Association of the EGFR-TKI or Cytotoxic Chemotherapy Efficacy with NF-κB and Transglutaminase 2 Expression Level in Non-Small Cell Lung Cancer

Jae-Heon Jeong

Department of Medicine
The Graduate School, Yonsei University

Association of the EGFR-TKI or Cytotoxic Chemotherapy Efficacy with NF-κB and Transglutaminase 2 Expression Level in Non-Small Cell Lung Cancer

Jae-Heon Jeong

Department of Medicine
The Graduate School, Yonsei University

Association of the EGFR-TKI or Cytotoxic Chemotherapy Efficacy with NF-κB and Transglutaminase 2 Expression Level in Non-Small Cell Lung Cancer

Directed by Professor Joo-Hang Kim

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Jae-Heon Jeong

June 2013

This certifies that the Doctoral Dissertation of Jae-Heon Jeong is approved

---

Thesis Supervisor : Joo-Hang Kim

Thesis Committee Member#1: Myung-Ju Ahn

Thesis Committee Member#2: Hyung Jung Kim

Thesis Committee Member#3: Yoon Soo Chang

Thesis Committee Member#4: Byoung Chul Cho

The Graduate School
Yonsei University

June 2013

ACKNOWLEDGEMENTS

I would first like to thank God, who makes all things possible. From the time I obtained my Master's degree until now, Prof. Joo Hang Kim directed, encouraged, and supported me to study lung cancer. For this support, I am deeply grateful. I am also thankful to Profs. Myung-Ju Ahn, Hyung Jung Kim, Yoon Soo Chang, and Byoung Chul Cho for their critical review of this manuscript and their valuable comments and advice. I would also like to express my gratitude to Profs. Jaejin Song and Hyosup Shim for their help. I would like to thank the faculty and staff in the Division of Medical Oncology and Hematology at Kyung Hee University School of Medicine, especially Profs. Si-Young Kim, Kyung Sam Cho, and Hwi-Joong Yoon for their encouragement and support. I am grateful to Ph.D. student S.M. Bakhtiar Ul Islam, Dr. Hye Ryun Kim, and Dr. Sun Min Lim for their laboratory work and data collection. I would also like to express my gratitude to Dr. Jungsil Ro, Dr. Jinsoo Lee, and Sooyoul Kim Ph D. for their encouragement to study transglutaminase 2 and drug development.

Finally, I wish to dedicate this doctoral thesis and the fruits of my research to my wife Yu Ri, my two lovely daughters, So-Yul and Ji-Yul, my mother- and father-in-law, and my late mother and father. If not for their encouragement and support, this thesis would not have been possible.

June 25, 2013

Jae-Heon Jeong

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Association of the EGFR-TKI or Cytotoxic Chemotherapy Efficacy with NF-κB and Transglutaminase 2 expression level in Non-Small Cell Lung Cancer

Jae-Heon Jeong

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Joo-Hang Kim)

Background: Transglutaminase 2 (TG2) is a cross-linking enzyme that is involved in drug resistance and the constitutive activation of nuclear factor κB (NF-κB), a proinflammatory transcription factor. We investigated the association between the clinical efficacy of epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) and cytotoxic chemotherapy and the expression of TG2 and NF-κB in non-small cell lung cancer (NSCLC).

Patients and methods: TG2 and NF-κB expression was immunohistochemically studied in 120 patients with NSCLC who underwent curative resection. Kaplan-Meier survival analyses and Cox regression analyses were used to estimate the association between TG2 and NF-κB expression and the clinical efficacy of chemotherapy and EGFR-TKI.

Results: The median age of the patients was 64 years (range, 41-84 years). One hundred two cases (85%) had adenocarcinoma, and 18 cases (15%) had other histologies. Eighty eight patients received adjuvant chemotherapy, 28 patients received platinum-based doublet chemotherapy as palliative therapy, and 29

patients received EGFR-TKI. Twenty-five of the patients were current smokers, 16 were former smokers, and 79 were never smokers. Fifty-five patients had an activating EGFR mutation, such as exon 19 deletion or L858R mutation. The median TG2 value was 50 (range, 0-300), and the median NF-κB value was 20 (range, 0-240). The score was obtained semiquantitatively by the multiplication of staining intensity and percentage of staining positive tumor cells. The overall response rate (ORR) for platinum-based doublet chemotherapy was 13.8%, and the disease control rate (DCR) was 69%. No patients achieved a complete response (CR), 14.3% achieved a partial response (PR), 57.1% achieved stable disease (SD), and 21.4% experienced progressive disease (PD). The ORR for EGFR-TKI was 24.1%, and the DCR was 58.6% (CR, 3.4%; PR, 20.7%; SD, 34.5%; PD, 34.5%). Among the 88 patients who received adjuvant chemotherapy, disease-free survival (DFS) did not differ between the low- and high-TG2 groups. Among the 28 patients who received palliative platinum-based doublet chemotherapy, progression-free survival (PFS) was longer in the low-TG2 group compared with the high-TG2 group, although this finding was not statistically significant (11.0 months *vs*. 7.0 months, $p = 0.330$). Among the patients who received EGFR-TKI ($n = 29$; seven first-line, 18 second-line, three third-line, and one fourth-line), PFS was significantly longer in the low-TG2 group compared with the high-TG2 group (11.0 months *vs*. 2.0 months, $p = 0.013$). In patients with wildtype EGFR ($n = 14$) treated with EGFR-TKI, PFS was longer in the low-TG2 group (9.0 months *vs*. 2.0 months, $p = 0.013$). We generated a small hairpin RNA of TG2 (sh TG2) expressing replication defective adenovirus. The newly engineered adenovirus showed a TG2-suppressing effect and sensitizing effect of gefitinib on cell viability and apoptosis.

Conclusion: The present results suggest that TG2 expression might be a

predictive factor associated with prolongation of PFS in patients with NSCLC who are treated with EGFR-TKI.

Keywords: Lung neoplasm, NF-κB, Transglutaminase 2

Association of the EGFR-TKI or Cytotoxic Chemotherapy Efficacy with NF-κB and Transglutaminase 2 expression level in Non-Small Cell Lung Cancer

Jae-Heon Jeong

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Joo Hang Kim)

I. INTRODUCTION

Lung cancer is the most common cause of cancer-related death in Korea. In 2011, 21,902 new cases (15,496 men and 6,406 women) of lung and bronchial cancer were diagnosed.[1] Approximately 85% of lung cancer cases are non-small cell lung cancer (NSCLC).

The nuclear factor-κB (NF-κB) pathway is associated with lung cancer carcinogenesis, epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) resistance, and cytotoxic chemotherapy resistance.[2-10] The NF-κB family of transcription factors consists of five members that can form homodimers or heterodimers in mammalian cells: RELA (also known as p65), REL (also known as c-REL), RELB, p50, and p52. In resting cells, NF-κB dimers are normally kept in an inactive state through an association with proteins of the inhibitor of NF-κB (IκB) family.

Transglutaminases comprise a family of Ca^{2+}-dependent enzymes involved in the posttranslational modification of proteins. They catalyze

cross-linking via ε-(γ-glutamyl)lysine isopeptide bonds or by incorporating primary amines at select glutamine residues. The eight TGs that have been identified in mammals and humans all require proteolytic propeptide cleavage, and three TGs (TG2, TG3, and TG5) are inhibited by guanosine triphosphate. Tissue transglutaminase (TG2 or tTG) is the most diversely functioning and ubiquitous member of the TG family.[11]

The interaction between TG2 and IκBα leads to the constitutive activation of NF-κB and confers protection against stress-induced cell damage through reactive oxygen species, inflammatory cytokines, and chemotherapeutic drugs. This suggests a new paradigm for the involvement of TG in NF-κB activation, which does not require kinase-ubiquitin-proteasome signaling for constitutive NF-κB activation. Interestingly, TG2 expression has been reported to correlate with drug resistance.[12-15] TG2 inhibition resulted in increased sensitivity to chemotherapeutic drugs, which may represent a potentially useful treatment for certain cancers.[16]

Methylation of the TG2 gene promoter can also predict the response to cisplatin treatment, and TG2 inhibition appears to be an effective cisplatin-sensitizing modality in NSCLC.[17] Furthermore, the level of TG2 expression has been significantly correlated with recurrence and shorter disease-free survival (DFS) in NSCLC.

The objectives of the present study were to immunohistochemically assess NF-κB and TG2 expression in NSCLC tumor samples and evaluate the relationships between NF-κB and TG2 expression and clinicopathological parameters in NSCLC. We also determined the prognostic value of NF-κB and TG2 in the survival of patients with resected NSCLC tumors and evaluated NF-κB and TG2 as predictive factors for cytotoxic chemotherapy and EGFR-TKI.

II. MATERIALS AND METHODS

1. Patients

We analyzed archival tissues and medical records from the lung cancer registry of Yonsei University College of Medicine, Seoul, Korea. One hundred twenty cases of resected NSCLC between 2004 and 2011 had EGFR mutation test results. Clinical data (i.e., age, gender, and stage) were obtained from medical records. All of the patients underwent curative surgical resection. The present study was approved by the Institutional Review Board of Severance Hospital (no. 4-2013-0079) and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

2. Preparation of tissue microarray and immunohistochemical staining

We selected patients who had paraffin-embedded tissue specimens available for immunohistochemical staining. The recipient blocks were made from purified agar in 3.8 × 2.2 × 0.5 cm frames. The paraffin donor blocks were prepared after a thorough evaluation of hematoxylin- and eosin-stained slides. Two adjacent areas of carcinoma from the matching donor blocks were transplanted to the recipient blocks using a 2-mm core needle. The tissue microarrays were sectioned at a thickness of 4 μm and stained using Ventana automated immunostainer Discovery XT (Ventana Medical Systems, Tucson, AZ, USA). The slides were dried at 60℃ for 1 h and deparaffinized using EZ Prep (Ventana Medical Systems) at 75℃ for 8 min. Cell conditioning was performed using CC1 solution (Ventana Medical Systems) at 100℃ for 48 min. NF-κB p65 (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and TG2 (1:150 dilution; NeoMarkers, Fremont, CA, USA) were diluted, treated, and incubated at 37℃ for 32 min. Signals were detected using the DAB Map Detection Kit (Ventana Medical Systems). Counterstaining was

performed with hematoxylin (Ventana Medical Systems) for 4 min at room temperature.[18]

3. Evaluation and scoring of immunohistochemically analyzed tissue sections

The immunostained tissue sections were evaluated and scored by one investigator (Dr. H.S. Shim) who was blind to the clinical and pathological information. For each case, 1,000 cells were assessed in three or four different fields at 400× magnification.

NF-κB expression was detected by the cytoplasmic brown staining of neoplastic cells with varying intensities. Positive NF-κB expression was defined as distinct cytoplasmic immunostaining. The staining intensity was scored on a 0-3 scale: 0 (no staining of cancer cells), 1 (weak staining), 2 (moderate staining), 3 (strong staining). The percentage of stained tumor cells was scored from 0% to 100%. These two scores were then multiplied.[19] NF-κB expression was defined as low if the result was less than the median value of the calculated products. NF-κB expression was defined as high if the product was greater than the median value. Lymphocytes within the tissue sections were used as positive internal controls, which showed positive nuclear staining in all cases.

Immunostaining for TG2 was graded semiquantitatively, considering both the staining intensity and percentage of positive tumor cells, by one pathologist who was blind to the clinicopathologic information. The staining intensity was scored on a 0-3 scale: 0 (no staining of cancer cells), 1 (weak staining), 2 (moderate staining), 3 (strong staining). The percentage of stained tumor cells was scored from 0% to 100%. These two scores were then multiplied.[19] TG2 expression was defined as low if the product was less than the median value of TG2 expression. TG2 expression was defined as high if

the product was more than the median value. TG2 expression was observed in cells with a normal structure, specifically in smooth muscle cells and blood vessel endothelial cells. However, no expression was found in alveolar or bronchial epithelial cells.

4. EGFR mutation testing

DNA was extracted from five 10-μm-thick paraffin sections that contained a representative portion of each tumor block using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). One hundred nanograms of DNA were then amplified in a 20-μL reaction solution that contained 2 μL of 10X buffer (Roche, Mannheim, Germany), 1.7-2.5 mmol/L $MgCl_2$, 0.3 μM of each of the primer pairs of EGFR gene, 250 μM deoxynucleoside triphosphate, and 2.5 units of DNA polymerase (Roche). The primer pairs were the following:

exon 18	forward, 5'-TCCAAATGAGCTGGCAAGTG
	reverse, 5'-CCACACAG-CAAAGCAGAAACTCAC
exon 19	forward, 5'-ATGTGGCACCATCTCACAAATTGCC
	reverse, 5'-CCACACAGCAAAGCAGAAACTCAC
exon 21	forward, 5'-GCTCAGAGCCTGGCATGAA
	reverse, 5'-CATCCTCCCCTGCATGTGT
exon 23	forward, 5'-TGAAGCAAATTGCCCAAGAC
	reverse, 5'-TGACATTTCTCCAGGGATGC

Amplifications were performed using 5-min initial denaturation at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C and a final extension at 72°C for 10 min. Polymerase chain reaction (PCR) products were then 2% gel-purified with a QIAgen gel extraction kit (Qiagen). DNA templates were processed for the DNA sequencing reaction

using ABI-PRISM BigDye Terminator version 3.1 (Applied Biosystems, Foster, CA, USA) with both forward and reverse sequence-specific primers. Twenty nanograms of purified PCR products were then used in a 20-μL sequencing reaction solution that contained 8 μL of BigDye Terminator v3.1 and 0.1 μM of the same PCR primer. Sequencing reactions were performed using 2-min initial denaturation at 96°C, followed by 25 cycles of 10 s at 94°C, 15 s at 50°C, and 3 min at 60°C. The sequence data of EGFR gene were generated with an ABI PRISM 3100 DNA Analyzer (Applied Biosystems)[20]. The sequences were analyzed using Sequencer 3.1.1 software (Applied Biosystems) to compare variations.

5. Cell lines and culture

PC-9 and PC-9GR cell lines were obtained from Dr. B.C. Cho. The cells were cultured in RPMI 1640 (HyClone, Logan, UT, USA) with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 26 mM sodium bicarbonate. The cells were maintained in a humidified environment that contained 5% CO_2 at 37˚C.

6. Antibodies and reagents

Antibodies to β-actin, TGase2, IκBα, E-cadherin, N-cadherin, and Vimentin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit was obtained from Promega (Madison, WI, USA), and the FITC Annexin V Apoptosis Detection Kit was purchased from BD Pharmigen (CA, USA). The NF-κB reporter assay kit was obtained from the Cignal reporter assay kit (Qiagen).

7. Designing shRNA against TGase-2 and cloning in adenoviral shuttle vector

The target sequence to construct an small hairpin RNA (shRNA) specific to TG-2 was 5'-GAGTACTTCCGCAATGAGTTTGGGG-3', which was obtained from Genolution Pharmaceuticals (South Korea). The loop sequence was 5'-TTCAAGAGA-3', which was originally used in 2002. The top strand
(5'-GATCCGAGTACTTCCGCAATGAGTTTGGGGTCTCCCCCAAACTC
ATTGCGGAAGTACTCTTTTTTA-3')
and bottom strand
(5'-AGCTTAAAAAAGAGTACTTCCGCAATGAGTTTGGGGGAGACCC
CAAACTCATTGCGGAAGTACTCG-3') of the TG2 shRNA was designed to clone in the shuttle vector. The two strands were annealed and cloned in a pSP72ΔE3-H1 adenovirus E3 shuttle vector known as pSP72ΔE3-H1-TG2.

8. Construction of recombinant adenovirus

The construction of the IX gene that contained the adenovirus vector known as dl324-IX was performed as described[21]. The E3 shuttle vector pSP72ΔE3-H1-TG2 was linearized with drdI, and the adenoviral vector dl324-IX was linearized by SpeI digestion. Both linearized vectors were co-transformed into *E. coli* BJ5183 for homologous recombination. A successful homologous recombinant adenoviral plasmid
(dl324-IX-ΔE3-H1-hshTG2) and the control plasmid
(dl324-IX-ΔE3-H1-shNC; negative control [NC]; scrambled sequences) were linearized with PacI and transected in human embryonic kidney 293 cells to generate a replication-incompetent adenovirus. The infectious adenovirus titer was determined by a limiting dilution assay using the AdEasy Adenoviral Vector System (Qbiogene, Carlsbad, CA, USA).

9. MTT assay

PC-9 and PC-9GR cells were exposed to gefitinib at different doses for 72 h following infection with 50 multiplicity of infection (MOI) dl324-IX-ΔE3-H1-hshTG2 virus or without infection. The growth inhibition of PC-9 and PC-9GR (gefitinib-resistant) cells was determined using a standard MTT assay kit.

10. Western blot analysis

PC-9 and PC-9GR cells were infected with recombinant adenovirus. After 48 h, the cells were lysed with 1X Laemmli lysis buffer (62.5 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, and 0.002% bromophenol blue), and the protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific, Fremont, CA, USA). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gels were electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Immunodetection was performed with anti-TGase, anti-IκB, anti-E-cadherin, anti-N-cadherin, anti-vimentin, and anti-β-actin antibodies using a chemiluminescent and fluorescent image analysis system (Syngene, Cambridge, UK).

11. Statistical analysis

The statistical analyses were performed using SPSS 18.0 software for Windows (SPSS, Chicago, IL, USA). The association between categorical variables, such as NF-κB and TG2 expression, was estimated using the χ^2 test or Fisher's exact test. Survival distribution was estimated using the Kaplan-Meier method. Significant differences in the probability of patients who experienced relapse, progression, or death between the strata were

evaluated using the log-rank test. Cox multiple regression analysis was used to assess the prognostic and predictive value of NF-κB, TG2, and other clinicopathological factors after adjusting for variables that resulted in significant differences.

Disease-free survival was determined from the date of surgery to the time of relapse. For the statistical analysis, overall survival (OS) and progression-free survival (PFS) were defined as the interval between the date of diagnosis to death or last follow-up visit and the interval between the start of palliative treatment (platinum-based doublet chemotherapy or EGFR-TKI) to clinical progression, death, or last follow-up visit if the disease had not progressed, respectively. The required sample size was estimated to be 118, assuming a hazard ratio (HR) of 1.75 for relapse after curative resection (α = 0.05, β = 0.2, 10% drop-out rate). The statistical analysis of the laboratory data was performed using GraphPad Prism 6 software.

III. RESULTS

1. Patient characteristics

The patient characteristics at baseline are shown in Table 1. A total of 60 men and 60 women were included in the study, aged 41 to 84 years (median, 64 years). One hundred two cases (85%) had adenocarcinoma, as well as sarcomatoid carcinoma (*n* = 1), squamous cell carcinoma (*n* = 3), large-cell carcinoma (*n* = 5), mucoepidermoid carcinoma (*n* = 1), giant-cell carcinoma (*n* = 3), and other histologies (*n* = 3). Among the 120 patients, 11 received neoadjuvant chemotherapy. Twenty-five of the patients (20.8%) were current smokers, 16 (13.3%) were former smokers, and 79 (65.8%) had never smoked. The calculated median TG2 expression value was 50 (range: 0-300), and the median NF-κB value was 20 (range, 0-240).

Table 1. Patient demographics and baseline characteristics (n = 120).

	Number of patients (n = 120) (%)
Sex: Male/Female	60/60 (50/50)
Age (years): Median (range)	64 (41 – 84)
ECOG PS: 0/1/2	12/102/6 (10/85/5)
Weight loss: Yes/ No	5/115 (95.8/4.2)
Smoking status: Current/Former/Never	25/16/79 (20.8/13.3/65.8)
Histology: Adenocarcinoma/Squamous cell carcinoma/Other	102/3/15 (85/2.5/12.5)
Staging: I/II/III/IV	47/22/45/6 (39.2/18.3/37.5/5.0)
Surgery type: Pneumonectomy/Lobectomy/Segmentectomy or wedge resection	5/111/4 (4.2/92.5/3.3)
Chemotherapy	11
Neoadjuvant chemotherapy:	3 (1 – 8)
Median number of cycles (range)	88
Adjuvant chemotherapy:	48/37/3 (54.6/42.0/3.3)
Cisplatin-based doublet/Carboplatin-based doublet/Non-platinum	4 (2 – 6)
Median number of cycles (range)	28
Palliative chemotherapy:	5/23 (17.9/82.1)
Cisplatin-based/Carboplatin-based	4 (1 – 6)
Median number of cycles (range)	29
EGFR tyrosine kinase:	20/8/1 (69/27.6/3.4)
Gefitinib/Erlotinib/Erlotinib plus sorafenib	
EGFR mutation	
Positive	55 (45.8)
Exon 19 deletion or L858R mutations/Other*	52/3 (94.5/5.5)
Negative/Unknown	62/3 (51.7/2.5)
TG2 level: Median (range)	50 (0–300)
NF-κB level: Median (range)	20 (0-240)

*Other mutation: 20 insertion (n = 2), Gly719Ser (c.2155G>A), and Arg775His (c.2327G>A) (n = 1)

Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; TG2, transglutaminase 2.

2. NF-κB and TG2 expression and the relationship between NF-κB, TG2 expression, and clinicopathologic characteristics in NSCLC

We examined NF-κB and TG2 expression in 120 cases of NSCLC. The NF-κB and TG2 staining mainly accumulated in the cytoplasm (Fig. 1). The median NF-κB staining value was 20 (range, 0-240), and the median TG2 staining value was 50 (range, 0-300). High TG2 expression was more frequently detected in non-adenocarcinoma samples. TG2 expression was also related to EGFR mutation status. More patients with wildtype EGFR exhibited high TG2 expression ($p = 0.017$; Table 2).

NF-κB expression was not different according to clinicopathological parameters (Table 3). No correlation was found between NF-κB and TG2 expression (χ^2 test, $p = 0.360$).

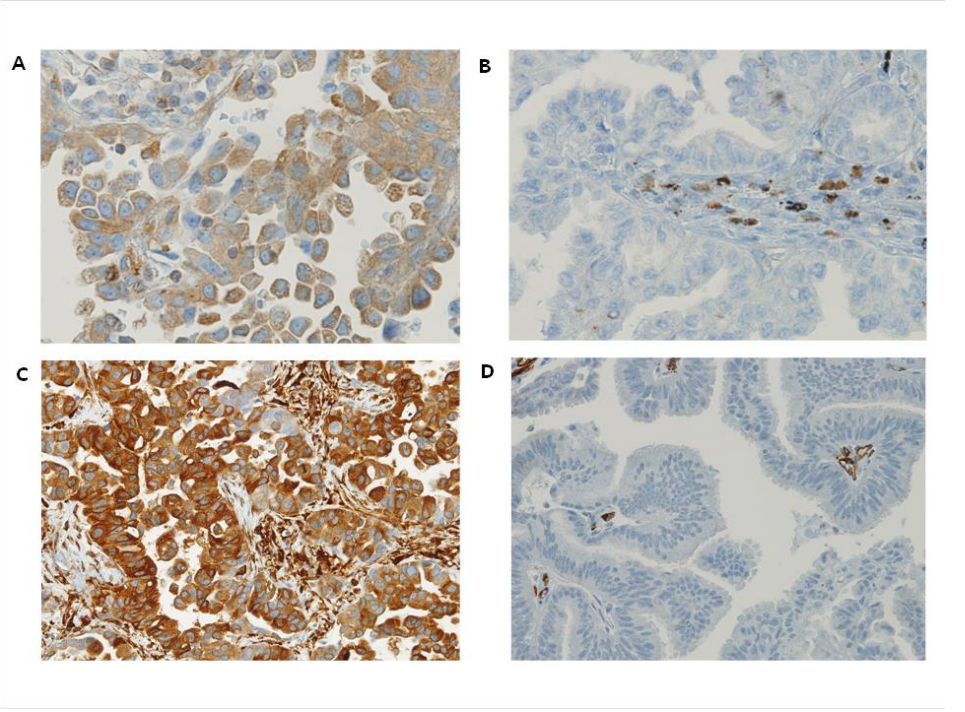

Figure 1. Immunohistochemical staining for NF-κB subunit p65 (A) and TG2 (C) in primary lung cancer. The cytoplasmic presence of TG2 in varying intensities is evident in cancer cells. The photographs show immunohistochemical-negative staining for the NF-κB subunit p65 (B) and TG2 (D) in primary lung cancer.

Table 2. Relationship between TG2 expression and clinicopathological parameters (n = 120).

	TG2 expression (n, %)		Total	p
	Low TG2	High TG2		
Age				
< 65 years	37 (55.2)	30 (44.8)	67	0.714
≥ 65 years	27 (50.9)	26 (49.1)	53	
ECOG PS				
0-1	62 (54.4)	52 (45.6)	114	0.416[F]
2	2 (33.3)	4 (66.7)	6	
Gender				
Male	28 (46.7)	32 (53.3)	60	0.200
Female	36 (60.0)	24 (40.0)	60	
Smoking history				
Smoker	21 (51.2)	20 (48.8)	41	0.847
Never smoker	43 (54.4)	36 (45.6)	79	
Histology				
Adenocarcinoma	58 (56.9)	44 (43.1)	102	0.077
Non-adenocarcinoma	6 (33.3)	12 (66.7)	18	
p-TNM stage				
Stage I	28 (60.9)	18 (39.1)	46	0.259
Stage II-IV	36 (48.6)	38 (51.4)	74	
Adjuvant chemotherapy				
Yes	43 (48.9)	45 (51.1)	88	0.147
No	21 (65.6)	11 (34.4)	32	
Weight loss				
Yes	3 (60)	2 (40)	5	1.000[F]
No	61 (53)	54 (47)	115	
Operation type				
Lobectomy	59 (53.2)	52 (46.8)	111	1.000
Pneumonectomy	3 (60.0)	2 (40)	5	
Segmentectomy and wedge resection	2 (50)	2 (50)	4	
EGFR mutation				
Yes	36 (65.5)	19 (34.5)	55	0.017[F]
No	26 (41.9)	36 (58.1)	62	
Unknown	2 (66.7)	1 (33.3)	3	

Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor.

Table 3. Relationship between NF-κB expression and clinicopathological parameters (n = 120).

	NF-κB expression (n, %)		Total	p
	Low NF-κB	High NF-κB		
Age				
< 65 years	33 (49.3)	34 (50.7)	67	0.359
≥ 65 years	31 (58.5)	22 (41.5)	53	
ECOG PS				
0-1	60 (52.6)	54 (47.4)	114	0.684[F]
2	4 (66.7)	2 (33.3)	6	
Gender				
Male	32 (53.3)	28 (46.7)	60	1.000
Female	32 (53.3)	28 (46.7)	60	
Smoking history				
Smoker	23 (56.1)	18 (43.9)	41	0.703
Never smoker	41 (51.9)	38 (48.1)	79	
Histology				
Adenocarcinoma	54 (52.9)	48 (47.1)	102	1.000
Non-adenocarcinoma	10 (55.6)	8 (44.4)	18	
p-TNM stage				
Stage I	21 (45.7)	25 (54.3)	46	0.194
Stage II-IV	43 (58.1)	31 (41.9)	74	
Adjuvant chemotherapy				
Yes	47 (53.4)	41 (46.6)	88	1.000
No	17 (53.1)	15 (46.9)	32	
Weight Loss				
Yes	3 (60)	2 (40)	5	1.000[F]
No	61 (53)	54 (47)	115	
Operation type				
Lobectomy	58 (52.3)	53 (47.7)	111	0.149[F]
Pneumonectomy	2 (40.0)	3 (60)	5	
Segmentectomy and wedge resection	4 (100)	0 (0)	4	
EGFR mutation				
Yes	27 (49.1)	28 (50.9)	55	0.637[F]
No	35 (56.5)	27 (43.5)	62	
Unknown	2 (66.7)	1 (33.3)	3	

Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor.

3. Correlation between NF-κB and TG2 expression and clinical outcome

Of the 120 patients studied, 76 relapsed, and 50 died. Median DFS was 24.0 months (95% confidence interval [CI], 16.6-31.4), with no difference in DFS according to NF-κB and TG2 expression (Fig. 2A, B). Median OS was not reached. The median follow-up duration was 38 months (range, 1.0-90.0 months).

To define the variables that predicted DFS and OS, we performed univariate analyses of NF-κB and TG2 expression and other clinicopathological factors. The clinicopathological factors included age, sex, Eastern Cooperative Oncology Group performance status (ECOG PS), weight loss, histology, stage, EGFR mutation status, and adjuvant chemotherapy. Univariate analyses indicated that stage, TG2 expression, age, sex, ECOG PS, weight loss, histology, and EGFR mutation were statistically significant for DFS and OS. Therefore, these variables were subsequently analyzed in a multivariate model (Table 4). Lower tumor stage (HR, 3.150; 95% CI, 1.830-5.423, $p < 0.001$) was significantly associated with better DFS. Older age (HR, 1.743; 95% CI, 0.942-3.226, $p = 0.007$), weight loss (HR, 3.732; 95% CI, 1.235-11.273, $p = 0.020$), non-adenocarcinomatous histology (HR, 3.057; 95% CI, 1.469-6.361, $p = 0.003$), and higher stage (HR, 4.093; 95% CI, 1.832-9.145, $p = 0.001$) were independent factors associated with worse OS. EGFR mutation status (HR, 0.458; 95% CI, 0.236-0.888, $p = 0.021$) was an independent factor associated with better OS. NF-κB and TG2 were not associated with DFS or OS.

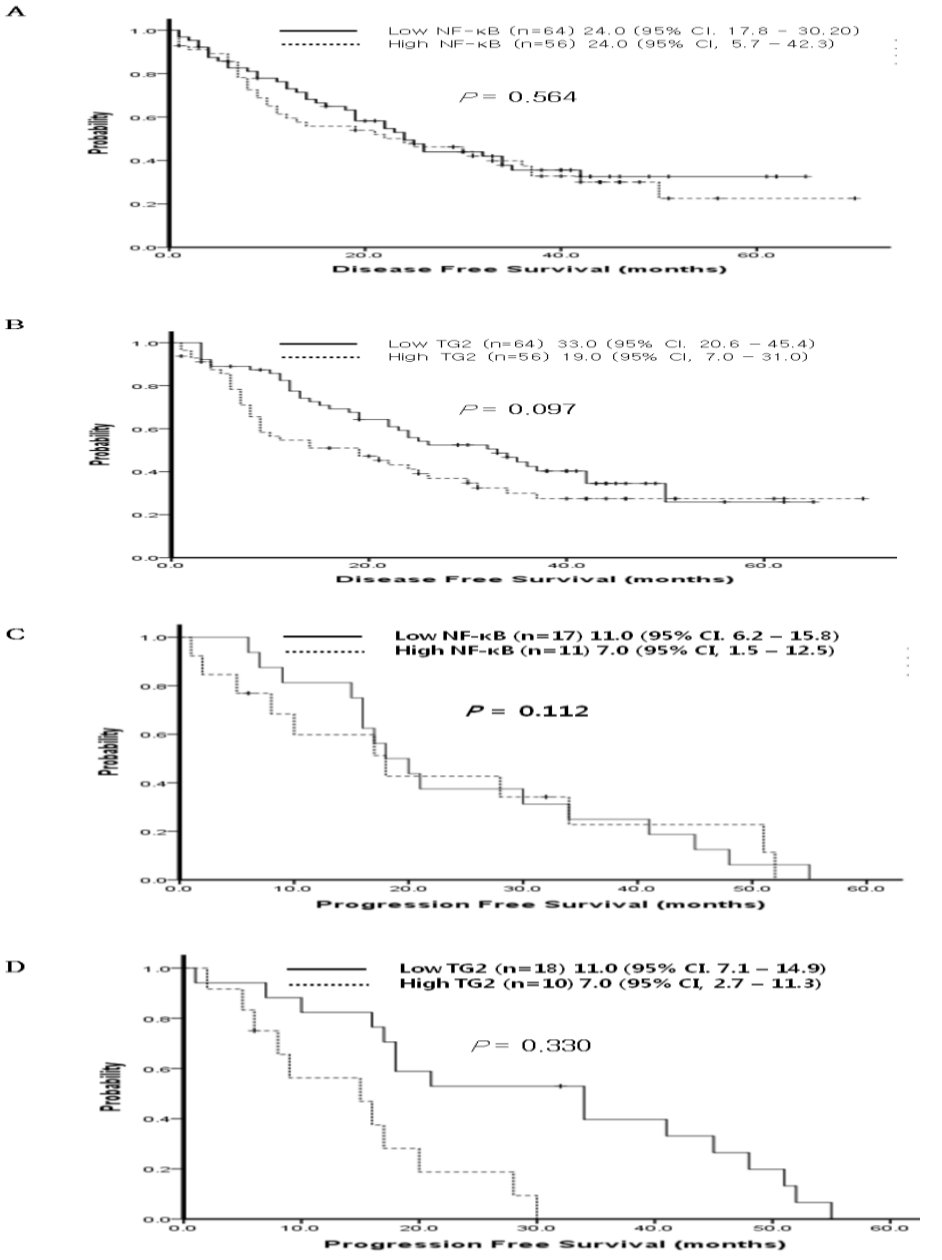

Figure 2. Kaplan-Meier plot that shows DFS according to NF-κB level (A) and TG2 level (B) and platinum-based doublet chemotherapy according to NF-κB level (C) and TG2 level (D). Disease-free survival and PFS were not different with regard to NF-κB levels (24 months *vs*. 24 months, $p = 0.564$, and 11.0 months *vs*. 7.0 months, $p = 0.112$, respectively) or TG2 levels (33.0 months *vs*. 19.0 months, $p = 0.097$, and 11.0 months *vs*. 7.0 months, $p = 0.330$, respectively).

Table 4. Univariate and multivariate analyses of prognostic factors for DFS and OS.

	Disease-free survival (n = 120)			Overall survival (n = 120)		
	Univariate		Multivariate	Univariate		Multivariate
	Median (months)	p	p (HR [95% CI])	Median (months)	p	p (HR [95% CI])
Age						
≤ 65 years	26.0	0.892		NR	0.037	0.077[1.743 (0.942 – 3.226)]
> 65 years	22.0			42.0		
Sex						
Male	19.0	0.524		44.0	0.037	0.665[0.875 (0.478 -1.602)]
Female	26.0			NR		
ECOG PS						
0-1	24	0.622		NR	0.047	0.967[1.026 (0.310 -3.392)]
2	21			25.0		
Smoking History						
Smoker						
Never-smoker	21	0.838		NR	0.428	
	24			NR		
Weight Loss						
No						
Yes	25.0	0.117		NR	<0.001	0.020[3.732 (1.235 – 11.273))
	19.0			20.0		
Histology						
Adeno	25.0	0.144		NR	0.002	0.003[3.057 (1.469 -6.361)]
Non-adeno	8			16.0		
Stage						
I						
II-IV						
	NR	<0.00	<0.001[3.150	NR	<0.001	0.001 [4.093
	15.0	1	(1.830-5.423)]	40.0		(1.832 -9.145)]
EGFR mutation						
No	19	0.725		44.0	0.010	0.005
Yes	32			NR		0.021[0.458 (0.236 -0.888)]
Unknown	7			17.0		0.081[4.521

						(0.829–24.643)]
TG2 level						
< 50	33.0	0.097	0.117[1.436	NR	0.010	0.359[1.328
≤ 50	19.0		(0.913-2.258)]	40.0		(0.724 -2.433)]
NF-κB level						
≤ 20	24.0	0.564		NR	0.282	
> 20	24.0			NR		
Adjuvant chemotherapy						
No	24.0	0.685		42	0.468	
Yes	24.0			NR		

Abbreviation: Adeno: adenocarcinoma, non-adeno: non-adenocarcinoma, NR: not reached, DFS: disease free survival, OS: overall survival; ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; TG2, transglutaminase 2.

4. Relationship between NF-κB and TG2 expression and clinical efficacy of palliative cytotoxic chemotherapy and EGFR-TKI treatment

Twenty-eight patients received platinum-based doublet chemotherapy as first-line treatment, and 29 received EGFR-TKI. Among the 28 patients treated with platinum-based doublet chemotherapy, 25 experienced disease progression, with median PFS of 9.0 months (95% CI, 6.6-11.4 months). The median PFS for patients with low TG2 expression tended to be longer compared with patients with high TG2 expression, but the difference was not significant (11.0 months *vs*. 7.0 months, $p = 0.330$; Fig. 2D). The response rates for platinum-based doublet chemotherapy were the following: 0% had complete response (CR), 14.3% had partial response (PR), 57.1% had stable disease (SD), and 21.4% had progressive disease (PD). Therefore, the overall response rate (ORR) was 13.8%, and the disease control rate (DCR) was 69%. Of the 29 patients treated with EGFR-TKI, 20 progressed with a median PFS of 6.0 months (95% CI, 3.8-8.2 months). The median PFS for patients with low TG2 expression was significantly longer compared with patients with high TG2 expression (11.0 months *vs*. 2.0 months, $p = 0.013$; Fig. 3C). However, no differences in survival were found with regard to EGFR mutation and NF-κB (Fig. 3A, B). Response rates for EGFR-TKI were the following: 3.4% had CR, 20.7% had PR, 34.5% had SD, 34.5% had PD, and 6.0% were unable to be evaluated. Therefore, the ORR was 24.1%, and the DCR was 58.6%.

To define the variables that predicted PFS for platinum-based doublet chemotherapy and EGFR-TKI treatment, the factors that were statistically significant in the univariate analyses (i.e., histology and TG2 expression) were analyzed in a multivariate model (Table 5). No factors were significantly associated with poor PFS in patients treated with platinum-based doublet chemotherapy. In patients treated with EGFR-TKI, only high TG2 expression

(HR, 2.637; 95% CI, 1.026-6.780, p = 0.044) was associated with poor PFS. No factors were associated with ORR or DCR (Table 6).

In EGFR mutation-positive cases treated with EGFR-TKI, no differences in PFS were found with regard to NF-κB and TG2 expression (Fig. 4A, B).

In wildtype EGFR cases treated with EGFR-TKI, no predictive factors were found for ORR or DCR. However, higher TG2 expression (HR, 5.915; 95% CI, 1.157-30.241, p = 0.033) was a statistically significant prognostic factor associated with longer PFS in the multivariate analysis (Table 7, Fig. 4C, D).

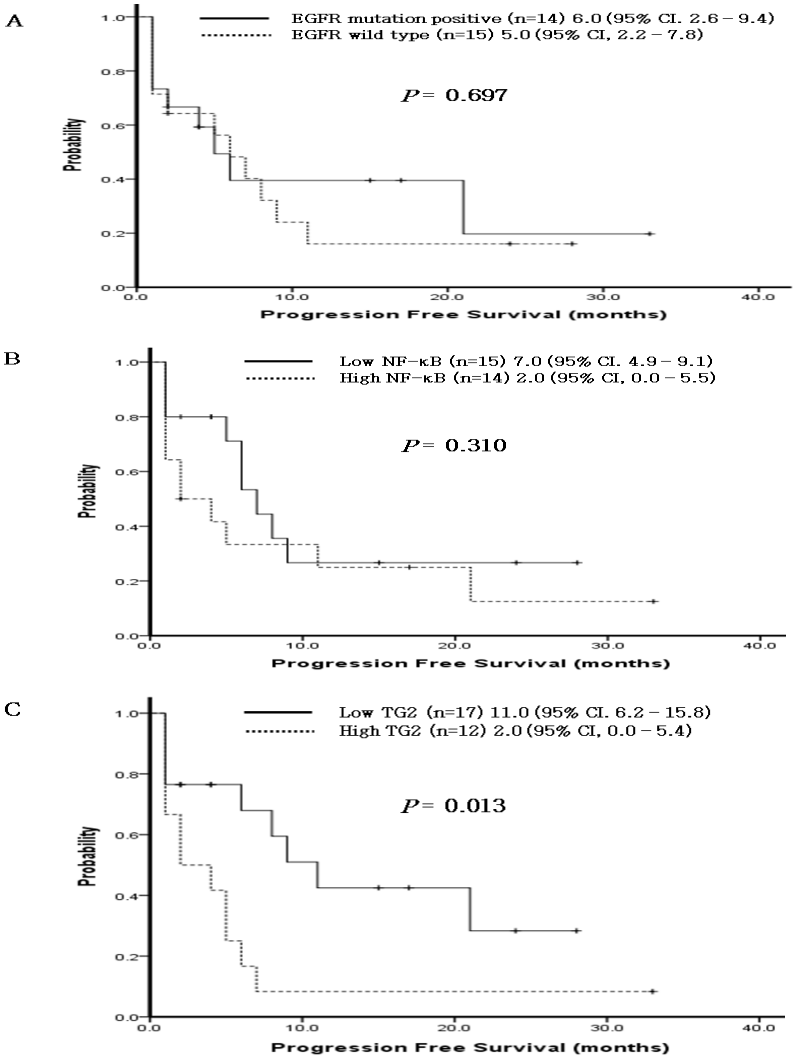

Figure 3. Kaplan-Meier plot that shows PFS (EGFR-TKI) according to EGFR mutation status, NF-κB level, and TG2 level (A-C) in patients treated with EGFR-TKI. In patients treated with EGFR-TKI, PFS was longer in the low TG2 group (11 months *vs*. 2 months, $p = 0.013$).

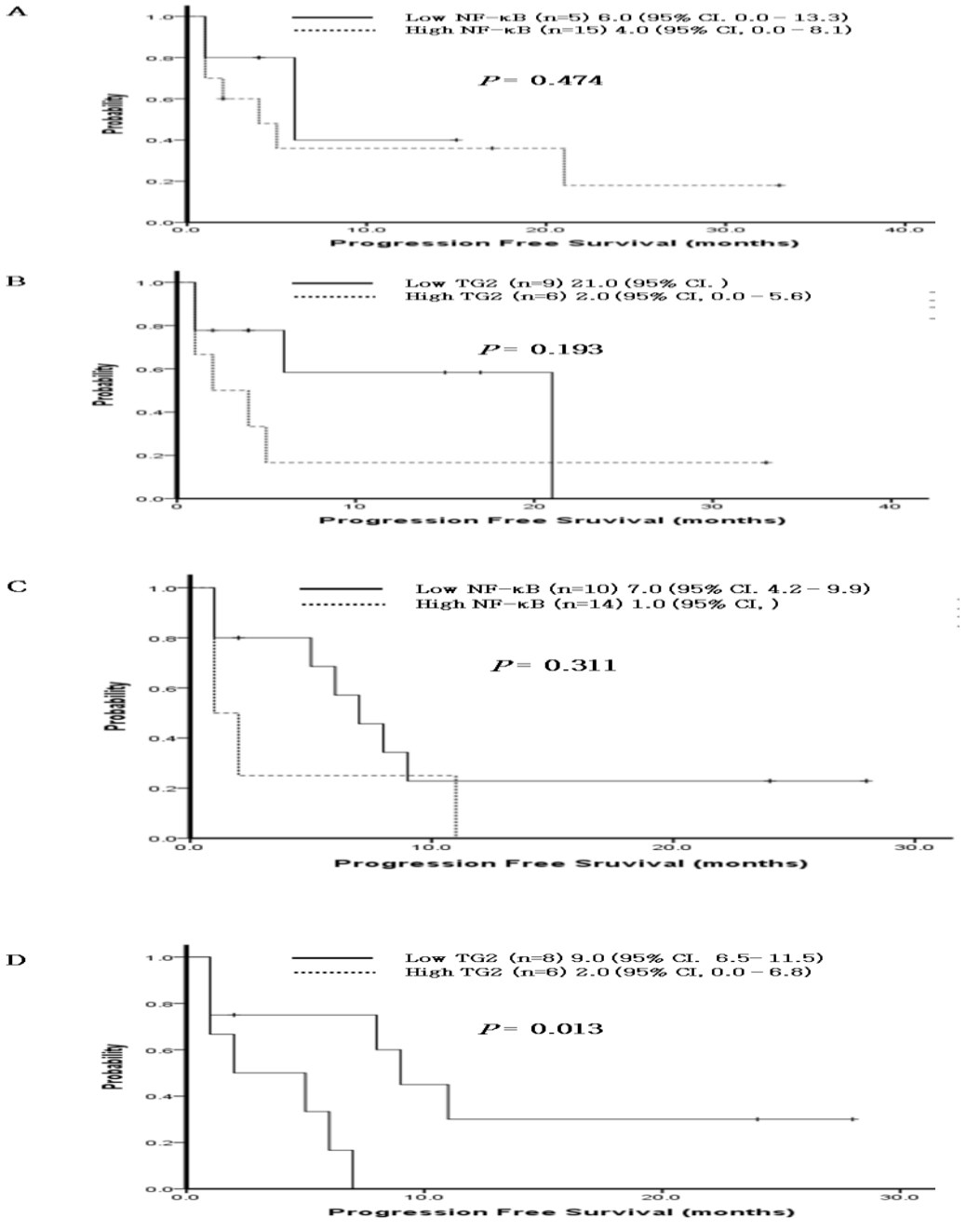

Figure 4. Kaplan-Meier plot that shows PFS (EGFR-TKI) according to NF-κB level and TG2 level in EGFR mutation-positive cases treated with EGFR-TKI (A, B) and wildtype EGFR cases treated with EGFR-TKI (C, D). In EGFR mutation-positive patients, PFS was not different according to NF-κB and TG2 levels. In wildtype EGFR patients, PFS was longer in the low TG2 group (9 months *vs.* 2 months, $p = 0.013$).

Table 5. Univariate and multivariate analyses of prognostic factors for PFS1 and PFS2.

	PFS1* (*n* = 28)			PFS2! (*n* = 29)		
	Univariate		Multivariate	Univariate		Multivariate
	Median (months)	*p*	*p* (HR [95% CI])	Median (months)	*p*	*p* (HR [95% CI])
Age						
≤ 65 years / > 65 years	8.0/9.0	0.872		5.0/6.0	0.850	
Sex						
Male/Female	8.0/11.0	0.204		4.0/7.0	0.473	
ECOG PS						
0/1-2	9.0/8.0	0.626		9.0/-	-	
Smoking history						
Smoker/Never-smoker	9.0/8.0	0.872		5.0/6.0	0.604	
Weight loss						
No/Yes	9.0/-	-		5.0/6.0	0.884	
Histology						
Adenocarcinoma	9.0	0.802		6.0	0.083	0.342 [1.905 (0.504 – 7.201)]
Non-adenocarcinoma	13.0			2.0		
Stage						
I/II-IV	8.0/9.0	0.457		11.0/6.0	0.677	
EGFR mutation						
No/Yes/Unknown	9.0/9.0/1.0	0.149		6.0/5.0	0.697	
TG2 level						
< 50 / ≥ 50	11.0/7.0	0.330		11.0/2.0	0.013	0.044 [2.637 (1.026 – 6.780)]
NF-κB level						
≤ 20 / > 20)	11.0/7.0	0.112		7.0/2.0	0.310	

*Progression-free survival in patients treated with palliative platinum-based doublet chemotherapy.
!Progression-free survival in patients treated with EGFR-TKI.
Abbreviations: PFS, progression-free survival; ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; TG2, transglutaminase 2.

Table 6. Tumor response and disease control with platinum-based doublet chemotherapy and EGFR-TKI treatment according to clinical and molecular variables.

	Platinum-based doublet (n = 29)				EGFR-TKI (n = 29)			
	Response rate		Disease control rate		Response rate		Disease control rate	
	n (%)	p	n (%)	p	n (%)	p	n (%)	p
Overall rate	4/28 (14.3)		20/28 (71.4)		6/29 (20.7)		16/28 (57.1)	
Age								
≤ 65 years	2/18 (11.1)	0.6[F]	13/18 (72.2)	1.00[F]	3/18 (16.7)	0.375[F]	10/18 (55.6)	0.72[F]
> 65 years	2/10 (20)		7/10 (70.0)		4/11 (36.4)		7/11 (63.6)	
Sex								
Male	2/13 (15.4)	1.0[F]	9/13 (69.2)	1.000[F]	3/16 (18.8)	0.667[F]	8/16 (50.0)	0.45
Female	2/15 (13.3)		11/15 (73.3)		4/13 (30.8)		9/13 (69.2)	
ECOG PS								
0 or 1	4/27 (14.8)	1.0[F]	19/27 (70.4)	1.0[F]	7/29 (24.1)		17/29 (58.6)	
2	0/1 (0)		1/1 (100)					
Smoking History								
Smoker	1/8 (12.5)	1.0[F]	6/8 (75.0)	1.00[F]	3/9 (33.3)	0.6[F]	5/9 (55.6)	1.00[F]
Never-smoker	3/20 (15.0)		14/20 (70.0)		4/20 (20.0)		12/20 (60.0)	
Weight Loss								
No	4/28 (14.3)		20/28 (71.4)		7/27 (25.9)	1.0[F]	16/27 (59.3)	1.0[F]
Yes					0/2 (0)		1/2 (50.0)	
Histology								
Adenocarcinoma	3/23 (13.0)	1.000[F]	18/23 (78.3)	0.123[F]	7/26 (26.9)	0.557[F]	16/26 (61.5)	0.55[F]
Non-adenocarcinoma	1/5 (20.0)		2/5 (40.0)		0/3 (0)		1/3 (33.3)	
Stage								
I	1/7 (14.3)	1.000[F]	6/7 (85.7)	0.382[F]	2/5 (40.0)	0.569[F]	3/5 (60.0)	1.00[F]
II-IV	3/21 (14.3)		14/21 (66.7)		5/24 (20.8)		14/24 (58.3)	
EGFR mutation								
No	4/13 (30.8)		8/13 (61.5)		2/14 (14.3)		7/14 (50.0)	
Yes	0/14 (0)	0.07[F]	12/14 85.7)	0.083[F]	5/15 (33.3)	0.390[F]	10/15 (66.7)	0.462
Unknown	0/1 (0)		0/1 (0)					
TG2 level								
< 50	1/18 (5.6)	0.12[F]	13/18 (72.2)	1.000[F]	6/17 (35.3)	0.187[F]	12/17 (70.6)	0.148[F]
≥ 50	3/10 (30.0)		7/10 (70.0)		1/12 (8.3)		5/12 (41.7)	
NF-κB level								
≤ 20	4/17 (23.5)		13/17 (76.5)		3/15 (20.0)		9/15 (60.0)	
> 20	0/11 (0)	0.13[F]	7/11 (63.6)	0.67[F]	4/14 (28.6)	0.68[F]	8/14 (57.1)	1.000

Abbreviations: EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; TG2, transglutaminase 2.

Table 7. Univariate and multivariate analyses of prognostic factors for PFS in wildtype EGFR patients and tumor response and disease control with EGFR-TKI treatment in wildtype EGFR patients.

	RR and PFS				PFS (n = 14)		
	Response rate		Disease control rate		Univariate		Multivariate
	n (%)	p	n (%)	p	Median (months)	p	p (HR [95% CI])
Overall Rate	2/14 (14.3)		7/14 (50)				
Age							
≤ 65 years	0/10 (0)	0.07[F]	4/10 (40)	0.56	2.0	0.135	
> 65 years	2/4 (50)		3/4 (75)		11.0		
Sex							
Male	0/8 (0)	0.165[F]	2/8 (25)	0.10[F]	1.0	0.311	
Female	2/6 (33.3)		5/6 (83.3)		7.0		
ECOG PS							
0 or 1	2/14 (14.3)		7/14 (50)				
Smoking History							
Smoker	0/4 (0)	1.00[F]	1/4 (25)		2.0		
Never-smoker	2/10 (20)		6/10 (60)	0.56	6.0	0.481	
Weight Loss							
No	2/12 (16.7)	1.00[F]	6/12 (50)	1.0[F]	5.0	0.937	
Yes	0/2 (0)		1/2 (50)		6.0		
Histology							
Adenocarcinoma	2/13 (15.4)	1.00[F]	6/13 (46.2)	1.0[F]	7.0	0.479	
Non-adenocarcinoma	0/1 (0)		1/1 (100)		5.0		
Stage							
I	1/3 (33.3)	0.40[F]	1/3 (33.3)	1.0[F]	1.0	0.415	
II-IV	1/11 (9.1)		6/11 (54.5)		7.0		
TG2 level							
< 50	2/8 (25)	0.48[F]	5/8 (62.5)	0.60[F]	9.0	0.013	0.033 [5.915 (1.157-30.241)]
≥ 50	0/6 (0)		2/6 (33.3)		2.0		
NF-κB level							
≤ 20	1/10(10)	0.51[F]	5/10 (50)		7.0		
> 20	1/4 (25)		2/4 (50)	1.0[F]	1.0	0.311	
Adjuvant chemotherapy							
No	0/2 (0)	1.00[F]	0/2 (0)	0.46[F]	1.0	0.448	
Yes	2/12 (16.7)		7/12 (58.3)		6.0		

Abbreviations: RR, response rate; PFS, progression-free survival; ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; TG2, transglutaminase 2.

5. Construction of replication-incompetent virus expressing TG2 shRNA

We identified an shRNA sequence that was shown to be effective in silencing human TG2 mRNA. In the present study, five other oligomers of TG2 shRNA and control shRNA (i.e., shRNA against luciferase) were validated using real-time PCR after the selection of appropriate TG2 target sequences and identification of the target sequence with maximal repression. To construct a defective adenovirus, the TG2 shRNA sequences were inserted into the pSP72ΔE3-H1 shuttle vector.

6. Effect of shTG2-expressing replication-incompetent adenovirus on TG2 protein expression

To demonstrate the effect of dl324-IX-ΔE3-H1-hshTG2 on TG2 suppression, PC9-GR cells were infected with dl324-IX-ΔE3-NC or dl324-IX-ΔE3-H1-hshTG2 viruses at an MOI of 20. Fig. 5 shows that dl324-IX-ΔE3-H1-hshTG2 adenovirus significantly reduced TG2 expression compared with dl324-IX-ΔE3-NC.

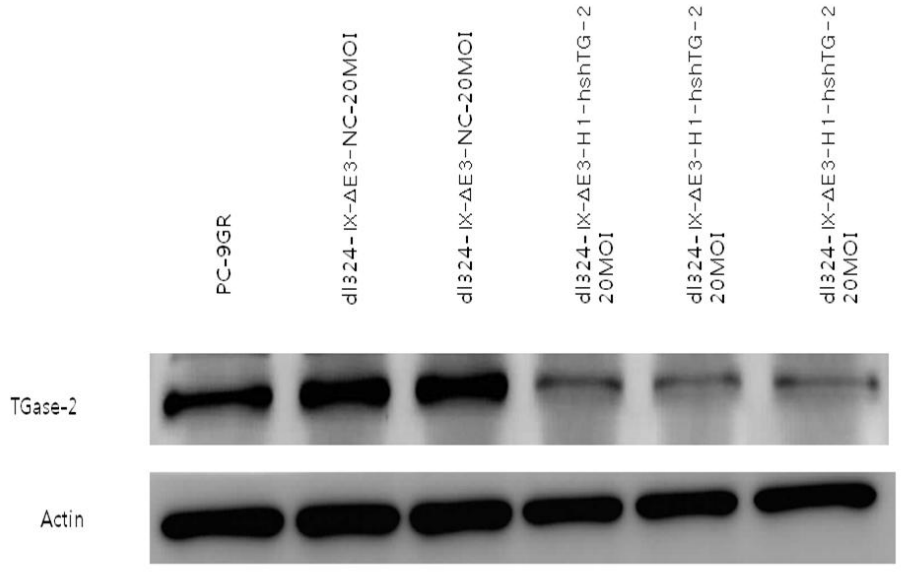

Figure 5. TG2 downregulation in the PC-9GR cell line according to target sequences. The experiments were performed in triplicate. The shTG2-expressing defective adenovirus showed a TG2-suppressing effect.

7. Sensitizing effect of apoptosis by TG2 shRNA-expressing adenovirus

To determine the sensitizing effect of TG2 suppression, we treated the PC9GR cell line with various concentrations of gefitinib and dl324-IX-ΔE3-H1-hshTG2 adenovirus. At all of the tested concentrations, dl324-IX-ΔE3-H1-hshTG2 adenovirus restored the sensitivity to gefitinib in the MTT assay (Fig. 6).

To investigate the mechanism of the cell-killing effect, we conducted a fluorescence-activated cell sorting analysis. Early apoptosis was observed at a gefitinib concentration of 5 μM. Late apoptosis was observed after treatment with dl324-IX-ΔE3-H1-hshTG2 adenovirus (Fig. 7).

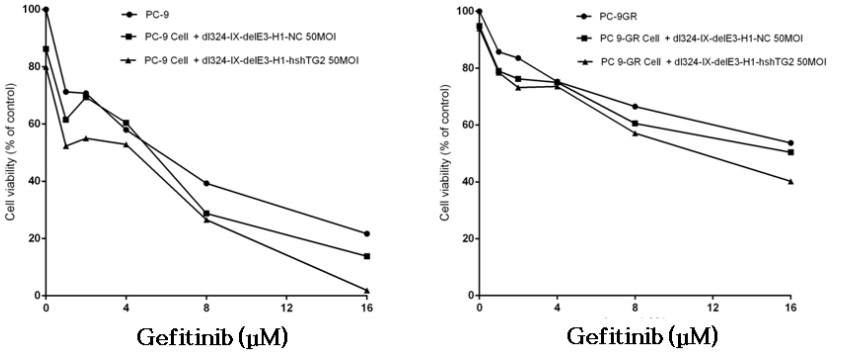

Figure 6**.** Cytotoxic effect of gefitinib (EGFR-TKI) in PC-9 and PC-9GR (gefitinib-resistant) cells. The cells were exposed to gefitinib at different doses for 72 h following infection with 50 MOI dl324-IX-ΔE3-H1-hsh TG2 virus or without infection. Growth inhibition in PC-9 and PC-9GR cells is shown. Cell viability was determined by the MTT assay (Cyto X Kit, LPS solution). The experiments were performed in triplicate. In PC-9 and PG-9GR cell lines, the defective shTG2 adenovirus showed slight sensitizing effects compared with the control adenovirus.

IV. DISCUSSION

Non-small cell lung cancer is an aggressive disease characterized by rapid progression and very poor patient survival. The majority of patients show resistance to conventional first-line cytotoxic chemotherapy. The present study demonstrated an association between drug resistance and TG2 expression in NSCLC through constitutive NF-κB activation. TG2 expression levels were associated with drug resistance in NSCLC cases treated with EGFR-TKI (p = 0.044).

Unfortunately, we could not demonstrate the prognostic value of NF-κB and TG2 with regard to DFS and OS in completely resected patients. No correlation was found between NF-κB and TG2 expression; therefore, TG2 appeared to be an independent predictive factor that was not associated with NF-κB expression. NF-κB is an inducible transcription factor, so immunohistochemistry cannot represent the accurate status of cell apoptosis. The present study analyzed an insufficient number of samples to prove an association between drug resistance and TG2 or NF-κB expression.

Several randomized Phase III studies recently compared EGFR-TKI treatment with cytotoxic chemotherapy and showed superior response rates and PFS in EGFR mutation-positive cases. Gefitinib and erlotinib have been shown to have comparable efficacy and a more favorable toxicity profile than cytotoxic chemotherapy in pretreated NSCLC. However, this was not the case in one study of wildtype EGFR NSCLC.[22-28] Furthermore, the present study showed that TG2 was significantly predictive of EGFR-TKI efficacy, especially in wildtype EGFR patients. TG2 causes the constitutive activation of NF-κB, a primary resistance regulator, through IκBα. TG2 is also an epithelial mesenchymal transition inducer involved in drug resistance.[29-32] Recently, the epithelial-mesenchymal transition has been suggested to be a

mechanism of resistance to EGFR-TKI, regardless of EGFR mutation status. TG2 might be important in this context, especially in wildtype EGFR cases. Recent data showed that patients with an epithelial phenotype benefited more from treatment with EGFR-TKIs in wildtype EGFR NSCLC cases, indicating that the epithelial-mesenchymal transition might be used as a marker to guide individualized EGFR-TKI therapy in this subpopulation.[33] Thus, TG2 may be a pharmaceutical target to overcome EGFR-TKI resistance. Future studies of combinations of EGFR-TKI (erlotinib or gefitinb) and TG2 inhibitors in several NSCLC cell lines and a xenograft model may help corroborate the present findings. Evaluating TG2 expression in patients with NSCLC could be a predictive marker for EGFR-TKI efficacy. Surgical samples were used for TG2 and NF-κB; therefore, the specimens did not necessarily reflect accurate NF-κB and TG2 status at the time of treatment (i.e., platinum-based doublet chemotherapy and EGFR-TKI).

We performed Cox regression analyses to determine the prognostic value of NF-κB and TG2 expression in NSCLC. Statistical significance was not achieved for either TG2 or NF-κB expression with regard to DFS or OS; such validation would be necessary to demonstrate the prognostic value of TG2 and NF-κB in large prospective studies. In adenocarcinoma, various types of driver mutations have been identified, and new targeted agents have been developed compared with squamous cell lung cancer. A previous study showed that TG2 was a more significant prognostic factor in squamous lung cancer.[34] Therefore, the role of TG2 in squamous NSCLC should be explored in future studies. In the present study, immunohistochemical scoring was performed by only one pathologist. Immunohistochemistry is known to be very subjective, which may have introduced bias into our study.

In the PC-9GR cell line that harbored the 19 deletion mutation but

acquired EGFR-TKI resistance, we confirmed a TG2-suppressing effect using shTG2-expressing defective adenovirus transfection. Co-treatment with gefitinib and the shTG2-expressing defective adenovirus facilitated a slight sensitizing effect of gefitinib, and TG2 suppression induced apoptosis.

V. CONCLUSION

In conclusion, TG2 may be a promising predictive indicator of platinum-based doublet or EGFR-TKI therapy in NSCLC. Future clinical studies with a large number of patients are needed to clarify the role of TG2 in NSCLC.

REFERENCES

1. Jung K-W, Park S, Kong H-J, Won Y-J, Lee JY, Park E-C, et al. Cancer Statistics in Korea: Incidence, Mortality, Survival, and Prevalence in 2008. Cancer Res Treat 2011;43:1-11.
2. Bivona TG, Hieronymus H, Parker J, Chang K, Taron M, Rosell R, et al. FAS and NF-[kgr]B signalling modulate dependence of lung cancers on mutant EGFR. Nature 2011;471:523-6.
3. Meylan E, Dooley AL, Feldser DM, Shen L, Turk E, Ouyang C, et al. Requirement for NF-[kgr]B signalling in a mouse model of lung adenocarcinoma. Nature 2009;462:104-7.
4. Bassères DS, Ebbs A, Levantini E, Baldwin AS. Requirement of the NF-κB Subunit p65/RelA for K-Ras–Induced Lung Tumorigenesis. Cancer Res 2010;70:3537-46.
5. Snchez-Prez I, Benitah SA, Martnez-Gomariz M, Lacal JC, Perona R. Cell stress and MEKK1-mediated c-Jun activation modulate NFkappaB activity and cell viability. Mol Biol Cell 2002;13:2933-45.
6. Mabuchi S, Ohmichi M, Nishio Y, Hayasaka T, Kimura A, Ohta T, et al. Inhibition of NFκB increases the efficacy of cisplatin in in vitro and in vivo ovarian cancer models. J Biol Chem 2004;279:23477-85.
7. Denlinger CE, Rundall BK, Keller MD, Jones DR. Proteasome inhibition sensitizes non-small-cell lung cancer to gemcitabine-induced apoptosis. Ann Thorac Surg 2004;78:1207-14.
8. Arlt A, Gehrz A, Muerkoster S, Vorndamm J, Kruse M-L, Folsch UR, et al. Role of NF-[kappa]B and Akt//PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death. Oncogene 2003;22:3243-51.
9. Patel NM, Nozaki S, Shortle NH, Bhat Nakshatri P, Newton TR, Rice S, et al. Paclitaxel sensitivity of breast cancer cells with constitutively active NF-kappaB is enhanced by IkappaBalpha super-repressor and parthenolide. Oncogene 2000;19:4159-69.
10. Motwani M, Delohery TM, Schwartz GK. Sequential dependent enhancement of caspase activation and apoptosis by flavopiridol on paclitaxel-treated human gastric and breast cancer cells. Clin Cancer Res 1999;5:1876-83.
11. Mehta K, Kumar A, Kim HI. Transglutaminase 2: A multi-tasking protein in the complex circuitry of inflammation and cancer. Biochem Pharmacol 2010;80:1921-9.

12. Antonyak MA, Miller AM, Jansen JM, Boehm JE, Balkman CE, Wakshlag J, et al. Augmentation of tissue transglutaminase expression and activation by epidermal growth factor inhibit doxorubicin-induced apoptosis in human breast cancer cells. Journal of biological chemistry 2004;279:41461-7.
13. Mehta K. High levels of transglutaminase expression in doxorubicin-resistant human breast carcinoma cells. Int J Cancer 1994;58:400-6.
14. Han JA, Park SC. Hydrogen peroxide mediates doxorubicin-induced transglutaminase 2 expression in PC-14 human lung cancer cell line. Exp Mol Med 1999;31:83-8.
15. Verma A, Mehta K. Tissue transglutaminase-mediated chemoresistance in cancer cells. Drug Resist Updat 2007;10:144-51.
16. Kim D-S, Park S-S, Nam B-H, Kim I-H, Kim S-Y. Reversal of drug resistance in breast cancer cells by transglutaminase 2 inhibition and nuclear factor-κB inactivation. Cancer Res 2006;66:10936-43.
17. Park K, Kim H, Lee J, Choi Y, Park S, Yang S, et al. Transglutaminase 2 as a cisplatin resistance marker in non-small cell lung cancer. J Cancer Res Clin Oncol 2010;136:493-502.
18. Shim HS, Yoon BS, Cho NH. Prognostic significance of paired epithelial cell adhesion molecule and E-cadherin in ovarian serous carcinoma. Hum Pathol 2009;40:693-8.
19. Pirker R, Pereira JR, von Pawel J, Krzakowski M, Ramlau R, Park K, et al. EGFR expression as a predictor of survival for first-line chemotherapy plus cetuximab in patients with advanced non-small-cell lung cancer: analysis of data from the phase 3 FLEX study. Lancet Oncol 2012;13:33-42.
20. Han S, Kim T, Hwang PG, Jeong S, Choi IS, Oh D, et al. Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small-cell lung cancer patients treated with gefitinib. J Clin Oncol 2005;23:2493-501.
21. Kim SY, Kim JH, Song JJ. c-Cbl shRNA-expressing adenovirus sensitizes TRAIL-induced apoptosis in prostate cancer DU-145 through increases of DR4/5. Cancer Gene Ther 2013;20:82-7.
22. Ciuleanu T, Stelmakh L, Cicenas S, Miliauskas S, Grigorescu AC, Hillenbach C, et al. Efficacy and safety of erlotinib versus chemotherapy in second-line treatment of patients with advanced, non-small-cell lung cancer with poor prognosis (TITAN): a

randomised multicentre, open-label, phase 3 study. Lancet Oncol 2012;13:300-8.

23. L. Vamvakas, S. Agelaki, N. K. Kentepozidis, A. Karampeazis, A. G. Pallis, C. Christophyllakis, et al. Pemetrexed (MTA) compared with erlotinib (ERL) in pretreated patients with advanced non-small cell lung cancer (NSCLC): Results of a randomized phase III Hellenic Oncology Research Group trial. J Clin Oncol 28:15s, 2010 (suppl; abstr 7519) 2010.
24. Kim ES, Hirsh V, Mok T, Socinski MA, Gervais R, Wu Y, et al. Gefitinib versus docetaxel in previously treated non-small-cell lung cancer (INTEREST): a randomised phase III trial. Lancet 2008;372:1809-18.
25. Lee DH, Park K, Kim JH, Lee J, Shin SW, Kang J, et al. Randomized Phase III trial of gefitinib versus docetaxel in non-small cell lung cancer patients who have previously received platinum-based chemotherapy. Clin Cancer Res 2010;16:1307-14.
26. Cufer T, Vrdoljak E, Gaafar R, Erensoy I, Pemberton K. Phase II, open-label, randomized study (SIGN) of single-agent gefitinib (IRESSA) or docetaxel as second-line therapy in patients with advanced (stage IIIb or IV) non-small-cell lung cancer. Anticancer Drugs 2006;17:401-9.
27. Maruyama R, Nishiwaki Y, Tamura T, Yamamoto N, Tsuboi M, Nakagawa K, et al. Phase III study, V-15-32, of gefitinib versus docetaxel in previously treated Japanese patients with non-small-cell lung cancer. J Clin Oncol 2008;26:4244-52.
28. Sun J-M, Lee KH, Kim S-W, Lee DH, Min YJ, Yun HJ, et al. Gefitinib versus pemetrexed as second-line treatment in patients with nonsmall cell lung cancer previously treated with platinum-based chemotherapy (KCSG-LU08-01). Cancer 2012;118:6234-42.
29. Cao L, Shao M, Schilder J, Guise T, Mohammad KS, Matei D. Tissue transglutaminase links TGF-[beta], epithelial to mesenchymal transition and a stem cell phenotype in ovarian cancer. Oncogene 2012;31:2521-34.
30. Kumar A, Xu J, Sung B, Kumar S, Yu D, Aggarwal B, et al. Evidence that GTP-binding domain but not catalytic domain of transglutaminase 2 is essential for epithelial-to-mesenchymal transition in mammary epithelial cells. Breast Cancer Res 2012;14:R4.
31. Chun-Yu L, Pei-Hsun T, Kandaswami CC, Geen-Dong C, Chia-Hsiung C, Chang-Jen H, et al. Role of tissue transglutaminase 2 in the acquisition of a mesenchymal-like

phenotype in highly invasive A431 tumor cells. Mol Cancer 2011;10:87-99.

32. Kumar A, Xu J, Brady S, Gao H, Yu D, Reuben J, et al. Tissue transglutaminase promotes drug resistance and invasion by inducing mesenchymal transition in mammary epithelial cells. PLoS One 2010;5:13390.

33. Shengxiang Ren, ChunXia Su, Xiaoxia Chen, Jiayu Li, Bing Li, Xuefei Li, et al. Association of epithelial to mesenchymal transition with efficacy of EGFR-TKIs in non-small cell lung cancer patients with EGFR wild type. J Clin Oncol 2012 (suppl; abstr 7535);30.

34. Choi C-M, Jang S-J, Park S-Y, Choi Y-B, Jeong J-H, Kim D-S, et al. Transglutaminase 2 as an independent prognostic marker for survival of patients with non-adenocarcinoma subtype of non-small cell lung cancer. Mol Cancer 2011;10:119.

ABSTRACT (IN KOREAN)

비소세포폐암에서 EGFR-TKI 또는 세포독성 항암제의 효과와 NF-κB 및 transglutaminase 2 발현과의 상관관계

<지도교수 김 주 항>

연세대학교 대학원 의학과

정 재 헌

배경: 트랜스글루타미나제 2 는 가교 효소로 약물 내성에 관여하며, 염증성 전사인자인 NF-κB 의 지속적인 활성화와 연관된다. 본 연구는 비소세포폐암 환자에서 트랜스글루타미나제 2와 NF-κB 의 표현 정도와 상피세포 성장인자 수용체 저해제와 세포독성 항암제 치료 효과와의 연관성을 살펴 보고자 계획되었다.

환자 및 방법: 트랜스글루타미나제 2와 NF-κB 의 표현 정도를 120명의 수술 받은 비소세포폐암 환자의 조직에서 살펴 보았다. 카플란 마이어 생존 분석과 비례 위험 회귀 분석 방법이 트랜스글루타미나제 2 와 NF-κB의 표현 정도가 세포 독성 항암제와 상피세포 성장인자 수용체 저해제 효과와의 연관성을 추정하기 위해 사용되었다.

결과: 환자들의 나이의 중앙값은 64세였다. (범위 41세 – 82세). 102명 (85%) 의 환자가 선암이었고, 18명의 (15%) 환자는 다른 조직형을 가지고 있었다. 88명의 환자가 수술 후 보조 항암 화학요법을

받았고, 29명의 환자가 백금을 기반으로 한 이제 항암 화학 요법을 받았다. 그리고 29명의 환자가 상피세포 성장인자 수용체 저해제로 치료 받았다. 흡연력은 25명이 현재 흡연자, 16명이 과거 흡연자 그리고 79명은 비흡연자였다. 상피세포 성장인자 수용체 돌연변이가 있는 환자는 55명이었다. 트랜스글루타미나제 2 의 중앙값은 50 (범위 0 – 300) 그리고 NF-kB 중앙값은 20이었다 (범위 0 to 240). 백금계 2제 항암화학 요범에 대한 반응율은 다음과 같다. 전체 반응율은 13.8% 였고, 질병 조절율은 69% 였다. (완전반응 0%, 부분 반응 13.8%, 안정성 병변 55.2%, 질병 진행 24.1%). 상피세포 성장인자 수용체 저해제에 대한 반응은 다음과 같았다. 전체 반응율은 24.1% 질병 조절율은 58.6% 였다 (완전 반응 3.4%, 부분 반응 20.7%, 안전성 병변 34.5%, 질병 진행 34.5%). 무병 생존율은 트랜스글루타미나제가 높은 그룹과 낮은 그룹에서 차이가 없었다. 그리고 고식적인 백금 기반 2제 항암 화학요법을 받은 환자들 (n=28) 에서 무진행 생존 기간이 트랜스글루타미나제가 낮은 그룹에서 더 길었다. (11.0 개월 대 7.0 개월, p = 0.330). 상피세포 성장인자 수용체 저해제를 받은 환자들 (n=29) 중에서는 (일차 치료 7명, 이차 치료 18 명, 삼차 치료 3명, 4차 치료 1명), 무진행 생존 기간이 트랜스글루타미나제 2가 높은 그룹에 비해 낮은 그룹에서 더 길었다. (11.0 개월 대 2.0 개월, p = 0.013). 상피세포 성장인자 수용체 저해제로 치료받은 야생형 상피세포 성장인자 수용체를 가지고 있는 환자들에서는 무진행 생존 기간이 트랜스글루타미나제 2가 높은 그룹에 비해서 낮은 그룹에서 더 길었다. (9.0 개월 대 2.0 개월, p=0.013).

shTG2 를 발현하는 defective 아데노바이러스는 트랜스글루타미나제 2의 발현을 억제하는 효과를 보여 주었다. 게피티닙과 shTG-2 를 발현하는 defective 아데노바이러스를 동시에 처리하였을 때, 세포 살상 효과를 증가시키는 것을 보여 주었고, TG2 의 발현이 낮을수록 게피티닙 감수성이 증가함을 추정할 수 있었다.

결론: 이 연구는 트랜스글루타미나제 2의 표현정도가 세포독성 항암제와 상피세포 성장인자 수용체 저해제의 효과를 예측하는 예측인자로서 역할을 할 수 있다는 것을 시사하고 있고 특히 야생형 상피세포성장인자를 가지고 있는 환자에서 더욱 그렇다고 할 수 있겠다.

핵심되는 말 : 폐암, NF-κB, transglutaminase2

PUBLICATION LIST

Jeong JH, Cho BC, Shim HS, Kim HR, Lim SM, Kim SK, et al. Transglutaminase 2 Expression Predicts Progression Free Survival in Non-Small Cell Lung Cancer Patients Treated with Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor J Korean Med Sci (In Press)

Kim SH, Shim HS, Cho J, Jeong JH, Hong YK, Sung JH, et al. A phase I trial of gefitinib and nimotuzumab in patients with advanced non-small cell lung cancer (NSCLC). Lung Cancer 2013;79:270-5.

Kim GM, Kim YS, Ae Kang Y, Jeong J, Kim SM, Hong YK, et al. Efficacy and toxicity of belotecan for relapsed or refractory small cell lung cancer patients. Journal of thoracic oncology 2012;7:731-6.

Choi C-M, Jang S-J, Park S-Y, Choi Y-B, Jeong J-H, Kim D-S, et al. Transglutaminase 2 as an independent prognostic marker for survival of patients with non-adenocarcinoma subtype of non-small cell lung cancer. Molecular Cancer 2011;10:119.

Jeong J, Cho BC, Sohn JH, Choi HJ, Kim SH, Lee YJ, et al. Belotecan for relapsing small-cell lung cancer patients initially treated with an irinotecan-containing chemotherapy: a phase II trial. Lung Cancer 2010; 70:77-81.