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Association of *BIM* Deletion Polymorphism and *BIM-γ* RNA Expression in NSCLC with *EGFR* Mutation

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Abstract. *Aim: This pilot study assessed the association of BIM deletion polymorphism and BIM RNA isoform in patients with EGFR-positive non-small cell lung cancer (NSCLC). Patients and Methods: The study included 33 patients with EGFR-positive NSCLC treated with gefitinib. BIM deletion polymorphism and BIM RNA isoform (EL/L/S/γ) were determined by polymerase chain reaction (PCR). Results: BIM-γ expression was significantly higher in patients with BIM deletion polymorphism than among those without BIM deletion polymorphism inside tumors (p=0.038) and around tumors (p=0.0024). Relative BIM-γ expression was significantly higher in patients with BIM deletion polymorphism than among those without BIM deletion polymorphism (p=0.0017). Patients with BIM-γ had significantly shorter progression-free survival than those without BIM-γ (median: 304 vs. 732 days; p=0.023). Conclusion: Expression of BIM-γ mRNA and BIM deletion polymorphism were strongly associated. BIM-γ overexpression may have a role in apoptosis related to EGFR-tyrosine kinase inhibitor.*

Activating mutations in epidermal growth factor receptor (*EGFR*) are promising targets in the treatment of non-small cell lung cancer (NSCLC) (1, 2). The frequency of *EGFR* mutations varies by population. In North America and Western Europe, approximately 5-10% of patients with adenocarcinoma harbor mutations, whereas in East Asia approximately 60-70% of never-smokers have *EGFR* mutations (3, 4). EGFR tyrosine kinase inhibitors (EGFR-TKIs) induce marked radiographic and clinical improvement in patients with *EGFR* mutations. EGFR-TKIs such as gefitinib, erlotinib, and afatinib are recommended for treating *EGFR*-mutated NSCLC (5, 6). NSCLC patients with such mutations who were treated with an EGFR-TKI as first-line therapy had longer progression-free survival (PFS) than those who received platinum-based chemotherapy (7-11). Therefore, detection of *EGFR* mutations in patients with metastatic NSCLC is important for selecting individualized therapies.

Treatment resistance invariably develops within 10 to 16 months after initial EGFR-TKI treatment (12). Approximately 60% of patients with acquired resistance to EGFR-TKIs had an *EGFR* T790M mutation (13, 14). Other reported mechanisms underlying resistance are *MET* amplification, in 5-10% of cases (15, 16), and small-cell cancer transformation, in fewer than 5% of cases (17). However, approximately 30% of patients with *EGFR*-active mutations do not exhibit an objective response to EGFR-TKI, which is known as primary resistance (18-22). Although the mechanisms of primary resistance have been investigated in several preclinical and retrospective studies, the clinical and molecular characteristics of such resistance remain poorly understood.

Abbreviations: BIM, BCL2-like 11; BCL, B-cell CLL/lymphoma 2; BH3, BCL2 homology domain 3; NSCLC, non–small-cell lung cancer; *EGFR*, epidermal growth factor receptor; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; FFPE, formalin-fixed paraffin-embedded; PFS, progression-free survival; OS, overall survival; PCR, polymerase chain reaction; RR, response rate; DCR, disease control rate; CTC, National Cancer Institute Common Terminology Criteria.

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Key Words: BIM, non-small cell lung cancer, epidermal growth factor receptor tyrosine kinase inhibitor.

BCL2-like 11 (*BIM*) is a pro-apoptotic member of the B-cell CLL/lymphoma 2 (BCL2) family of proteins (23, 24) and is a key modulator of apoptosis triggered by EGFR-TKIs (25, 26). Faber *et al*. (27) used quantitative real-time polymerase chain reaction (PCR) and *BIM* immunohistochemistry to investigate *BIM* and β-actin RNA expression in pre-treatment tumors from 24 patients with *EGFR*-mutant lung cancer. The response rate to EGFR-TKIs was 44% in patients with low BIM expression and 77% in those with high *BIM* expression, although the difference was not significant. Recent data from the European Tarceva (EURTAC) trial showed that PFS and overall survival (OS) were shorter in patients with low/intermediate *BIM* mRNA levels in primary tumors than in those with high mRNA levels (PFS: 7.2 *vs*. 12.9 months, p=0.0003; OS: 22.1 *vs*. 28.6 months, p=0.0364) (28).

Ng *et al*. (29) reported a common intronic deletion polymorphism in the gene encoding *BIM*. This polymorphism switched *BIM* splicing from exon 4 to exon 3, which resulted in increased expression of *BIM* RNA isoforms lacking the proapoptotic BCL2-homology domain 3 (BH3), such as *BIM-γ*. The *BIM* isoforms with a BH3 domain were *BIM-EL*, *L*, and *S*. This *BIM* deletion polymorphism was absent in individuals from African and European populations but was present in 12% of an Asian population (29). After EGFR-TKI treatment, PFS was significantly shorter in patients with *BIM* deletion polymorphism than in those without this polymorphism, which suggests that reduced expression of *BIM* with a BH3 domain is associated with unfavorable response to EGFR-TKIs (29-33). However, few studies have examined the association between *BIM* polymorphism and expression of *BIM* RNA isoforms such as *BIM-EL*, *L*, *S*, and *γ*.

The present study investigated the association between BIM polymorphism and expression of the *BIM* RNA isoforms *BIM-γ* and *BIM-EL/L/S* in lung tissue from patients with *EGFR*-positive NSCLC.

Patients and Methods

Clinical samples. We studied 33 patients with *EGFR* mutation-positive NSCLC who were treated with EGFR-TKIs during the period from January 2008 to January 2016. *BIM* isoform and *BIM* deletion polymorphism were investigated by real-time PCR analysis of 33 formalin-fixed paraffin-embedded (FFPE) slides of surgical specimens of lung tissue.

Detection of BIM deletion polymorphism. To identify *BIM* deletion polymorphism, we performed 2 types of PCR analysis, using the method of Ng *et al*. (22). In brief, we used a single primer set that contained the deletion area in intron 2, as well as 2 separate primer sets designed for wild-type and deletion alleles. The DNA was subjected to PCR amplification using primers designed to detect the deletion site (2,903 bp) in intron 2 of the *BCL2L11* gene. The resulting PCR products from the deletion (1,285 bp) and wild-type (4,188 bp) alleles were analyzed on agarose gels. In addition, the PCR products for the deletion (177 bp) and wild-type (174 bp) alleles were analyzed on agarose gels (30).

Detection of BIM-EL/L/S and BIM-γ. An miRNeasy FFPE Kit (Qiagen KK, Tokyo, Japan) was used to extract total RNA (including miRNA) from the FFPE sections of tumor tissue and non-tumor tissue. The extracted RNA was stored at −80˚C until use. cDNA was synthesized using PrimeScriptRT MasterMix (PerfectRealTime, Takara Bio Inc., Otsu, Japan). Quantitative real-time PCR was performed in a Thermal Cycler Dice Real Time System TP800 (Takara Bio Inc.), using SYBR Premix Ex Taq II (Tli RNaseH Plus, Takara Bio Inc.).

Quantification of BIM, BIM-EL/L/S, and BIM-γ. The quantitative real-time PCR primers (forward and reverse) used Perfect Real Time Primer (Takara Bio Inc.). To correct for differences in quality and quantity between samples, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The targets were obtained from the same mRNA preparations. Relative expression of *BIM-EL/L/S* and *BIM-γ* in mRNA from tissue sections inside and around tumors, as normalized to the reference gene (*GAPDH* mRNA), was calculated by using the KCL22 cell line for calibration.

Clinical outcomes. We retrospectively analyzed the clinical characteristics, response rate (RR), disease control rate (DCR), and toxicity of gefitinib in patients with and without BIM-γ. We then estimated PFS and overall survival (OS) in the same groups. The PFS of patients treated with EGFR-TKI was assessed from the date gefitinib therapy started to the first sign of disease progression, as determined by computed tomographic or magnetic resonance imaging, according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. OS was defined as the interval from the date of diagnosis until death from any cause.

Statistical analysis. Statistical analyses were conducted using the SPSS software for Windows, version 12.0 (SPSS Inc., Tokyo, Japan). Differences in relative expressions of *BIM*, *BIM-EL/L/S*, and *BIM-γ* between patients with and without *BIM-γ* were compared with the Wilcoxon rank sum test. Differences in clinical characteristics, RR, and DCR, frequency of *BIM* deletion polymorphism, and *BIM-γ* expression between patients with and without *BIM-γ* were compared using the Fisher exact test. Survival curves were drawn by the Kaplan–Meier method, and statistical analysis was performed using the log-rank test. A p-value of less than 5% was considered statistically significant.

This single-center study was conducted at Toho University Omori Medical Center (Tokyo, Japan) and was approved by its Human Genome/Gene Analysis Research Ethical Committee (authorization number, 24-1).

Results

BIM deletion polymorphism in EGFR-positive NSCLC. We analyzed *BIM* deletion polymorphism in 33 patients with *EGFR* mutation-positive NSCLC who were treated with gefitinib. *BIM* deletion polymorphism was present in 4 of the 33 patients (12.1%); heterozygous deletion was noted in all 4 patients (Table I).

Table I. *Patient characteristics (N=33).*

Age (years) range	25-82
Mean	64.7
Gender	
Male	26
Female	7
ECOG Performance status	
0	21
1	10
2	2
Histological pattern	
Ad	33
Clinical stage	
Rec	33
EGFR mutation at primary site	
19del	16
L858R	15
G719C	2
BIM deletion polymorphism	
Yes	4
No	29
Line of gefitinib therapy	
First	16
Second	16
Third	1

ECOG: Eastern Cooperative Oncology Group; Rec: recurrence after surgical resection; Ad: adenocarcinoma; *EGFR*: epidermal growth factor receptor; L858R: exon 21 L858R; 19del: exon 19 deletion; G719C: exon 18 G719C.

Table II. *Clinical response and adverse events after EGFR-TKI therapy (N=33).*

	Patients with *BIM-γ* (N=8)	Patients without *BIM-γ* (N=25)	*p*-Value
Clinical response (%)			
RR	62.5	52	0.60
DCR	100	92	0.41
All adverse events (%)			
Rash	50.0	32.0	0.35
Diarrhea	37.5	24.0	0.45
AST/ALT	0	8.0	0.30
Appetite loss	37.5	16.0	0.20
Pneumonitis	0	12.0	0.30
CTC Grade 3-5 (%)			
Rash	12.5	8.0	0.69
Diarrhea	0	8.0	0.41
AST/ALT	0	4.0	0.56
Appetite loss	0	0	-
Pneumonitis	0	8.0	0.30

RR: Response rate, DCR: disease control rate, CTC: National Cancer Institute Common Terminology Criteria.

Table III. *Association of BIM-EL/L/S and BIM-γ mRNA expression (N=33).*

	BIM-EL/L/S			
	Inside tumor	Around tumor	Both sites	None
BIM-γ				
Inside tumor	3	0	2	0
Around tumor	1	0	1	1
Both sites	0	0	0	0
None	8	3	6	8

Clinical characteristics of patients with and without BIM deletion polymorphism. There was no significant difference in RR, DCR, or incidences of adverse events between patients with (n=8) and without (n=25) *BIM-γ* (Table II).

Association of BIM-EL/L/S and BIM-γ expression. Expression of BIM-EL/L/S mRNA was detected inside the tumor in 12 patients, around the tumor in 3 patients, and at both sites in 9 patients; 9 patients had no such expression. Expression of *BIM-γ* mRNA was detected inside the tumor in 5 patients and around the tumor in 3 patients; 25 patients had no such expression. There was no association between *BIM-EL/L/S* and *BIM-γ* expression (Table III). Relative expression was significantly higher for *BIM-γ* than for *BIM-EL/L/S* (276±163.6 *vs*. 12±15.1, p=0.0018) (Figure 1).

Association of BIM deletion polymorphism and BIM-EL/L/S expression. We compared *BIM-EL/L/S* expression in relation to the frequency of *BIM* polymorphism inside and/or around tumors. There was no significant difference in *BIM-EL/L/S* expression in any comparison (Table IV).

Association of BIM deletion polymorphism and BIM-γ expression. We compared the frequency of *BIM* deletion polymorphism and *BIM-γ* expression inside and/or around tumors. *BIM-γ* expression was significantly more frequent in patients with *BIM* deletion polymorphism than in those without *BIM* polymorphism inside tumors (p=0.038) and around tumors (p=0.0024). Absence of *BIM-γ* expression was significantly more frequent in patients without *BIM* polymorphism than in those with *BIM* polymorphism (p=0.00016) (Table V). Relative *BIM-γ* expression was significantly higher in patients with *BIM* deletion polymorphism than in those without *BIM* deletion polymorphism (p=0.0017; Figure 2).

Survival and indicators of shorter PFS. We estimated PFS and OS in patients with and without *BIM-γ*. Patients with *BIM-γ* had significantly shorter PFS than those without *BIM-γ* (median: 304 *vs*. 732 days; p=0.023; Figure 3). There was no significant difference in OS (median: 1,345 *vs*. 1,552 days, p=0.24; Figure 4).

Table IV. *Association of BIM deletion polymorphism and BIM-EL/L/S expression (N=33).*

	BIM polymorphism		*p*-Value
	Positive (N=4)	Negative (N=29)	
BIM-EL/L/S			
Inside tumor	2	10	0.55
Around tumor	1	2	0.23
Both sites	0	9	0.19
None	1	8	0.91

Table V. *Association of BIM deletion polymorphism and BIM-γ expression (n=33).*

	BIM polymorphism		*p*-Value
	Positive (n=4)	Negative (n=29)	
BIM-γ			
Inside tumor	2	3	0.038
Around tumor	2	1	0.0024
Both sites	0	0	-
None	0	25	0.00016

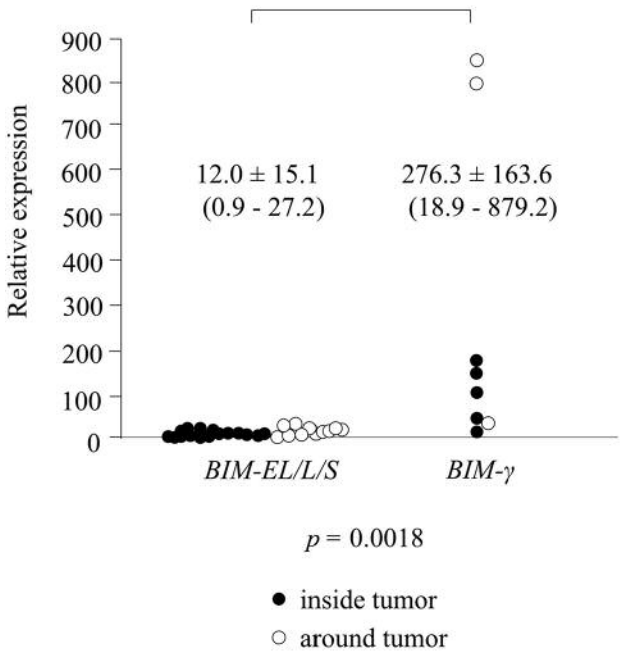

Figure 1. *Relative expression was significantly higher for BIM-γ than for BIM-EL/L/S (276.3±163.6 vs. 12.0±15.1, p=0.0018).*

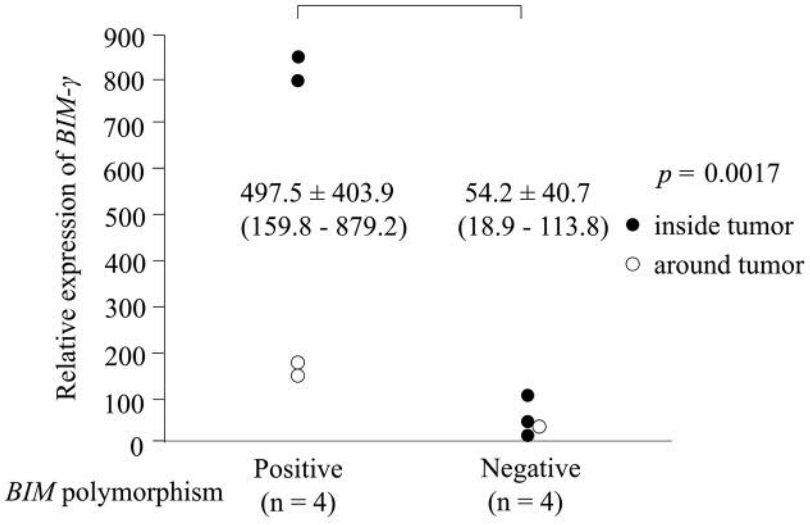

Figure 2. *Frequency of BIM-γ expression was significantly higher in patients with BIM polymorphism than in those without BIM polymorphism (p=0.0017).*

Discussion

The *BIM* deletion polymorphism is located in intron 2 of the *BIM* gene and results in expression of *BIM* isoforms lacking the BH3 domain, such as *BIM-γ*. However, we detected both mRNA *BIM-γ* and *BIM-EL/L/S* expression in and around tumors in patients with and without *BIM* deletion polymorphism. We found no association between *BIM-EL/L/S* and *BIM-γ* expression, regardless of the status of BIM deletion polymorphism. Furthermore, relative expression was

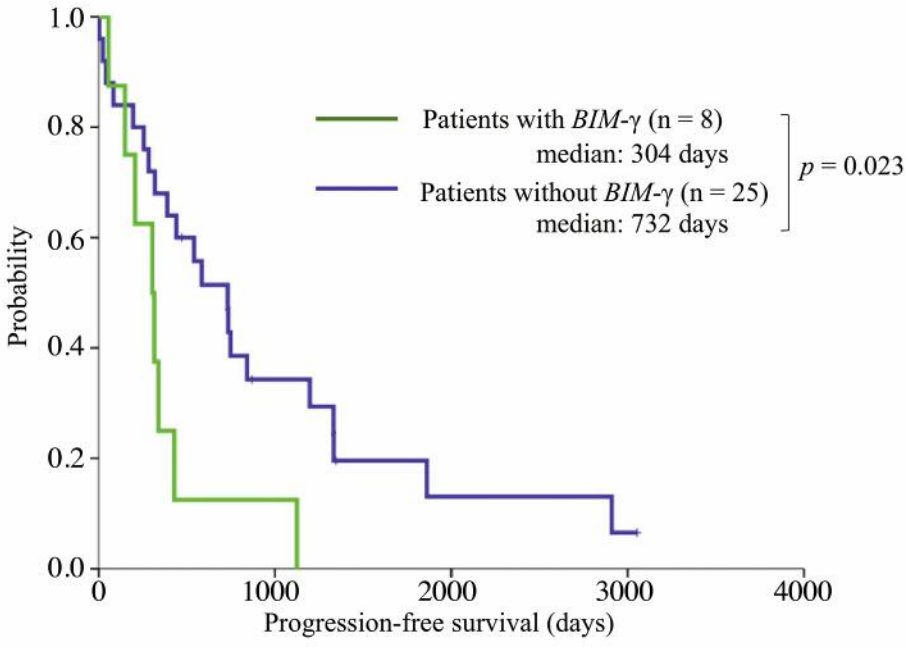

Figure 3. *Kaplan-Meier curves for progression-free survival. Patients with BIM-γ had significantly shorter progression-free survival than those without BIM-γ (median: 304 vs. 732 days; p=0.023).*

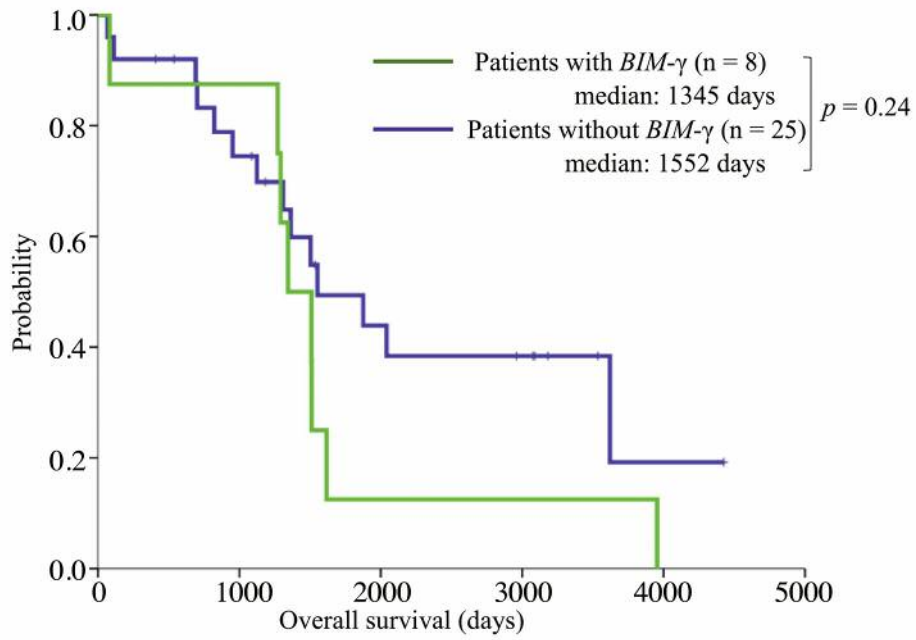

Figure 4. *Kaplan-Meier curves for overall survival. Overall survival did not significantly differ between patients with and without BIM-γ (median: 1,345 vs. 1,552 days; p=0.24).*

significantly higher for *BIM-γ* than for *BIM-EL/L/S* (276±163.6 *vs.* 12±15.1, p=0.0018). Faber *et al.* (24) reported that BIM levels were important in determining response to targeted therapies in patients with solid tumors. This finding is consistent with research showing that cancer cells are sensitive to small changes in *BIM* protein concentration.

BIM-γ, a *BIM* isoform that lacks the BH3 domain, is up-regulated in most prostate cancer cell lines (34). *BIM-γ* inhibits clonal growth in prostate cancer and promotes apoptosis. Interestingly, *BIM-γ* was found in 13.7% (4 out of 29) of the present specimens without *BIM* deletion polymorphism. Relative *BIM-γ* expression in patients without *BIM* polymorphism was significantly lower than in those with *BIM* deletion polymorphism (p=0.0017). This suggests that, among the *BIM* isoforms, overexpression of *BIM-γ* suppresses TKI-related apoptosis. Further study of the mechanism of *BIM-γ* expression is warranted.

One hypothesis is that *BIM* deletion polymorphism itself results in relative resistance to EGFR-TKIs. Kuroda *et al*. (35) showed that cancer cells were sensitive to small changes in *BIM* protein concentrations and that *BIM* protein concentration had a dose-dependent effect on apoptosis and the degree of TKI resistance (35). We compared the frequency of *BIM* deletion polymorphism and *BIM-γ* inside and/or around tumors. Patients with *BIM* deletion polymorphism had significantly higher *BIM-γ* expression inside tumors (p=0.038) and around tumors (p=0.0024) than those without *BIM* deletion polymorphism. Absence of *BIM-γ* expression was significantly more frequent in patients without BIM polymorphism than among those with BIM polymorphism (p=0.00016). These findings suggest a strong association between an imbalance in *BIM* isoforms and *BIM* deletion polymorphism.

Clinical characteristics, response to EGFR-TKIs, and incidences of adverse events due to EGFR-TKI did not significantly differ among patients with and without *BIM-γ*. Thus, clinical characteristics are not sufficient to identify patients with and without *BIM-γ*. However, our analysis of PFS and OS in patients with and without *BIM-γ* showed that PFS was significantly shorter in patients with *BIM-γ* than in those without *BIM-γ* (median: 304 *vs*. 732 days; p=0.023). Future studies should attempt to clarify the association between *BIM-γ* and PFS in patients receiving gefitinib.

The major limitation of this study is that it was a retrospective single-center study with a small sample size. A large-scale multicenter study is thus needed in order to statistically confirm the validity of our results. Clinical application of our results would require a prospective study of patients receiving gefitinib for *EGFR* mutation-positive NSCLC with or without *BIM-γ* overexpression. Bean *et al*. (36) reported that *BIM* act as sentinels that interconnect kinase signaling networks and the mitochondria-dependent apoptotic program. Karachaliou *et al*. (37) reported that *BIM* and *mTOR* mRNA expression levels predict the outcome of erlotinib therapy in *EGFR*-mutant NSCLC. Future studies should examine the associations of *BIM-γ* with *PUMA*, *mTOR*, and other apoptosis markers.

In conclusion, the present study is the first to show that *BIM-γ* expression was strongly associated with *BIM* deletion polymorphism and that *BIM-γ* overexpression was associated with TKI-related apoptosis. These findings may be useful in developing treatment strategies for patients receiving EGFR-TKIs for *EGFR* mutation-positive NSCLC.

Conflicts of Interest

The Authors declare no conflicts of interest.

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