

# Clinical Significance of *BIM* Deletion Polymorphism in Non–Small-Cell Lung Cancer with Epidermal Growth Factor Receptor Mutation

Kazutoshi Isobe, MD, PhD,\* Yoshinobu Hata, MD, PhD,† Naobumi Tochigi, MD, PhD,‡ Kyohei Kaburaki, MD,\* Hiroshi Kobayashi, MD,\* Takashi Makino, MD,† Hajime Otsuka, MD,† Fumitomo Sato, MD,† Fumiaki Ishida, MD,\* Naoshi Kikuchi, MD,\* Nao Hirota, MD,\* Keita Sato, MD,\* Go Sano, MD,\* Keishi Sugino, MD, PhD,\* Susumu Sakamoto, MD, PhD,\* Yujiro Takai, MD, PhD,\* Kazutoshi Shibuya, MD, PhD,‡ Akira Iyoda, MD, PhD,† and Sakae Homma, MD, PhD\*

**Background:** Germline alterations in the proapoptotic protein Bcl-2–like 11 (*BIM*) can have a crucial role in tumor response to treatment. To determine the clinical utility of detecting *BIM* deletion polymorphism in non–small-cell lung cancer positive for epidermal growth factor receptor (*EGFR*) mutation, we examined outcomes of patients with and without *BIM* alterations.

**Methods:** We studied 70 patients with *EGFR* mutation-positive non–small-cell lung cancer who were treated with an EGFR tyrosine kinase inhibitor between January 2008 and January 2013. *BIM* deletion was analyzed by polymerase chain reaction in 58 samples of peripheral blood and 24 formalin-fixed paraffin-embedded slides of surgical specimens (20 of lung tissue and four of brain tissue); both blood and tissue specimens were available for 12 patients. We retrospectively analyzed clinical characteristics, response rate, toxicity, and outcomes among patients with and without *BIM* deletion.

**Results:** *BIM* deletion was present in 13 of 70 patients (18.6%). There were no significant differences between patients with and without *BIM* deletion in clinical characteristics, rate of response to EGFR tyrosine kinase inhibitor, or incidence of adverse events. Patients with *BIM* deletion had significantly shorter progression-free survival (PFS) than those without *BIM* deletion (median, 227 versus 533 days;  $p < 0.001$ ). Multivariate Cox regression analysis showed that *BIM* deletion was an independent indicator of shorter PFS (hazard ratio, 3.99; 95% confidence interval, 1.864–8.547;  $p < 0.001$ ).

**Conclusions:** Polymerase chain reaction successfully detected *BIM* deletion in samples of peripheral blood and formalin-fixed paraffin-embedded slides of surgical specimens. *BIM* deletion was the most important independent prognostic factor in shorter PFS.

Departments of \*Respiratory Medicine, †Chest Surgery, and ‡Surgical Pathology, Toho University Omori Medical Center, Tokyo, Japan.

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Address for correspondence: Kazutoshi Isobe, MD, PhD, Department of Respiratory Medicine, Toho University Omori Medical Center, 6-11-1 Omori-Nishi, Ota-ku, Tokyo 143-8541, Japan. E-mail: kazutoshiisobe@aol.com

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An activating mutation of the epidermal growth factor receptor (*EGFR*) gene is a promising target in the treatment of non–small-cell lung cancer (NSCLC).<sup>1,2</sup> The frequency of *EGFR* mutations depends on the population studied. In North America and Western Europe, approximately 10% of patients with NSCLC harbor mutations, whereas in East Asia approximately 30% have *EGFR* mutations.<sup>3,4</sup> EGFR tyrosine kinase inhibitors (EGFR-TKI) such as gefitinib and erlotinib are recommended for treating *EGFR* mutation-positive NSCLC.<sup>5,6</sup> NSCLC patients with such mutations who were treated with EGFR-TKI as first-line therapy had longer progression-free survival (PFS) than did those who received platinum-based chemotherapy.<sup>7–10</sup> Therefore, detection of *EGFR* mutations in patients with metastatic NSCLC is important in selecting individualized therapies.

However, most patients develop a recurrence within 10 to 16 months after initial EGFR-TKI treatment.<sup>11</sup> Approximately 50% of patients with acquired resistance to EGFR-TKI were found to have the *EGFR* T790M mutation.<sup>12,13</sup> Other reported mechanisms responsible for acquired resistance are *MET* amplification, in 5% to 10% of cases,<sup>14,15</sup> and small-cell cancer transformation, in less than 5% of cases.<sup>16</sup> However, the mechanisms responsible for acquired EGFR-TKI resistance are not known in approximately 30% to 40% of patients.<sup>11</sup>

Bcl-2–like 11 (*BIM*) is a proapoptotic member of the B-cell CLL/Lymphoma 2 (Bcl-2) family of proteins<sup>17,18</sup> and has emerged as a key modulator of apoptosis triggered by EGFR-TKI.<sup>19,20</sup> Low expression levels of *BIM* in primary tumors are reported to be associated with shorter PFS in patients treated with EGFR-TKI.<sup>21</sup> Recently, Ng et al.<sup>22</sup> 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* isoforms lacking the proapoptotic Bcl-2-homology

domain 3 (BH3). After TKI exposure, cells with the *BIM* deletion polymorphism showed decreased induction of exon-4-containing transcripts and, consequently, impaired BH3-domain-dependent apoptosis. This germline alteration could have a crucial role in determining how a tumor responds to treatment. However, few studies have examined the clinical usefulness of detecting *BIM* deletion polymorphism or the clinical characteristics of *EGFR* mutation-positive NSCLC.

To determine the clinical utility of detecting *BIM* deletion polymorphism in patients with *EGFR* mutation-positive NSCLC, we examined the outcomes of patients with and without *BIM* alterations.

## PATIENTS AND METHODS

### Polymerase Chain Reaction

To detect *BIM* deletion polymorphism, we performed two types of polymerase chain reaction (PCR) analysis, according to the method of Ng et al.<sup>22</sup> In brief, we used a single primer set that contains the deletion area in intron 2 and two separate primer sets designed for wild-type and deletion alleles. The DNA was subjected to PCR amplification using primers designed to detect deletion site (2903 bp) in intron 2 of the *BCL2L1* gene. The resulting PCR products from the deletion (1285 bp) and wild-type (4188 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 gel. We analyzed 20 cell lines, including the KCL-22 cell (which was reported to have the *BIM* deletion),<sup>22</sup> and 30 DNA samples from healthy Japanese volunteers.

### Clinical Samples

We studied 70 patients with *EGFR* mutation-positive NSCLC who were treated with EGFR-TKI during the period from January 2008 to January 2013. *BIM* deletion polymorphism was analyzed by PCR in 58 samples of peripheral blood (cell-free DNA in 34, leukocyte DNA in 35) and on 24 formalin-fixed paraffin-embedded (FFPE) slides of surgical specimens (20 specimens of lung tissue and four of brain tissue); both blood and tissue specimens were available for 12 patients. To confirm the validity of PCR analysis of two types of samples, we compared the results for *BIM* deletion polymorphism identified in lung tissue on FFPE slides with those from peripheral blood (cell-free DNA or leukocyte DNA) from the same patients ( $n = 12$ ). DNA was extracted from FFPE slides using the QIAamp FFPE Tissue Kit (QIAGEN KK, Tokyo, Japan). DNA extraction blood samples were diluted in lysis solution to lyse the red cells and the white cell fraction was pelleted and washed once in phosphate-buffered saline. DNA was extracted from the white cell pellets using the QIAamp DNA mini Kit (QIAGEN KK, Tokyo, Japan).

### Clinical Outcomes

We retrospectively analyzed the clinical characteristics, response rate (RR), disease control rate (DCR), and toxicity of EGFR-TKI in patients with and without *BIM* deletion polymorphism. Toxicity was assessed according to National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.

We estimated PFS and overall survival (OS) in patients with and without *BIM* deletion polymorphism. The PFS of patients treated with EGFR-TKI was assessed from the date EGFR-TKI therapy was started to the earliest sign of disease progression as determined by findings from computed tomography or magnetic resonance imaging, according to the Response Evaluation Criteria in Solid Tumors. OS was defined as the period from the date of diagnosis until death from any cause.

### Statistical Analysis

Statistical analyses were conducted using SPSS software for Windows, version 12.0 (SPSS, Tokyo, Japan). Differences in clinical characteristics, RR, DCR, and adverse events between patients with and without *BIM* deletion polymorphism were compared using Fisher's exact test. Survival curves were drawn by the Kaplan-Meier method, and statistical analysis was performed using the log-rank test.

We used univariate analysis and multivariate Cox regression analysis to identify factors associated with shorter PFS. The investigated prognostic factors were age, sex (male versus female), performance status (2 versus 1 versus 0), brain metastasis (yes versus no), bone metastasis (yes versus no), pulmonary metastasis (yes versus no), liver metastasis (yes versus no), lymph node metastasis (yes versus no), *EGFR* mutation (major mutations [L858R and exon 19 deletion] versus minor mutations [other mutations]), EGFR-TKI response (partial response versus stable disease), smoking history (pack-years), and *BIM* deletion (yes versus no).

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

### Detection of *BIM* Deletion in Cell Lines and Healthy Volunteers

Using the two types of PCR analysis, we analyzed 20 cell lines and 30 DNA samples from healthy Japanese volunteers. Among the 20 cell lines, only KCL-22 showed *BIM* deletion. As for DNA samples, *BIM* deletion polymorphism was present in six of the 30 (20%) healthy volunteers. There was no discordance between the two types of PCR analysis.

**TABLE 1.** Presence of *BIM* Deletion in Patients with *EGFR* Mutation-Positive NSCLC ( $n = 70$ )

Patients with <i>BIM</i> Deletion		Patients without <i>BIM</i> Deletion	Frequency of <i>BIM</i> Deletion
Heterozygous Deletion	Homozygous Deletion		
12	1	57	18.6%

## Validation between Blood Samples and FFPE Slides

We confirmed the validity between blood samples (leukocyte DNA in 12 and cell-free DNA in four) and FFPE slides of surgical specimens (lung tissue in 12): *BIM* deletion was detected in three of 12 patients (25%). There was no discordance between the two sample types.

## Detection of *BIM* Deletion on EGFR-Positive NSCLC

We analyzed *BIM* deletion polymorphism in 70 patients with *EGFR* mutation-positive NSCLC who were treated with EGFR-TKI. *BIM* deletion polymorphism was present in 13 of the 70 patients (18.6%); homozygous deletion was noted in one and heterozygous deletion in 12. For

**TABLE 2.** Patient Characteristics (*n* = 70)

	Patients with <i>BIM</i> Deletion ( <i>n</i> = 13)	Patients without <i>BIM</i> Deletion ( <i>n</i> = 57)	<i>p</i>
Age	63.8±6.7	65.4±14.1	0.64
Sex male/female	4/9	15/42	0.74
PS 0/1/2	7/3/3	31/21/5	0.29
Pathological type			
Ad/Sq	13/0	50/7	0.18
Clinical stage			
IV/Rec	7/6	27/30	0.67
Smoking history			
Current/former/never	1/3/9	2/14/41	0.79
Pack-years	11.1±17.5	10.5±24.5	0.90
<i>EGFR</i> mutation			
19Del/L858R/Other	6/7/0	27/28/2	0.78
First EGFR-TKI			
Gefitinib/erlotinib	13/0	52/5	0.26
Cytotoxic chemotherapy			
0/1/2/3/≥4 regimen(s)	4/1/2/3/3	14/16/6/8/13	0.60
Mean ± SD	2±1.63	2.1±1.97	0.17
Chemotherapy after TKI (+/-)			
Platinum doublet	2/11	10/47	0.82
Single agent	7/6	21/36	0.25
TKI rechallenge	2/11	15/42	0.40
Site of metastasis (+/-)			
Brain	6/7	22/35	0.62
Bone	7/6	17/40	0.09
Liver	2/11	2/55	0.09
Pulmonary	6/7	17/40	0.26
Lymph nodes	4/9	10/47	0.28
No. of metastases (+/-)			
Organs ≥ 2	7/6	30/27	0.93
Organs ≥ 3	2/11	7/50	0.76

PS, performance status; *EGFR*, epidermal growth factor receptor; 19Del, exon 19 deletion; L858R, exon 21 L858R; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; Ad, adenocarcinoma; Sq, squamous cell carcinoma; Rec, recurrence after surgical resection.

the one case of homozygous deletion, PCR analysis using the primer set for the wild-type allele showed no amplification (Table 1).

## Comparison between Patients with and without *BIM* Deletion Polymorphism

There were no significant differences in the clinical characteristics, RR, DCR, or incidence of adverse events between patients with (*n* = 13) and without (*n* = 57) *BIM* deletion polymorphism (Tables 2 and 3).

## Survival and Indicators of Shorter PFS

We estimated PFS and OS in patients with and without *BIM* deletion polymorphism. The patients with *BIM* deletion polymorphism had significantly shorter PFS than did those without *BIM* deletion polymorphism (median, 227 versus 533 days; *p* < 0.001; Fig. 1). There was no significant difference in OS (median, 1176 versus 1363 days; *p* = 0.27; Fig. 2). Multivariate Cox regression analysis showed that *BIM* deletion polymorphism was the strongest independent indicator of shorter PFS (hazard ratio [HR], 3.99; 95% confidence interval [CI], 1.864–8.547; *p* < 0.001; Table 4).

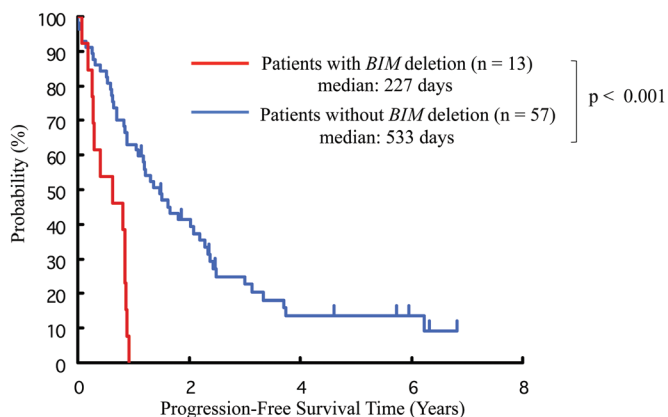
## DISCUSSION

*BIM* deletion polymorphism is a germline alteration that affects EGFR-TKI-related apoptosis.<sup>17,18</sup> In a study that screened 2597 healthy individuals, *BIM* deletion polymorphism was present in 12.3% of East Asians but absent in Africans and Europeans.<sup>22</sup> In the present study, *BIM* deletion polymorphism was present in 13 of 70 Japanese patients (18.6%) with *EGFR* mutation-positive NSCLC and in six of 30 healthy Japanese volunteers (20%), a statistically insignificant difference. The

**TABLE 3.** Comparison of Clinical Response and Adverse Events after EGFR-TKI Therapy

	Patients with <i>BIM</i> Deletion ( <i>n</i> = 13)	Patients without <i>BIM</i> Deletion ( <i>n</i> = 57)	<i>p</i>
Clinical response (%)			
RR	61.5	64.9	0.81
DCR	100	91.2	0.26
All adverse events (%)			
Rash	61.5	47.3	0.36
Diarrhea	38.5	22.8	0.24
AST/ALT	0	8.8	0.27
Appetite loss	15.3	14.0	0.90
Pneumonitis	0	14.0	0.15
CTC grade 3–5 (%)			
Rash	7.7	3.5	0.49
Diarrhea	0	5.3	0.39
AST/ALT	0	3.5	0.49
Appetite loss	0	1.8	0.63
Pneumonitis	0	7.0	0.32

RR, response rate; DCR, disease control rate; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CTC, National Cancer Institute Common Terminology Criteria.

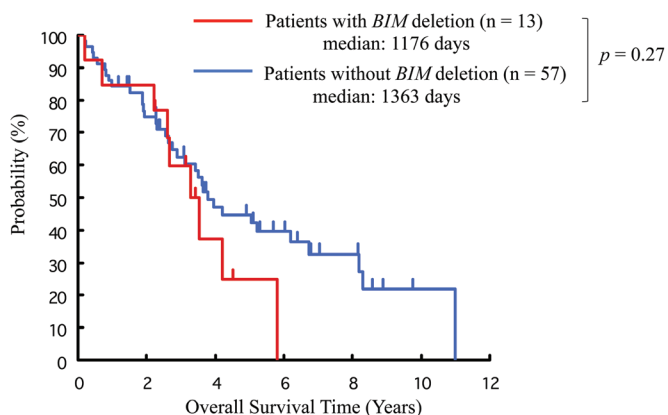


**FIGURE 1.** Patients with *BIM* deletion polymorphism had significantly shorter progression-free survival than did those without *BIM* deletion polymorphism (median, 227 versus 533 days;  $p < 0.001$ ).

overall frequency of *BIM* deletion polymorphism in our study (19%,  $n = 100$ ) was higher than that noted in a previous report.<sup>22</sup>

Tagawa et al.<sup>23</sup> reported homozygous *BIM* deletions in patients with mantle-cell lymphoma, and homozygous *BIM* deletion was found in 0.5% of East Asians.<sup>24</sup> Among the present 70 Japanese patients with NSCLC, one (1.4%) had homozygous deletion and 12 had heterozygous deletion. Future studies should investigate the characteristics of patients with homozygous *BIM* deletion polymorphism to determine if this genotype results in worse clinical outcomes when compared with heterozygous *BIM* deletion.

There were no significant differences between clinical characteristics, response to EGFR-TKI, or incidences of adverse events due to EGFR-TKI among patients with and without *BIM* deletion polymorphism. Thus, it is difficult to distinguish between patients with and without *BIM* deletion polymorphism on the basis of clinical characteristics alone. No patient with *BIM* deletion developed EGFR-TKI-related pneumonitis. *BIM* knockdown was reported to prevent FOXO3 (i.e., FKHL1, a member of the forkhead transcription factor subfamily)-mediated overproduction of reactive



**FIGURE 2.** There was no significant difference in overall survival between patients with and without *BIM* deletion polymorphism (median, 1176 versus 1363 days;  $p = 0.27$ ).

**TABLE 4.** Indicators of Shorter PFS after EGFR-TKI Treatment

Parameter	HR	95% CI	<i>p</i>
Univariate Cox regression analysis			
Sex (male vs. female)	1.50	1.022–3.413	0.04
Bone metastasis (yes vs. no)	2.11	1.187–3.755	0.007
Smoking history (pack-years)	1.012	1.002–1.022	0.017
<i>BIM</i> deletion	4.03	1.944–8.340	<0.001
Multivariate Cox regression analysis			
<i>BIM</i> deletion	3.99	1.864–8.547	<0.001

HR, hazard ratio; CI, confidence interval.

oxygen species and apoptosis.<sup>25</sup> *BIM* deletion polymorphism might affect EGFR-TKI-related lung injury by preventing overproduction of reactive oxygen species. Further studies are needed to clarify the relationship between EGFR-TKI-related pneumonitis and *BIM*.

*BIM* deletion polymorphism, a germline alteration, is thought to be associated with intrinsic resistance to EGFR-TKI and would likely result in primary resistance and no response to treatment. However, the present clinical outcomes were probably due to acquired resistance: when compared with patients without *BIM* polymorphism, those with *BIM* deletion polymorphism had similar RRs and DCRs but shorter PFS. The reasons for these findings remain to be investigated. It has been hypothesized that EGFR-TKI-induced apoptosis does not completely depend on the *BIM* pathway and that tumor response to EGFR-TKI in patients with *BIM* deletion might depend on other proapoptotic regulators, which could have less-prolonged clinical activity than those of the *BIM* pathway. A second hypothesis is that *BIM* deletion polymorphism itself results in relative resistance to EGFR-TKI. Kuroda et al.<sup>26</sup> showed that cancer cells were sensitive to small changes in *BIM* protein concentrations and that *BIM* protein concentration exerted a dose-dependent effect on apoptosis and the degree of TKI resistance.<sup>26</sup> In a report by Faber et al.,<sup>21</sup> PFS was shorter (4.7 versus 13.7 mo,  $p = 0.007$ ) among patients with low *BIM* RNA expression, which appeared to correlate with high *BIM* protein expression on immunohistochemistry. The RR after EGFR-TKI was worse among patients with low *BIM* RNA expression (44%) than among those with high *BIM* RNA expression (77%), although the difference was not statistically significant. Patients with *BIM* deletion polymorphism could be regarded as “carriers” that have varied *BIM* expression and clinical responses that are modulated by genetic or epigenetic interactions, a possibility that warrants further study. Although cells with *BIM* deletion polymorphism show decreased induction of exon-4-containing transcripts after TKI exposure,<sup>22</sup> the response after prolonged TKI exposure should be investigated.

Ng et al.<sup>22</sup> reported that patients with *BIM* deletion polymorphism had significantly shorter PFS than did patients without *BIM* deletion polymorphism after EGFR-TKI treatment (6.6 versus 11.9 mo,  $p = 0.0027$ ), but they did not report RR or OS. Our present study in a Japanese population yielded similar

results: *BIM* deletion polymorphism was an independent indicator of shorter PFS. However, there was no significant difference in OS among patients with and without *BIM* deletion polymorphism. Multivariate Cox regression analysis showed that indicators of shorter OS were EGFR-TKI-related pneumonitis (HR, 3.52; 95% CI, 1.190–3.860;  $p = 0.023$ ), brain metastasis (HR, 2.14; 95% CI, 1.099–4.165;  $p = 0.025$ ), and smoking history (HR, 1.001; 95% CI, 1.000–1.001;  $p = 0.026$ ). EGFR-TKI-related pneumonitis developed only in patients without *BIM* deletion polymorphism ( $n = 8$ , 14%) but has a detrimental effect on chemotherapy given after pneumonitis. Thus, EGFR-TKI-related pneumonitis might have reduced OS among the present patients without *BIM* deletion, which possibly explains the lack of a significant difference in OS between patients with and without *BIM* deletion polymorphism in the present study.

BH3-mimetic drugs<sup>22</sup> and histone deacetylase inhibitors<sup>24</sup> may be able to surmount *BIM*-associated resistance to EGFR-TKI. Our findings suggest that although there was no significant difference in RR or OS among patients with and without *BIM* deletion polymorphism, the addition of these drugs might prolong PFS. However, this study was a retrospective study at a single center. A prospective multicenter study should be conducted to investigate the clinical significance of *BIM* deletion polymorphism on EGFR-TKI therapy. In addition, EGFR-TKI-related pneumonitis should be considered in any randomized prospective study of the clinical benefit of BH3-mimetic drugs or histone deacetylase inhibitors for patients with *BIM* deletion polymorphism.

In conclusion, *BIM* deletion polymorphism, a germline alteration, was successfully detected by PCR analysis of samples of peripheral blood and FFPE slides of surgical specimens, thus providing a minimally invasive and convenient detection method. *BIM* deletion polymorphism was the strongest indicator of shorter PFS among patients with EGFR mutation-positive NSCLC treated with EGFR-TKI. Our results indicate that new treatment strategies should be established for patients with *BIM* deletion polymorphism.

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