ≥4 signals in <10% (low trisomy)
3	3 signals in ≥40% and ≥4 signals in <10% (high trisomy)
4	≥4 signals in 10% to 40% (low polysomy)
5	≥4 signals in ≥40% (high polysomy)
6	*EGFR* gene cluster ≥10% or ratio EGFR/CEP7 ≥2 or ≥15 signals in ≥10% (amplification)

EGFR, epidermal growth factor receptor; CEP7, centromeric probe of chromosome 7.

Four serial sections were taken from each TMA block for hematoxylin and eosin staining, IHC for EGFR, SISH for *EGFR* and CEP7, and *EGFR* mutation analysis. Tumor subtyping and staging were carried out according to the 2015 World Health Organization (WHO) classification of lung adenocarcinoma [2] and guidelines of American Joint Committee on Cancer (AJCC) Tumor Staging [19]. This study was approved by the institutional review board of Yeungnam University Hospital (YUH 2017-01-006).

Immunohistochemistry for EGFR

TMA blocks were cut into 3-μm thick sections. IHC for EGFR was performed using automated Benchmark XT (Ventana, Tuscon, AZ, USA). The primary antibody was a mouse monoclonal anti-EGFR (pre-diluted, CONFIRM™, clone 3C6, Ventana). TMA sections were deparaffinized in xylene with protease 1 to digest proteins for 8 min, incubated with antibody at room temperature for 20 min with UltraWash, and counterstained with hematoxylin for 4 min and bluing reagent for 4 min. The staining for EGFR was scored from 0 to 3+ and defined as follows: 0, no staining; 1+, faint cytoplasmic staining in >10% of tumor cells; 2+, moderate membranous staining; 3+, strong membranous staining (**Figure 1**). 0 and 1+ were considered IHC negative and 2+ and 3+ were considered IHC positive [20-22].

Single-color silver-enhanced in situ hybridization analysis

Two tissue sections of 3-μm thickness per case were prepared for SISH analysis. Paraffin tissue sections on glass slides were baked at 65°C for 20 min before the deparaffinization step with EZ Prep™ (Ventana) at 69°C for 4 min. Deparaffinized tissue sections were pretreated with a combination of heat treatment using Cell Conditioner 2 (pH8.4 EDTA buffer, Ventana) and ISH Protease 3 (Ventana) to unmask DNA targets. For *EGFR* gene detection, the INFORM® *EGFR* DNA Probe (Ventana) dinitrophenyl (DNP)-labeled DNA probe was applied to the glass slide for the hybridization step at 52°C for 6 h. Subsequently, three stringency wash steps were performed at 72°C with 2× standard sodium citrate and tissue sections were incubated with rabbit anti-DNP antibody (Ventana) for 20 min. The metallic silver deposit representative of *EGFR* ISH signal was developed using a silver acetate, hydroquinone, and H_2O_2 reaction in the presence of horseradish peroxidase (HRP) using the UltraView™ SISH Detection Kit (Ventana). For CEP 7 detection, the INFORM® Chromosome 7 Probe (Ventana) dinitrophenyl (DNP)-labeled probe was applied to the glass slide for the hybridization at 44°C for 2 h. After three stringency wash steps at 59°C with 2× sodium citrate and sodium chloride buffer (Ventana), tissue sections were incubated with rabbit anti-DNP antibody (Ventana) for 20 min and the metallic silver deposit representative of the CEP 7 signal was developed using a silver acetate, hydroquinone, and H_2O_2 reaction in the presence of HRP using the UltraView™ SISH Detection Kit (Ventana). Lastly, the slides were counterstained with hematoxylin II for 8 min and Bluing Reagent (Ventana) for 4 min.

EGFR and CEP7 positivity *were* assessed using more than 50 non-overlapping nuclei per case. *EGFR* SISH scores were defined according to the Colorado scoring system (**Table 1**). Only scores of 5 and 6 (high polysomy or amplification) were considered SISH positive (**Figure 2**) [15, 16, 23].

EGFR DNA sequencing

For *EGFR* mutation analysis, five paraffin-embedded sections of 10-μm thickness, representing a portion of each tumor block, were

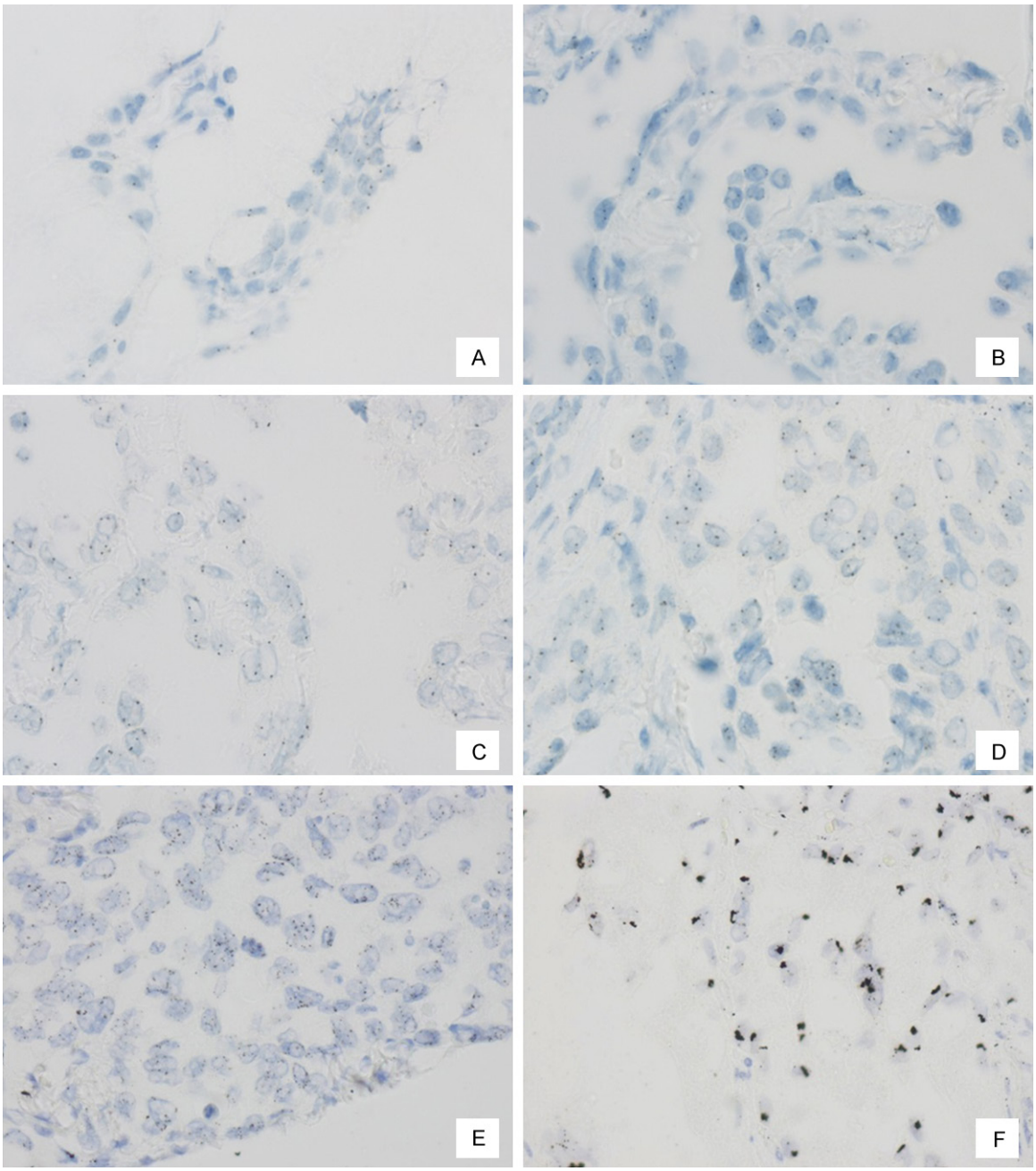

Figure 2. Silver in situ hybridization for EGFR. (A) score1 (disomy), (B) score 2 (low trisomy), (C) score 3 (high trisomy), (D) score 4 (low polysomy), (E) score 5 (high polysomy), (F) score 6 (amplification).

obtained. DNA extraction, polymerase chain reaction (PCR) amplification, and direct sequencing of *EGFR* exons 18-21 were performed as previously described [24]. DNA was extracted using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany). *EGFR* exons were amplified from the DNA (100 ng) by PCR in a 20 μL solution containing 2 μL of 10× buffer (Roche, Mannheim, Germany), 1.7-2.5 mM of $MgCl_2$, 0.3 μM of each *EGFR* primer pair, 250 μM of deoxynucleotide triphosphates, and 2.5 U of DNA polymerase (Roche). The following primers were used: exon 18, forward: 5'-TCCAAATGAGCTGGCAAGTG-3', reverse: 5'-TCCCAAACACTCAGTGAAACAAA-3'; exon 19, forward: 5'-ATGTGGCACCATCTCACAATTGCC-3', reverse: 5'-CCACACAGCAAAGCAGAAACTCAC-3'; exon 20, forward: 5'-CATTCATGCGTCTTCACCTG-3', reverse: 5'-CATATCCCCATG-

Table 2. Clinicopathologic parameters of 88 patients and results of EGFR immunohistochemistry, *EGFR* SISH, and *EGFR* mutation analysis

	Number (%)	EGFR IHC positive			*EGFR* SISH positive			*EGFR* mutation		
		Present	Absent	*P* value	Present	Absent	*P* value	Present	Absent	*P* value
Gender				0.001			0.045			0.884
Male	45 (51.1)	14 (31.1)	31 (68.9)		13 (28.9)	32 (71.1)		14 (31.1)	31 (68.9)	
Female	43 (48.9)	2 (4.7)	41 (95.3)		5 (11.6)	38 (88.4)		14 (32.6)	29 (67.4)	
Smoking				0.001			0.045			0.755
Yes	45 (51.1)	14 (31.1)	31 (68.9)		13 (28.9)	32 (71.1)		15 (33.3)	30 (66.7)	
No	43 (48.9)	2 (4.7)	41 (95.3)		5 (11.6)	38 (88.4)		13 (30.2)	30 (69.8)	
Histologic type				0.279			0.771			0.078
Lepidic predominant	10 (11.4)	0 (0)	10 (100)		0 (0)	10 (100)		4 (40.0)	6 (60.0)	
Acinar predominant	34 (38.6)	8 (23.5)	26 (76.5)		10 (29.4)	24 (70.6)		11 (32.4)	23 (67.6)	
Papillary predominant	19 (21.6)	3 (15.8)	16 (84.2)		4 (21.1)	15 (78.9)		11 (57.9)	8 (42.1)	
Solid predominant	13 (14.8)	4 (30.8)	9 (69.2)		3 (23.1)	10 (76.9)		1 (7.7)	12 (92.3)	
Micropapillary predominant	2 (2.3)	1 (50.0)	1 (50.0)		1 (50.0)	1 (50.0)		1 (50.0)	1 (50.0)	
Invasive mucinous	10 (11.4)	0 (0)	10 (100)		0 (0)	10 (100)		0 (0)	10 (100)	
Primary tumor (pT)				0.430			0.450			0.081
pT1a	13 (14.8)	1 (7.7)	12 (92.3)		2 (15.4)	11 (84.6)		7 (53.8)	6 (46.2)	
pT1b	35 (39.8)	7 (20.0)	28 (80.0)		10 (28.6)	25 (71.4)		10 (28.6)	25 (71.4)	
pT2a	30 (34.1)	6 (20.0)	24 (80.0)		5 (16.7)	25 (83.3)		10 (33.3)	20 (66.7)	
pT2b	3 (3.4)	0 (0)	3 (100)		0 (0)	3 (100)		0 (0)	3 (100)	
pT3	7 (8.0)	2 (28.6)	5 (71.4)		1 (14.3)	6 (85.7)		1 (14.3)	6 (85.7)	
Lymph node metastasis (N)				0.030			0.069			0.653
N0	69 (78.4)	9 (13.0)	60 (87.0)		11 (15.9)	58 (84.1)		21 (30.4)	48 (69.6)	
N1	16 (18.2)	6 (37.5)	10 (62.5)		6 (37.8)	10 (62.5)		6 (37.5)	10 (62.5)	
N2	3 (3.4)	1 (33.3)	2 (66.7)		1 (33.3)	2 (66.7)		1 (33.3)	2 (66.7)	
Stage				0.053			0.618			0.360
IA	41 (46.6)	5 (12.2)	36 (87.8)		9 (22.0)	32 (78.0)		14 (34.1)	27 (65.9)	
IB	21 (23.9)	3 (14.3)	18 (85.7)		2 (9.5)	19 (90.5)		7 (33.3)	14 (66.7)	
IIA	17 (19.3)	5 (29.4)	12 (70.6)		5 (29.4)	12 (70.6)		6 (35.3)	11 (64.7)	
IIB	4 (4.5)	1 (25.0)	3 (75.0)		0 (0)	4 (100)		0 (0)	4 (100)	
IIIA	5 (5.7)	2 (40.0)	3 (60.0)		2 (40.0)	3 (60.0)		1 (20.0)	4 (80.0)	

Values are presented as number (%). *EGFR, epidermal growth factor receptor*; IHC, immunohistochemistry; SISH, silver-enhanced in situ hybridization.

GCAAACTC-3'; and exon 21, forward: 5'-GCTCAGAGCCTGGCATGAA-3', reverse: 5'-CATCCTCCCCTGCATGTGT-3'. Amplifications were performed using a 5-min initial denaturation step at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C, with a final 10-min extension at 72°C. All PCR products were purified from a 2% agarose gel using a QIAgen gel extraction kit (Qiagen), and used as templates for DNA sequencing with both forward and reverse sequence-specific primers. For sequencing, each purified PCR product (20 ng) was used in a 20 μL sequencing reaction solution containing 8 μL of BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) and 0.1 μM of the appropriate PCR primers. Sequencing was performed with 25 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C, using an ABI PRISM 3100 DNA Analyzer (Applied Biosystems). The sequencing data were analyzed using Sequencer 3.1.1. software (Applied Biosystems).

Statistical analysis

Statistical analysis was performed using SPSS version 18.0 for Windows (SPSS Inc., Chicago, USA). A Chi-square test or Fisher's exact test was used for comparisons between EGFR protein expression, *EGFR* SISH, *EGFR* mutation, and clinicopathological parameters. The κ coefficient was calculated for comparison of *EGFR* SISH and EGFR protein expression. Overall survival was analyzed using the Kaplan-Meier curve method with a log-rank test. A *P* value of <0.05 was considered statistically significant.

Table 3. Correlations among *EGFR* mutation status, *EGFR* SISH status, and *EGFR* immunohistochemistry

Case	Histologic subtype	*EGFR* mutation	*EGFR* SISH	EGFR IHC
1	Papillary predominant	Exon 19, deletion	Negative	Negative
2	Papillary predominant	Exon 19, deletion	Positive	Negative
3	Acinar predominant	Exon 21, L858R	Negative	Negative
4	Acinar predominant	Exon 21, L858R	Negative	Negative
5	Lepidic predominant	Exon 21, L858R	Negative	Negative
6	Acinar predominant	Exon 19, deletion	Positive	Positive
7	Papillary predominant	Exon 21, L858R	Negative	Negative
8	Papillary predominant	Exon 19, deletion	Positive	Positive
9	Micropapillary predominant	Exon 21, L858R	Positive	Positive
10	Solid predominant	Exon 19, deletion	Negative	Negative
11	Acinar predominant	Exon 19, deletion	Negative	Negative
12	Papillary predominant	Exon 18, G719A	Negative	Negative
13	Papillary predominant	Exon 19, deletion	Negative	Negative
14	Lepidic predominant	Exon 19, deletion	Negative	Negative
15	Acinar predominant	Exon 21, L858R	Negative	Negative
16	Acinar predominant	Exon 19, deletion	Positive	Negative
17	Papillary predominant	Exon 21, L858R	Positive	Positive
18	Acinar predominant	Exon 19, deletion	Positive	Positive
19	Acinar predominant	Exon 21, L858R	Negative	Negative
20	Lepidic predominant	Exon 19, deletion	Negative	Negative
21	Acinar predominant	Exon 19, deletion	Negative	Negative
22	Papillary predominant	Exon 19, deletion	Negative	Negative
23	Papillary predominant	Exon 19, deletion	Negative	Negative
24	Lepidic predominant	Exon 19, deletion	Negative	Negative
25	Papillary predominant	Exon 19, deletion	Positive	Positive
26	Papillary predominant	Exon 21, L858R	Negative	Negative
27	Acinar predominant	Exon 21, L858R	Negative	Negative
28	Acinar predominant	Exon 19, deletion	Negative	Negative

EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; SISH, silver-enhanced in situ hybridization.

Results

Patients' characteristics

Patients' characteristics are summarized in **Table 2**. Patient age ranged from 36 to 79 years (mean, 62.5 years). There were 45 male patients and 43 female patients. Smokers represented 51.1% (45/88) and non-smokers represented 48.9% (43/88) of cases. According to WHO histologic classification of lung adenocarcinoma, 11.4% (10/88) were lepidic predominant adenocarcinoma, 38.6% (34/88) acinar predominant adenocarcinoma, 21.6% (19/88) papillary predominant adenocarcinoma, 14.8% (13/88) solid predominant adenocarcinoma (13/88), 2.3% (2/88) micropapillary predominant adenocarcinoma (2/88), and 11.4% (10/88) invasive mucinous adenocarcinoma. According to the seventh edition of AJCC TNM staging, 14.8% (13/88) were pT1a, 39.8% (35/88) pT1b, 34.1% (30/88) pT2a, 3.4% (3/88) pT2b, and 8.0% (7/88) pT3. Sixty-nine patients (78.4%) were classified as N0, 16 patients (18.2%) as N1, and three (3.4%) patients as N2. All patients were classified as M0.

EGFR protein expression

Sixteen of 88 tumors showed EGFR protein expression (18.4%) (**Figure 1**). EGFR protein expression was significantly correlated with gender (P=0.001), smoking status (P=0.001), and N stage (P=0.03). EGFR protein expression was not significantly correlated with histologic subtype (P=0.279), pT (P=0.430), or stage (P=0.053) (**Table 2**).

EGFR SISH

Twelve (13.6%) of 88 cases were scored as 1, 25 cases (28.4%) were scored as 2, six cases (6.8%) were scored as 3, 27 cases (30.7%) were scored as 4, 14 cases (15.9%) were scored as 5 (high polysomy), and four cases (4.6%) were scored as 6 (amplification). Eighteen (20.5%) of 88 were *EGFR* SISH positive (**Figure 2**). All remaining 70 cases (79.5%) were *EGFR* SISH negative. *EGFR* SISH positivity was significantly correlated with gender (P=0.045) and smoking status (P=0.045). *EGFR* SISH positivity was not significantly correlated with histologic subtype (P=0.771), pT (P=0.450), N (P=0.069), or stage (P=0.618) (**Table 2**).

EGFR mutation

Twenty-eight (31.8%) of 88 tumors were determined to have *EGFR* mutations. The mutation types are summarized in **Table 3**. An exon 19 deletion was present in 60.7% (19/28) of *EGFR*

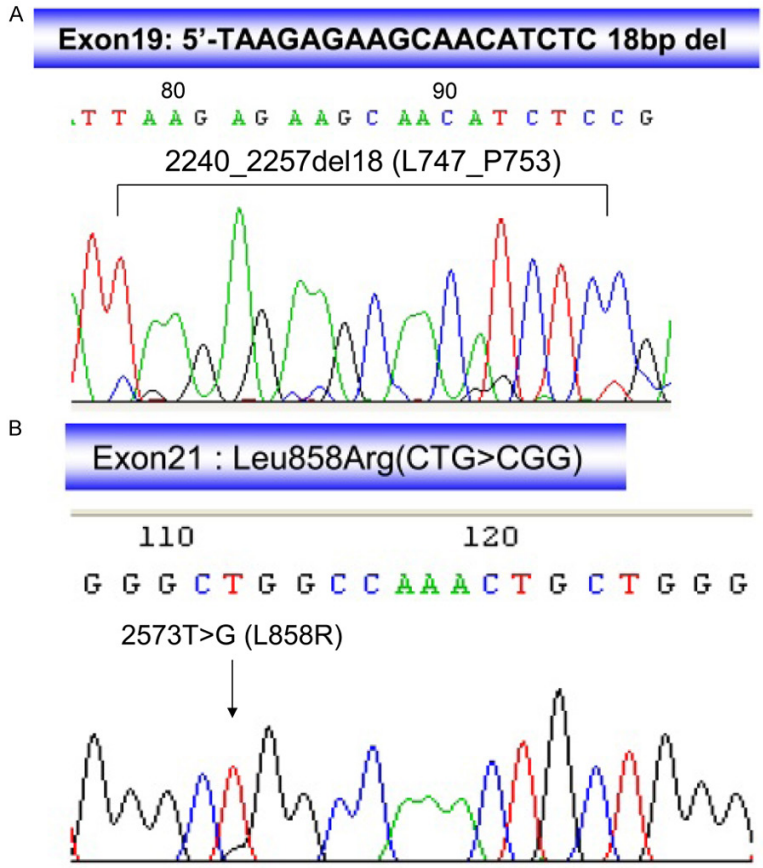

Figure 3. DNA sequencing of *EGFR* mutations. A. There was c.2240_2257del18 (delL747_P753) mutation in exon 19. B. There was a c.2573T>G (L858R) mutation in exon 21.

Table 4. Correlation between EGFR immunohistochemistry and *EGFR* SISH

		EGFR SISH		
		Positive	Negative	*P* value
EGFR IHC	Positive	14 (15.9)	2 (2.3)	0.000
	Negative	4 (4.5)	68 (77.3)	

Values are presented as number (%). *EGFR, epidermal growth factor receptor*; IHC, immunohistochemistry; SISH, silver-enhanced in situ hybridization.

Table 5. Correlation between EGFR mutations and *EGFR* SISH

		EGFR SISH		
		Positive	Negative	*P* value
EGFR mutation	Positive	8 (9.1)	20 (22.7)	0.197
	Negative	10 (11.4)	50 (56.8)	

Values are presented as number (%). *EGFR, epidermal growth factor receptor*; SISH, silver-enhanced in situ hybridization.

mutation patients, exon 21 L858R mutation in 35.7% (10/28), and exon 18 G709A mutation in 3.6% (1/28) (**Figure 3**). Of the 28 cases with *EGFR* mutations, 28.6% (8/28) demonstrated *EGFR* SISH positivity and 21.4% (6/28) showed EGFR protein expression. Of eight *EGFR* SISH positive cases, 75% (6/8) had an exon 19 deletion. According to histologic subtypes, *EGFR* mutations were present in 39.3% (11/28) of papillary predominant adenocarcinomas, 39.3% (11/28) of acinar predominant adenocarcinomas, 14.2% (4/28) of lepidic predominant adenocarcinomas, 3.6% (1/28) of solid predominant adenocarcinomas, and 3.6% (1/28) of micropapillary predominant adenocarcinomas. *EGFR* mutations were not significantly correlated with gender (*P*=0.884), smoking (*P*=0.755), histologic subtype (*P*=0.078), pT (*P*=0.081), N (*P*=0.653), or stage (*P*=0.360) (**Table 2**).

Correlation between EGFR protein expression, EGFR SISH, and EGFR mutations

EGFR protein expression showed a significant correlation with *EGFR* SISH positivity (*P*<0.001) (**Table 4**). There was high concordance between *EGFR* GCN and EGFR protein expression with a κ coefficient of 0.781. There was no significant relation between *EGFR* SISH positivity and *EGFR* mutation status (*P*=0.197) (**Table 5**).

Survival according to EGFR protein expression, EGFR SISH, and EGFR mutation

The overall survival was compared according to EGFR protein expression, *EGFR* SISH positivity, and *EGFR* mutations. The overall survival was not significantly correlated with EGFR protein expression (*P*=0.713), *EGFR* SISH positivity (*P*=0.681), or *EGFR* mutation status (*P*=0.323) (**Figure 4**).

Discussion

The aim of the current study was to investigate the relationship between *EGFR* GCN variation detected by SISH and *EGFR* mutation status,

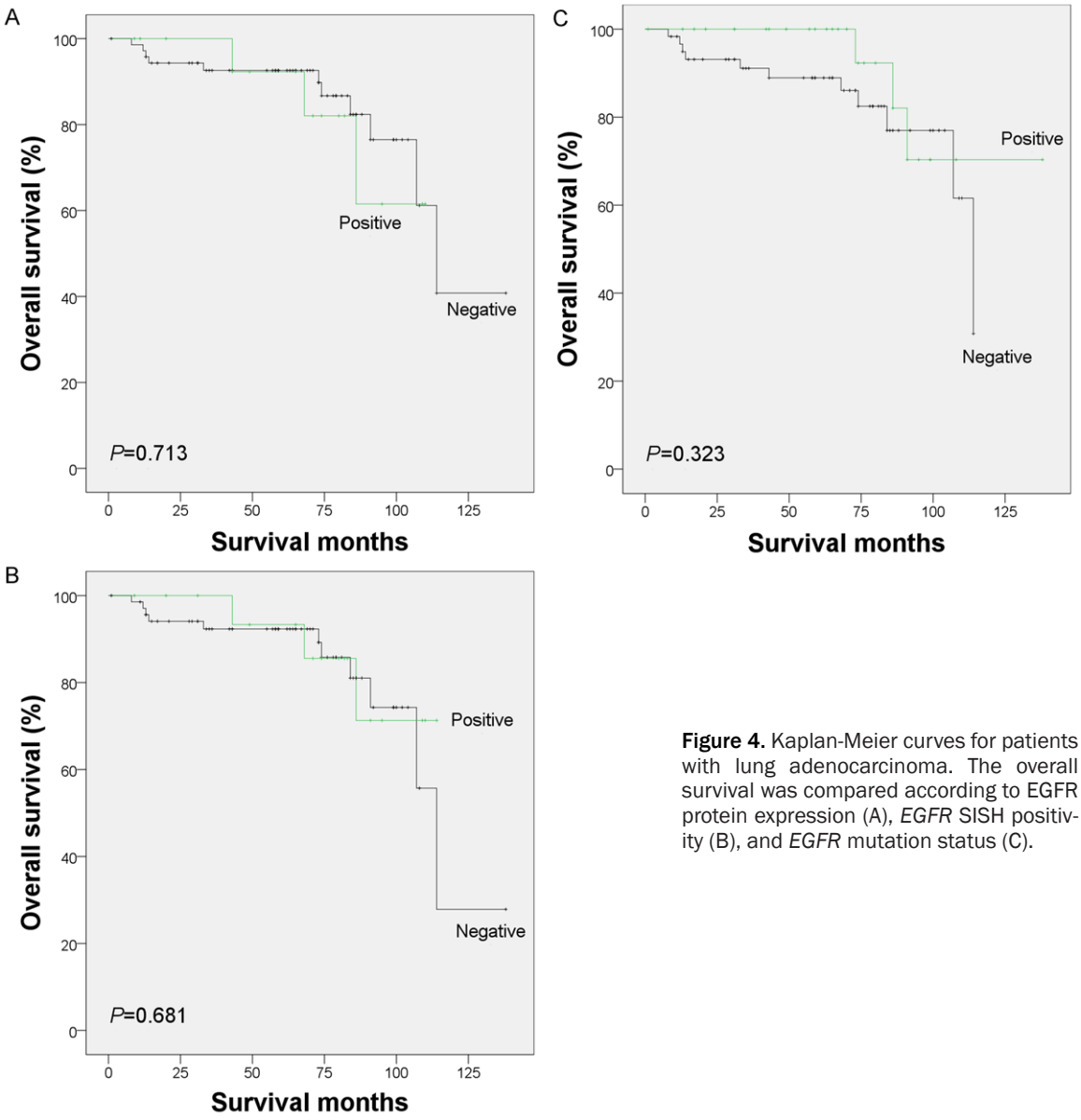

Figure 4. Kaplan-Meier curves for patients with lung adenocarcinoma. The overall survival was compared according to EGFR protein expression (A), *EGFR* SISH positivity (B), and *EGFR* mutation status (C).

protein expression, and clinicopathologic parameters in lung adenocarcinoma.

EGFR protein expression in lung adenocarcinoma has been reported to be positive in 10.9% to 51.6% of cases [25, 26]. In the present study, EGFR protein expression was present in 18.2% of the samples. This difference might be attributed to different antibodies, antigen retrieval methods, different criteria for positivity, and heterogeneity of the samples. EGFR protein expression was significantly associated with gender and smoking status. In the present study, *EGFR* SISH positivity occurred in 20.5% of cases. *EGFR* SISH positivity was also significantly associated with gender and smoking status.

EGFR gene alterations are important driver mutations in lung adenocarcinoma. The point mutation at exon 21, codon 858 (L858R) and the in-frame shift deletion in exon 19 account for approximately 90% of all mutations [2]. In the present study, *EGFR* mutations were present in 31.8% of cases. Among the patients with *EGFR* mutations, exon 19 deletions were found in 60.7% and exon 21 mutations were present in 35.7% of the samples, respectively. Consistent with the observation of other studies, our study showed that the majority of *EGFR* muta-

tions were found in exon 19 and 21. *EGFR* mutations are frequently detected in cases with lepidic and papillary growth and are associated with thyroid transcription factor-1 (TTF-1) positivity [2]. In the present study, *EGFR* mutations were predominantly found in patients with papillary adenocarcinoma and acinar adenocarcinoma. Liang et al. [17] found that *EGFR* mutations are more frequent in non-smoking patients. In the current study, *EGFR* mutations were not associated with smoking status.

Studies on the relationship between *EGFR* mutations and *EGFR* GCN gains have been reported. In one study, EGFR protein expression, *EGFR* GCN, and *EGFR* mutations were closely related [17]. However, in another study, *EGFR* copy number demonstrated low sensitivity and poor positive predictive value, such that it could not be reliably used to predict EGFR mutation status or to select patients for EGFR mutation testing [18]. Wulf et al. [16] found a strong association between FISH or SISH positivity and *EGFR* gene mutation and EGFR protein expression in non-small cell lung cancer (NSCLC). In the present study, there was a strong association between EGFR protein expression and *EGFR* SISH positivity, but no association between *EGFR* mutation status and *EGFR* SISH positivity. These results suggest that high polysomy and amplification of the *EGFR* gene are associated with EGFR immunohistochemical staining, but cannot predict *EGFR* mutation status [25].

In the present study, the Colorado score system for definition of EGFR SISH positivity was used. Cappuzzo et al. [15] established a definition for *EGFR* FISH positivity in patients with NSCLC by linking *EGFR* FISH findings to response to an EGFR TKI. The advantage of the Colorado scoring system lies in the clear definition of *EGFR* positivity (scores 5 and 6) and the evaluation of tumor heterogeneity in *EGFR* patterns, which can be challenging in many cases [16]. SISH for *EGFR* GCN assessment in routine diagnosis of NSCLC would be of great value because SISH only requires a bright-field microscope and can therefore be more easilyinterpreted by surgical pathologists when compared to FISH [16]. However, the SISH method should be evaluated in larger patient cohorts. Inter-laboratory and inter-observer reliability also need to be investigated [16]. In addition, standard methods for detecting *EGFR* mutations need to be established in the future.

Shan et al. [27] found that *EGFR* amplification was an indicator of better response to EGFR TKI treatment. However, Chang et al. [28] found that *EGFR* amplification was correlated with *EGFR* mutation status, but had a lower association with TKI responsiveness. Sholl et al. [29] found that lung adenocarcinomas with *EGFR* amplifications had a significantly worse prognosis. In the present study, EGFR protein expression was correlated with higher N stage. EGFR protein expression, *EGFR* SISH positivity, and *EGFR* mutations had no association with overall survival. However, this study was limited by the relatively small sample size. Further studies are necessary to clarify the relationship between *EGFR* mutations and clinicopathologic parametersin a larger series of lung adenocarcinoma cases.

In conclusion, *EGFR* SISH positivity is associated with EGFR protein expression. However, *EGFR* copy number gains, detected by SISH, are not significantly related to *EGFR* mutation status. Therefore, *EGFR* SISH positivity cannot be used as a substitute for *EGFR* mutation analysis.

Disclosure of conflict of interest

None.

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References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.

[2] Travis WD, Noguchi M, Yatabe Y, Brambilla E, Nicholson AG, Aisner SC, Austin JHM, Devesa SS, Hirsch FR, Ladanyi M, Meyerson M, Mino-Kenudson M, Powell CA, Rami-Porta E, Rekhtman N, Riely G, Russell P, Samet J, Scagliotti G, Thunnissen E, To KF, Tsuta K, van Schil P, Warth A, Wistuba II and Yankelevitz D. Adenocarcinoma. In: WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart. Travis WD, Brambilla E, Burke AP, Marx A, Nicholson

AG, editors. 4th edition. Lyon: IARC Press; 2015. pp. 26-37.
[3] Sholl LM. Biomarkers in lung adenocarcinoma: a decade of progress. Arch Pathol Lab Med 2014; 139: 469-480.
[4] Kang JU, Koo SH, Kwon KC, Park JW and Jung SS. Gain of the EGFR gene located on 7p12 is a frequent and early event in squamous cell carcinoma of the lung. Cancer Genet Cytogenet 2008; 184: 31-37.
[5] Pennock S and Wang Z. Stimulation of cell proliferation by endosomal epidermal growth factor receptor as revealed through two distinct phases of signaling. Mol Cell Biol 2003; 23: 5803-5815.
[6] Oda K, Matsuoka Y, Funahashi A and Kitano H. A comprehensive pathway map of epidermal growth factor receptor signaling. Mol Syst Biol 2005; 1: 2005.0010.
[7] Bethune G, Bethune D, Ridgway N andXu Z. Epidermal growth factor receptor (EGFR) in lung cancer: an overview and update. J Thorac Dis 2010; 2: 48-51.
[8] Inamura K, Ninomiya H, Ishikawa Y and Matsubara O. Is the epidermal growth factor receptor status in lung cancers reflected in clinicopathologic features? Arch Pathol Lab Med 2010; 134: 66-72.
[9] Ohsaki Y, Tanno S, Fujita Y, Toyoshima E, Fujiuchi S, Nishigaki Y, Ishida S, Nagase A, Miyokawa N, Hirata S and Kikuchi K. Epidermal growth factor receptor expression correlates with poor prognosis in non-small cell lung cancer patients with p53 overexpression. Oncol Rep 2000; 7: 603-607.
[10] Siegelin MD and Borczuk AC. Epidermal growth factor receptor mutations in lung adenocarcinoma. Lab Invest 2014; 94: 129-137.
[11] Roengvoraphoj M, Tsongalis GJ, Dragnev KH and Rigas JR. Epidermal growth factor receptor tyrosine kinase inhibitors as initial therapy for non-small cell lung cancer: focus on epidermal growth factor receptor mutation testing and mutation-positive patients. Cancer Treat Rev 2013; 39: 839-850.
[12] Chan BA and Hughes BG. Targeted therapy for non-small cell lung cancer: current standards and the promise of the future. Transl Lung Cancer Res 2015; 4: 36-54.
[13] Eberhard DA, Giaccone G and Johnson BE. Biomarkers of response to epidermal growth factor receptor inhibitors in non-small cell lung cancer working group: standardization for use in the clinical trial setting. J Clin Oncol 2008; 26: 983-994.
[14] Fritzsche FR, Bode PK, Moch H, Kristiansen G, Varga Z and Bode B. Determination of the-Her-2/neugene amplification status in cytologic breast cancer specimens using automated silver-enhanced in-situ hybridization (SISH). Am J Surg Pathol 2010; 34: 1180-1185.
[15] Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, Haney J, Witta S, Danenberg K, Domenichini I, Ludovini V, Magrini E, Gregorc V, Doglioni C, Sidoni A, Tonato M, Franklin WA, Crino L, Bunn PA Jr and Varella-Garcia M. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst 2005; 97: 643-655.
[16] Wulf MA, Bode B, Zimmermann D, Rufibach K, Weder W, Moch H, Soltermann A and Tischler V. Silver-enhanced in situ hybridization for determination of EGFR copy number alterations in non-small cell lung cancer. Am J Surg Pathol 2012; 36: 1801-1808.
[17] Liang Z, Zhang J, Zeng X, Gao J, Wu S and Liu T. Relationship between EGFR expression, copy number and mutation in lung adenocarcinomas. BMC Cancer 2010; 10: 376.
[18] Russell PA, Yu Y, Do H, Clay TD, Moore MM, Wright GM, Conron M, Wainer Z, Dobrovic A, and McLachlan SA. EGFR gene copy number alterations are not a useful screening tool for predicting EGFR mutation status in lung adenocarcinoma. Pathology 2014; 46: 32-36.
[19] Edge S, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A, III, editors. AJCC cancer staging manual. 7th edition. New York: Springer; 2011. pp.253-270.
[20] Brevet M, Arcila M and Ladanyi M. Assessment of EGFR mutation status in lung adenocarcinoma by immunohistochemistry using antibodies specific to the two major forms of mutant EGFR. J Mol Diagn 2010; 12: 169-176.
[21] Hasanovic A, Ang D, Moreira AL and Zakowski MF. Use of mutation specific antibodies to detect EGFR status in small biopsy and cytology specimens of lung adenocarcinoma. Lung Cancer Amst Neth 2012; 77: 299-305.
[22] Wen YH, Brogi E, Hasanovic A, Ladanyi M, Soslow RA, Chitale D, Shia J and Moreira AL. Immunohistochemical staining with EGFR mutation-specific antibodies: high specificity as a diagnostic marker for lung adenocarcinoma. Mod Pathol 2013; 26: 1197-1203.
[23] Varella-Garcia M, Diebold J, Eberhard DA, Geenen K, Hirschmann A, Kockx M, Nagelmeier I, Rüschoff J, Schmitt M, Arbogast S and Cappuzzo F. EGFR fluorescence in situ hybridisation assay: guidelines for application to non-small-cell lung cancer. J Clin Pathol 2009; 62: 970-977.
[24] Jang TW, Oak CH, Chang HK, Suo SJ and Jung MH. EGFR and KRAS mutations in patients with adenocarcinoma of the lung. Korean J Intern Med 2009; 24: 48-54.

[25] Li AR, Chitale D, Riely GJ, Pao W, Miller VA, Zakowski MF, Rusch V, Kris MG, and Ladanyi M. EGFR mutations in lung adenocarcinomas: clinical testing experience and relationship to EGFR gene copy number and immunohistochemical expression. J Mol Diagn 2008; 10: 242-248.

[26] Dacic S, Flanagan M, Cieply K, Ramalingam S, Luketich J, Belani C and Yousem SA. Significance of EGFR protein expression and gene amplification in non-small cell lung carcinoma. Am J Clin Pathol 2006; 125: 860-865.

[27] Shan L, Wang Z, Guo L, Sun H, Qiu T, Ling Y, Li W, Li L, Liu X, Zheng B, Lu N and Ying J. Concurrence of EGFR amplification and sensitizing mutations indicate a better survival benefit from EGFR-TKI therapy in lung adenocarcinoma patients. Lung Cancer Amst Neth 2015; 89: 337-342.

[28] Chang JW, Liu HP, Hsieh MH, Fang YF, Hsieh MS, Hsieh JJ, Chiu YT, Tsai HY, Chen YH, Chen YT, Hsu HY, Chen YT, Tsai SF, Chen YR, Hsi BL and Huang SF. Increased epidermal growth factor receptor (EGFR) gene copy number is strongly associated with EGFR mutations and adenocarcinoma in non-small cell lung cancers: a chromogenic in situ hybridization study of 182 patients. Lung Cancer Amst Neth 2008; 61: 328-339.

[29] Sholl LM, Yeap BY, Iafrate AJ, Holmes-Tisch AJ, Chou YP, Wu MT, Goan YG, Su L, Benedettini E, Yu J, Loda M, Jänne PA, Christiani DC and Chirieac LR. Lung adenocarcinoma with EGFR amplification has distinct clinicopathologic and molecular features in never-smokers. Cancer Res 2009; 69: 8341-8348.