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Phase I study of vorinostat with gefitinib in BIM deletion polymorphism/epidermal growth factor receptor mutation double-positive lung cancer

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Abstract

Patients with epidermal growth factor receptor (*EGFR*)-mutated non-small cell lung cancer (NSCLC) harboring *BIM* deletion polymorphism (*BIM* deletion) have poor responses to EGFR TKI. Mechanistically, the *BIM* deletion induces preferential splicing of the non-functional exon 3-containing isoform over the functional exon 4-containing isoform, impairing TKI-induced, BIM-dependent apoptosis. Histone deacetylase inhibitor, vorinostat, resensitizes *BIM* deletion-containing NSCLC cells to EGFR-TKI. In the present study, we determined the safety of vorinostat-gefitinib combination and evaluated pharmacodynamic biomarkers of vorinostat activity. Patients with

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This study was registered with UMIN Clinical Trials Registry (UMIN00001519) and ClinicalTrials.gov (NCT02151721).

EGFR-mutated NSCLC with the *BIM* deletion, pretreated with EGFR-TKI and chemotherapy, were recruited. Vorinostat (200, 300, 400 mg) was given daily on days 1-7, and gefitinib 250 mg was given daily on days 1-14. Vorinostat doses were escalated based on a conventional 3 + 3 design. Pharmacodynamic markers were measured using PBMC collected at baseline and 4 hours after vorinostat dose on day 2 in cycle 1. No dose-limiting toxicities (DLT) were observed in 12 patients. We determined 400 mg vorinostat as the recommended phase II dose (RP2D). Median progression-free survival was 5.2 months (95% CI: 1.4-15.7). Disease control rate at 6 weeks was 83.3% (10/12). Vorinostat preferentially induced *BIM* mRNA-containing exon 4 over mRNA-containing exon 3, acetylated histone H3 protein, and proapoptotic BIM_{EL} protein in 11/11, 10/11, and 5/11 patients, respectively. These data indicate that RP2D was 400 mg vorinostat combined with gefitinib in *BIM* deletion/*EGFR* mutation double-positive NSCLC. *BIM* mRNA exon 3/exon 4 ratio in PBMC may be a useful pharmacodynamic marker for treatment.

KEYWORDS

BIM deletion polymorphism, EGFR-TKI, NSCLC, resistance, vorinostat

1 | INTRODUCTION

The majority of patients with non-small cell lung cancer (NSCLC) with epidermal growth factor receptor (*EGFR*)-activating mutations, such as exon 19 deletion and an L858R point mutation, show marked responses to EGFR-TKI, such as gefitinib, erlotinib, afatinib, and osimertinib.[1-5] However, 20%-30% of patients with *EGFR*-activating mutations show intrinsic resistance to EGFR-TKI. Molecular mechanisms of the intrinsic resistance, including a pre-existing EGFR-T790M resistance mutation-positive clone and the activation of alternative pathways, such as hepatocyte growth factor (HGF)/MET, have been discovered.[6]

Decreased activity of BIM, also known as Bcl-2-like protein 11, a proapoptotic molecule that belongs to the Bcl-2 family, has been recognized as an important mechanism mediating intrinsic resistance to EGFR-TKI. BIM upregulation is essential for the induction of apoptosis in lung cancer cells with *EGFR* mutations treated with first-generation EGFR-TKI, and low BIM protein levels are associated with resistance to EGFR-TKI.[7,8] Underlying the importance of BIM in EGFR-TKI resistance, a functional *BIM* deletion polymorphism, specifically, a 2903-bp deletion in intron 2, was discovered in East Asian individuals (13%-18%) and found to confer inferior responses to EGFR-TKI.[9,10] Subsequently, the *BIM* deletion was also found in South American patients with NSCLC (15.7%).[11] Mechanistically, the *BIM* deletion results in the mutually exclusive splicing of exon 3 over the BH3-encoding (proapoptotic) exon 4 in *BIM* pre-mRNA and leads to the production of an inactive BIM protein isoform (BIM_{γ}) lacking the BH3 domain. In turn, this reduces the expression of the proapoptotic BIM protein isoform (BIM_{EL}) in *EGFR*-mutant lung cancer cell lines following TKI exposure and is sufficient to confer TKI resistance.[9] Several meta-analyses have reported an association between the *BIM* deletion polymorphism and shorter progression-free survival (PFS) in patients with NSCLC harboring *EGFR* mutations who received either gefitinib or erlotinib treatment.[12-17] Therefore, restoration of BIM activity in patients with the *BIM* deletion may be an important strategy to overcome intrinsic resistance to EGFR-TKI in *EGFR*-mutated NSCLC.

Vorinostat (suberoylanilide hydroxamic acid [SAHA]), has been approved in 20 countries to date, including Japan, for cutaneous T-cell lymphoma as monotherapy. It is a small-molecule inhibitor of histone deacetylase (HDAC) that causes the acetylation of histone proteins, including histone H2, and induces cell differentiation, cell cycle arrest, and apoptosis in several types of tumor cells.[18] We previously reported that the combined use of vorinostat and gefitinib was able to preferentially induce transcription of the proapoptotic exon 4-containing *BIM* isoform over the inactive exon 3-containing isoform, thus resensitizing *BIM* deletion-containing *EGFR*-mutated NSCLC cell lines to TKI in vitro and in vivo.[19] Two clinical trials combining TKI and vorinostat have been conducted: a phase I/II study in patients with advanced NSCLC, regardless of the presence/absence of *EGFR* mutation in Korea,[20] and a phase I/II study in patients with advanced *EGFR*-mutated NSCLC after EGFR-TKI progression in Spain.[21] However, neither combination regimen showed significant efficacy in these populations, suggesting the need for improved patient selection and the development of pharmacodynamic biomarkers of vorinostat activity.

Based on our preclinical findings, we designed the present phase I study, named VICTORY-J "Vorinostat-Iressa Combined Therapy on Resistance by *BIM* Polymorphism in *EGFR* Mutant Lung Cancer", to evaluate the safety of combined therapy with vorinostat and gefitinib, and to determine the maximum tolerated dose

(MTD) and recommended phase II dose (RP2D) of vorinostat combined with a fixed dose of gefitinib for patients with *EGFR*-mutated NSCLC harboring the *BIM* deletion polymorphism.[22] In addition, we conducted pharmacodynamic analyses to identify biomarkers of vorinostat activity.

2 | MATERIALS AND METHODS

2.1 | Study design

This study was an open-label, multi-institutional phase I dose escalation study in patients with *EGFR*-mutated (exon 19 deletion and L858R mutation) NSCLC with a *BIM* deletion polymorphism. Primary endpoint was to determine MTD, which was defined as the highest dose level at which two or fewer of six patients experienced dose-limiting toxicity (DLT).

Three to six patients were enrolled at each dose level of vorinostat. With a fixed dose of gefitinib, dose escalation of vorinostat was used, in accordance with a conventional 3 + 3 design using an escalation scheme (Figure S1). Initially, three patients were enrolled at the first level. If one or two patients experienced DLT, an additional three patients were enrolled to that level. If three of six patients experienced DLT, the previous dose level was declared the MTD. If two or fewer of the six patients experienced DLT, dose escalation was permitted to continue. After termination of protocol treatment, the patients were allowed any further treatment and followed until death over a period of at least 1 year. This study was conducted in accordance with the International Committee for Harmonization Good Clinical Practice (ICH-GCP) guidelines and the Declaration of Helsinki. The study protocol was approved by the institutional review boards of all participating institutions. Written informed consent was provided by all patients before registration. This study was registered with UMIN Clinical Trials Registry (UMIN00001519) and ClinicalTrials.gov (NCT02151721).

2.2 | Patient eligibility

Prior to enrolment in the study, patients had to fulfil all of the following criteria: histologically or cytologically diagnosed NSCLC (excluding squamous cell carcinoma); NSCLC of clinicopathological stage IIIB or IV for which radical radiation therapy was impractical or there was a recurrence after surgery; *EGFR* mutations (deletion of exon 19 and L858R mutation of exon 21) for which the clinical benefits of an EGFR-TKI (gefitinib, erlotinib, or afatinib) were recognized by testing methods that were listed by the national health insurance; history of treatment with an EGFR-TKI (gefitinib, erlotinib, or afatinib) and a history of pathological deterioration during treatment; history of treatment with cytotoxic anticancer agents (not including pre- or postoperative chemotherapy in the previous 1 year or more from the day of final dose); confirmed *BIM* polymorphism by the PCR fragment analytical method and the sequence method at the central laboratory; a lesion measurable according to the RECIST guidelines version 1.1; 20 years of age and older; ECOG Performance Status (PS) 0 or 1; estimated life expectancy of 12 or more weeks; provision of written informed consent to participate in the present study; and adequate hematological, liver, renal, and respiratory function within 14 days before entry.

Patients were excluded for any of the following reasons: received a cytotoxic anticancer agent within the past 4 weeks, received an EGFR-TKI within the past 7 days, or surgery or radiotherapy for a primary tumor or mediastinum within the past 6 months; interstitial lung disease or history thereof, radiation pneumonitis treated with corticosteroids or a history thereof; detection of known resistance mutations of EGFR (eg, T790M).

Between March 2014 and February 2017, 527 patients with advanced *EGFR*-mutated NSCLC were screened for *BIM* deletion polymorphism. Among them, 77 patients (14.6%) had *BIM* deletion polymorphism (75 patients [14.2%] were heterozygous and two patients [0.4%] were homozygous) and a total of 12 patients were enrolled in the present study.

2.3 | Treatment and assessment

Vorinostat and gefitinib were purchased from Taiho Pharmaceutical and AstraZeneca, respectively. Vorinostat (level 1: 200 mg, level 2: 300 mg, level 3: 400 mg) was given orally once daily on days 1-7, and 250 mg gefitinib was given orally once daily in a cycle of 14 days; this continued until the criteria for respite, dosage reduction, or discontinuation of the protocol treatment were met.

Toxicities were graded in accordance with the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. DLT was defined as follows: grade ≥1 intestinal lung disease; grade ≥4 neutropenia lasting 5 days or more; febrile neutropenia; grade ≥3 thrombocytopenia requiring platelet transfusion; grade ≥4 thrombocytopenia; any grade uncontrollable skin toxicity; grade ≥3 nonhematological toxicity. DLT was evaluated during the first two cycles (14 days per cycle) of therapy. Secondary endpoints were the pharmacokinetics and pharmacodynamics of vorinostat and gefitinib, PFS, overall survival (OS), response rate (RR), duration of response and complete response, disease control rate (DCR), and incidence of adverse events defined by CTCAE version 4.0.

We assessed the objective tumor response in accordance with version 1.1 of the revised RECIST guidelines.[23] At baseline, we carried out imaging of the chest and abdominal by computed tomography (CT) and brain magnetic resonance imaging (MRI) or CT within 28 days before randomization. After the start of treatment, assessments were carried out at 6-week intervals during the first 24 weeks and at 12-week intervals after this period. Brain MRI or CT was required to follow the same schedule, but only for patients with brain metastasis at the time of enrolment (Table S1). Investigators reviewed the images of patients. PFS was defined as the time from the start of protocol treatment to the first occurrence of progression or to death. OS was defined as the

time from the start of protocol treatment to death. DCR was defined as the proportion of patients who achieved complete response, partial response, and stable disease for at least 6 weeks.

2.4 | Statistical analysis

The population analyzed for the primary endpoint included the enrolled patients with complete safety data on DLT during the first two cycles.

2.5 | Genotyping of the *BIM* deletion polymorphism

Cellular DNAs were extracted from patients' PBMC using a DNeasy Blood and Tissue Kit (Qiagen). To recognize the presence of the wild-type and deletion alleles, we conducted PCR as reported previously.[19] Primer sequences used were as follows. Forward: 5′-CCACCAATGGAAAAGGTTCA-3, reverse: 5′-CTGTCATTTCTCCCCACCAC-3′ for detection of wild-type *BIM*; and forward: 5′- CTGTCATTTCTCCCCACCAC-3′, reverse: 5′-GGCACAGCCTCTATGGAGAA-3′ for identification of the *BIM* deletion polymorphism. The primer pairs yielded PCR products of 362 and 284 bp, respectively.

2.6 | Pharmacokinetic analysis

Blood sampling for pharmacokinetics was carried out pretreatment and post-treatment (0.5, 1, 2, 4, 6, 8, 10, 24, 48, and 72 hours after the first dose). Concentration of gefitinib and vorinostat was measured in plasma and serum, respectively.[24,25]

2.7 | Pharmacodynamic analysis

Patients' PBMC were sampled at baseline and 4 hours after giving vorinostat on day 2 in cycle 1. Total RNA and proteins were extracted from PBMC using lysis buffer and RNeasy PLUS Mini kit (Qiagen), respectively. Reverse transcription of the collected RNAs was done using SuperScript VILO cDNA synthesis Kit and Master Mix (Invitrogen). Expression of *BIM* mRNA was quantitatively measured by ViiA 7 Real-Time PCR System (Applied Biosystems) using the following primers: *BIM* exon 2A (forward: 5′-ATGGCAAAGCAACCTTCTGATG-3′; reverse: 5′-GGCTCTGTCTGTAGGGAGGT-3′), *BIM* exon 3 (forward: 5′-CAATGGTAGTCATCCTAGAGG-3′; reverse: 5′-GACAAAATGCTCAAGGAAGAGG-3′), *BIM* exon 4 (forward: 5′-TTCCATGAGGCAGGCTGAAC-3′; reverse: 5′-CCTCCTTGCATAGTAAGCGTT-3′) and β-actin (forward: 5′-GGACTTCGAGCAAGAGATGG-3′; reverse: 5′-AGCACTGTGTTGGCGTACAG-3′).

Proteins harvested from PBMC were separated by SDS-PAGE. The proteins were transferred onto PVDF membranes (Bio-Rad); the membranes were immersed in StartingBlock T20 (TBS) Blocking Buffer (ThermoFisher Scientific) for 1 hour at approximately 20°C, and by incubation for longer than 8 hours at 4°C with antibodies against acetylated histone H3 (Lys27), BIM, and β-actin (Cell Signaling Technology). After three washes in Tris-buffered saline with polyoxyethylene sorbitan monolaurate (TBST), the membranes were incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies. Proteins labeled with secondary antibodies were visualized by using SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (ThermoFisher Scientific).

3 | RESULTS

3.1 | Patients and safety

From June 2014 to February 2017, 12 patients were enrolled into this study. Patient characteristics are summarized in Table 1. Previously treated EGFR-TKI in each patient are shown in Table S2.

Planned dose escalation was completed without reaching the MTD. DLT were not observed in any patients. Treatment-related adverse events are summarized in Table 2. The most common adverse events were diarrhea (92%), anorexia (75%), oral mucositis (58%), rash, weight loss, dysgeusia, nausea (50%), vomiting, and malaise (42%). Treatment-related grade 3 adverse events included grade 3 hypokalemia (17%), lung infection and thrombocytopenia (8%) (Table 3). No treatment-related death or grade 4 adverse events were observed.

Adverse events that resulted in drug cessation were observed in four cases: one at level 1 (pneumonia); two at level 2 (diarrhea, vomiting, fever, alanine aminotransferase [ALT] elevation, and loss of appetite); and one at level 3 (neutropenia). One adverse event resulting in vorinostat dose reduction was observed at level 2 (thrombocytopenia).

Based on these data, RP2D was determined as 250 mg gefitinib daily and 400 mg/day vorinostat biweekly.

3.2 | Efficacy

Median PFS and OS of 12 patients at levels 1-3 were 156.5 days (5.2 months; 43-479 days) and 673 days (22.4 months; 411 days–not reached), respectively (Figure 1). Disease control (stable disease assessed for at least 6 weeks) was achieved in 10 out of 12 (83.3%) patients with a history of progressive disease during EGFR-TKI treatment (Table 4).

3.3 | Pharmacokinetics

Median T_{max} of vorinostat in the 200, 300, and 400 mg groups was 2, 4, and 4 hours, respectively (Table 5, Figure S2); $T_{1/2}$ (mean ± SD) was 3.6 ± 3.4, 1.5 ± 0.5, and 2.0 ± 1.1 hours, respectively, and C_{max} (mean ± SD) was 602 ± 166, 661 ± 33, and 1010 ± 335 nmol/L, respectively. AUC_{last} (mean ± SD) was 2340 ± 753, 2430 ± 348, and

TABLE 1 Characteristics of patients with *EGFR*-mutated NSCLC harboring *BIM* deletion polymorphism

	Total	Level 1 (200 mg)	Level 2 (300 mg)	Level 3 (400 mg)
Analysis of population	12	3	3	6
Gender				
Male/Female	7/5	1/2	2/1	4/2
Age, y				
Median (range)	69.5 (51-84)	73.0 (60-78)	71.0 (51-76)	68.0 (62-84)
Smoking status Brinkman index				
Never	5	2	2	1
30≤ <1000	4	0	1	3
1000≤	3	1	0	2
Performance status				
0/1	7/5	2/1	1/2	4/2
Histology				
Adenocarcinoma/Other	12/0	3/0	3/0	6/0
BIM polymorphism				
Heterozygote/Homozygote	11/1	3/0	3/0	5/1
EGFR mutation				
Exon19 deletion/Exon21 L858R	8/4	2/1	2/1	4/2
Duration from previous EGFR-TKI treatment (days)				
Median (range)	58 (8-574)	274 (8-518)	11 (8-191)	58 (14-574)

Abbreviations: EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer.

TABLE 2 Number of patients with treatment-related adverse events

	Total	Level 1			Level 2			Level 3		
Analysis of population	**12**	**3**			**3**			**6**		
Grade (CTCAE version 4.0)		**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Diarrhea	11 (92%)	3	0	0	2	0	0	3	3	0
Anorexia	9 (75%)	0	2	0	1	1	0	4	1	0
Oral mucositis	7 (58%)	0	2	0	1	1	0	3	0	0
Rash acneiform	6 (50%)	1	0	0	0	1	0	3	1	0
Weight loss	6 (50%)	0	2	0	2	0	0	1	1	0
Dysgeusia	6 (50%)	2	0	0	1	0	0	1	2	0
Nausea	6 (50%)	0	1	0	1	0	0	2	1	1
Vomiting	5 (42%)	2	0	0	1	0	0	2	0	0
Malaise	5 (42%)	1	0	0	2	0	0	2	0	0
Hypokalemia	3 (25%)	0	0	0	0	0	0	1	0	2
Dry skin	3 (25%)	0	1	0	1	0	0	1	0	0
Paronychia	3 (25%)	0	1	0	0	0	0	1	1	0

4790 ± 2040 nmol·h/L, respectively, and AUC_{inf} (mean ± SD) was 2430 ± 809, 2480 ± 407, and 4810 ± 2040 nmol·h/L, respectively.

Median T_{max} of gefitinib in the 200, 300, and 400 mg groups was 4, 4, and 4.1 hours, respectively (Figure S3); $T_{1/2}$ (mean ± SD) was 20.2 ± 7.3, 25.1 ± 5.5, and 25.2 ± 11.4 hours, respectively, and C_{max} (mean ± SD) was 344 ± 44, 240 ± 98, and 295 ± 253 ng/mL, respectively. AUC_{last} (mean ± SD) was 4524 ± 779, 3398 ± 1050, and 3853 ± 3004 ng·h/L, respectively, and AUC_{inf} (mean ± SD) was 8674 ± 3064, 7333 ± 1808, and 9537 ± 8064 ng·h/L, respectively. There was no significant difference between the three groups.

TABLE 3 Number of patients with ≥grade 3 treatment-related adverse events

	Grade 3
Nausea	1 (Level 3)
Hypokalemia	2 (Level 3)
Thrombocytopenia	1 (Level 3)
Lung infection	1 (Level 2)
Peripheral neuropathy	1 (Level 3)

These results indicated that vorinostat was unlikely to affect the pharmacokinetics of gefitinib.

3.4 | Pharmacodynamics

Pharmacodynamic analyses of PBMC obtained at baseline and 4 hours after giving vorinostat and gefitinib on day 2 in cycle 1 were carried out. As a measure of the effects of vorinostat on the expression and splicing of *BIM* transcripts, we evaluated, by using real-time qPCR, *BIM* exon 2 expression as a surrogate expression marker for all *BIM* transcripts. In addition, we also separately evaluated the expression of *BIM* exon 3 and exon 4, which represent the BIM isoform that lacks the proapoptotic BH3 domain and the isoform that has the proapoptotic BH3 domain, respectively. In two cases, (VK-02) and (VS-02), we were unable to measure these parameters owing to poor quality of the mRNA and protein, respectively.

We found that the *BIM* mRNA exon 3/exon 4 ratio was decreased by treatment with gefitinib and vorinostat in all 11 patients assessed (Figure 2A,B). As reported, HDAC inhibitors, alone or in combination with a TKI, may cause the expression of proapoptotic exon 4-containing *BIM* transcripts to increase by significantly more than the expression of exon 3-containing *BIM* transcripts.[19,26,27] We also noted that VI-03, the only patient in this study that was homozygous for the *BIM* deletion polymorphism, had the highest expression of exon 3-containing transcripts, as well as the highest exon 3/exon 4 ratio at baseline. This was in line with our previous findings that cells that were homozygous for the *BIM* deletion polymorphism tended to have higher expression of exon 3-containing transcripts than of the proapoptotic exon 4-containing transcripts.[9,26]

Mean odds ratio of exon 3/exon 4 before and after treatment with gefitinib and vorinostat was 1.9376 (1.5988 at level 1, 1.8436 at level 2, and 2.1974 at level 3), and the results showed a significant upward trend in terms of a vorinostat dose-dependent increase in proapoptotic exon 4-containing *BIM* transcripts (Figure 2C).

In addition, we also assessed the acetylation status of histone H3 as a measure of the inhibitory effect of vorinostat on HDAC activity.[28] We also evaluated the protein expression of the pro-apoptotic BIM isoform, BIM_{EL}, as an indicator of the ability of vorinostat to enhance the proapoptotic isoforms of BIM. As expected, vorinostat markedly increased the protein expression of acetylated histone H3 in 10/11 patients (91%) (3/3 at level 1, 3/3 at level 2, and 4/5 at level 3) (Figure 3). Moreover, vorinostat increased proapoptotic BIM protein (BIM_{EL}) expression in five of 11 patients (45.5%) (1/3 at level 1, 3/3 at level 2, and 1/5 at level 3). Summary of the pharmacokinetic and pharmacodynamic analyses for each patient is shown in Table S3.

These results indicated that vorinostat could stimulate histone acetylation through the inhibition of HDAC. Furthermore, vorinostat increased the expression of proapoptotic exon 4-containing *BIM* transcript and protein in the majority of patients with *EGFR*-mutated NSCLC with *BIM* deletion polymorphism. This observation was in line with a previous report on the use of HDAC inhibitors alone or in

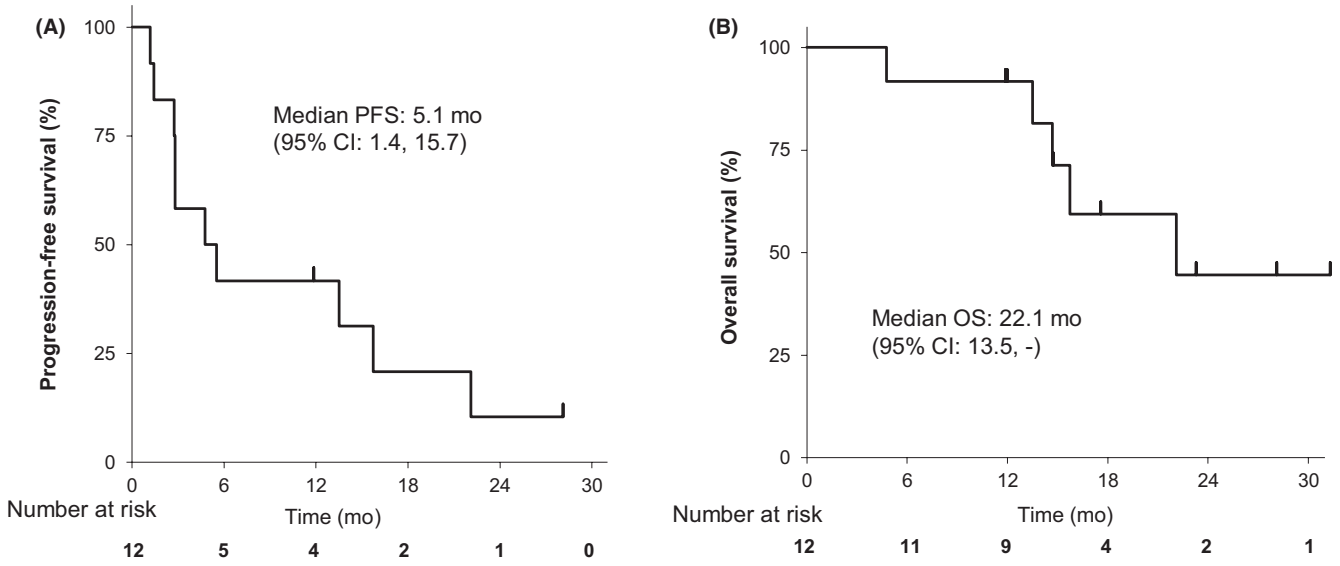

FIGURE 1 Progression-free survival (PFS) and overall survival (OS) of patients with *BIM* deletion-positive epidermal growth factor receptor-mutated non-small cell lung cancer treated with gefitinib combined with vorinostat. Kaplan-Meier curves for PFS (A) and OS (B) of 12 patients are shown. The 95% confidence intervals were calculated by using the Brookmeyer and Crowley method

TABLE 4 Summary of tumor response

Total	Best overall response by RECIST					SD ≥6 wk	DCR	DCR 95% CI
	CR	PR	SD	PD	NE			
12	0	0	10	2	0	10	83.3%	0.52, 0.98

Abbreviations: CI, confidence interval; CR, complete response; DCR, disease-control rate; NE, not evaluable; PD, progressive disease; PR, partial response; SD, stable disease.

combination with a TKI, which can increase the protein expression of BIM.[19,26,27] Therefore, these data have shown the technical feasibility of monitoring these parameters as markers of vorinostat activity.

4 | DISCUSSION

In the present study, we determined the RP2D of vorinostat, 400 mg/day biweekly, in combination with gefitinib for patients with *BIM* deletion polymorphism-positive *EGFR*-mutated NSCLC. Two recent clinical trials of vorinostat combined with either gefitinib or erlotinib showed the feasibility of combined therapy in NSCLC. In the Spanish phase I/II trial, vorinostat plus erlotinib (150 mg daily) was evaluated in erlotinib-refractory *EGFR*-mutated NSCLC.[21] In the Korean phase I/II trial, vorinostat plus gefitinib (250 mg daily) was evaluated in unselected NSCLC.[20] Both trials determined the recommended dose of vorinostat as 400 mg/day biweekly, but drug concentrations in the plasma or serum were not evaluated. Therefore, the pharmacokinetic interactions of vorinostat and EGFR-TKI have been unknown until now. In our study, we showed that selection of patients with both an *EGFR* mutation and the *BIM* deletion polymorphism could be carried out in a clinical setting, and that vorinostat 400 mg/day biweekly was the recommended dose for further studies in *BIM* deletion polymorphism/*EGFR* mutation-positive populations in NSCLC. Furthermore, our pharmacokinetic analyses showed that vorinostat did not affect the dynamics of gefitinib in these populations.

The most important and informative findings in our study are the pharmacodynamic analyses. We found that vorinostat, in combination with gefitinib, induced acetylated histone H3 protein expression, as well as a decrease in the *BIM* mRNA exon 3/exon 4 ratio in PBMC. These results have provided proof of concept that the combined therapy can mitigate the functional effects of the *BIM* deletion polymorphism, which, to the best of our knowledge, remains the most common resistance-conferring germline variant in *EGFR*-mutated NSCLC.[17] Moreover, we were also able to show increased expression of a proapoptotic BIM protein isoform (BIM_{EL}) in primary patient material. Interestingly, while combined therapy resulted in an increase in protein expression of acetylated histone H3, as well as a decrease in the *BIM* exon 3/exon 4 ratio, not all individuals who showed these changes had a concomitant increase in BIM_{EL} protein. The reason for this discrepancy is unclear at present, but may encompass both technical and biological explanations. For example, although PBMC samples were harvested and handled according to a common protocol, protein degradation may have occurred between blood sampling and protein purification. For example, in one case (VS-02), although we detected a decrease in the *BIM* exon 3/exon 4 ratio, we were unable to detect BIM_{EL} protein. As a result, the *BIM* mRNA exon 3/exon 4 ratio may be a more robust pharmacodynamic biomarker for vorinostat activity than the measurement of BIM_{EL} protein.

TABLE 5 Pharmacokinetic parameters

			C_{max} (ng/mL)	AUC_{last} (ng•h/L)	AUC_{inf} (ng•h/L)	T_{max} (h)	$t_{1/2}$ (h)
Gefitinib	250 mg	No. of patients	12	12	11	12	11
		Median (range)	250 (148-802)	3430 (1880-9850)	8270 (3190-23 400)	4.0 (3.8-8)	22.6 (13.3-42.6)
		Mean (SD)	294 (181)	3910 (2140)	8700 (5430)	4.6 (1.3)	23.8 (8.6)
			C_{max} (nmol/L)	AUC_{last} (nmol•h/L)	AUC_{inf} (nmol•h/L)	T_{max} (h)	$t_{1/2}$ (h)
Vorinostat	Level 1 (200 mg)	No. of patients	3	3	3	3	3
		Median (range)	615 (430-762)	2720 (1470-2830)	2830 (1500-2960)	2.0 (1.1-4.0)	1.8 (1.6-7.5)
		Mean (SD)	602 (166)	2340 (753)	2430 (809)	2.4 (1.5)	3.6 (3.4)
	Level 2 (300 mg)	No. of patients	3	3	3	3	3
		Median (range)	643 (641-699)	2250 (2210-2830)	2260 (2240-2950)	4.0 (1.8-4.0)	1.3 (1.1-2.1)
		Mean (SD)	661 (33)	2430 (348)	2480 (407)	3.3 (1.3)	1.5 (0.5)
	Level 3 (400 mg)	No. of patients	6	6	6	6	6
		Median (range)	944 (695-1630)	4250 (2960-8610)	4270 (3010-8650)	4.0 (0.9-4.1)	2.1 (0.8-3.1)
		Mean (SD)	1010 (335)	4790 (2040)	4810 (2040)	3.5 (1.3)	2.0 (1.1)

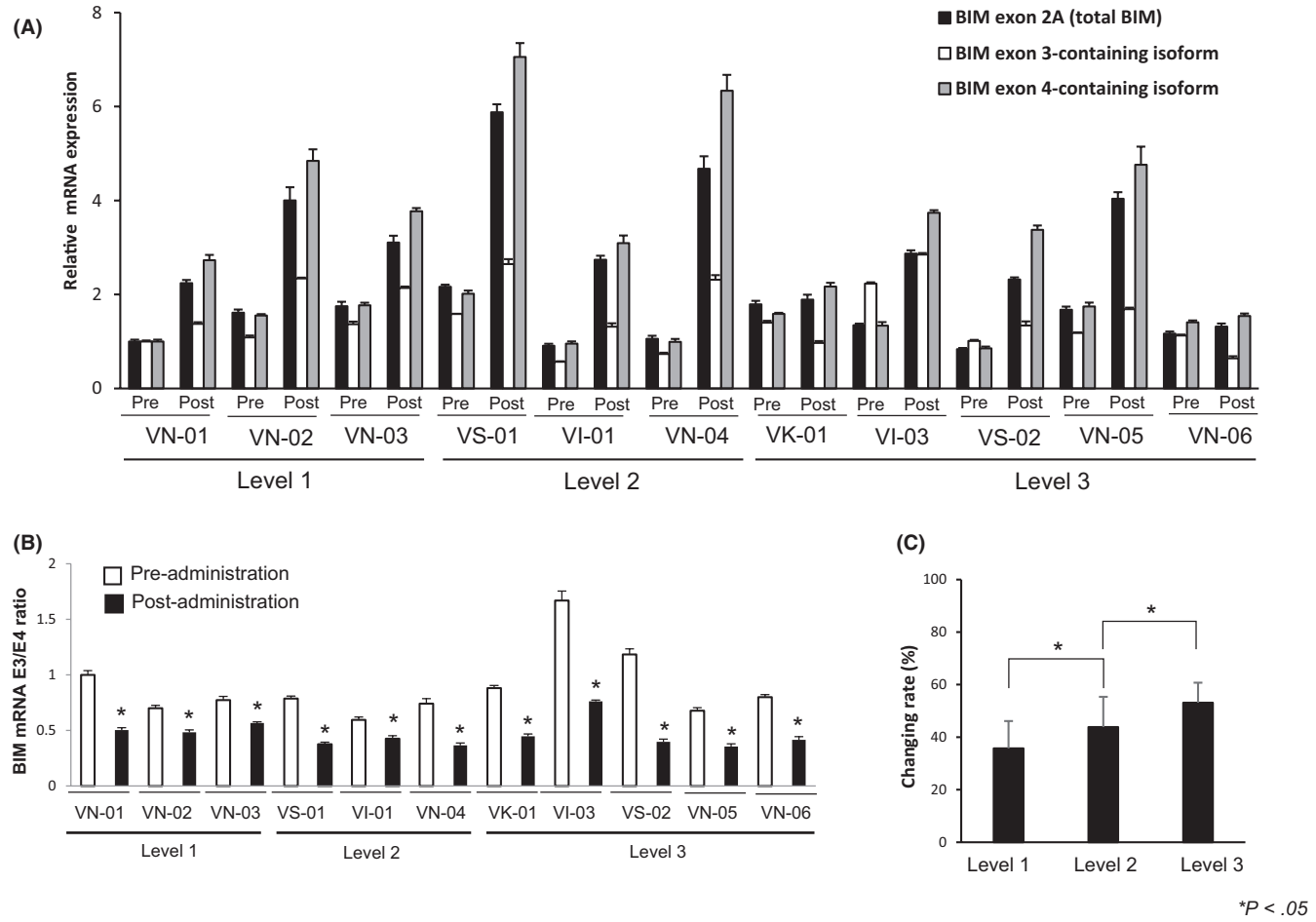

FIGURE 2 Pharmacodynamic analysis of vorinostat through transcription of *BIM* mRNA isoforms in PBMC. A, PBMC were harvested at baseline and 4 h after giving gefitinib and 200 mg (n = 3), 300 mg (n = 3), and 400 mg (n = 5) vorinostat on day 2 of cycle 1. RNA was purified from PBMC and mRNA expression of BIM exon 2A (representative of total *BIM* mRNA expression), exon 3 (representative of the inactive isoform of *BIM* mRNA), and exon 4 (representative of the proapoptotic isoform of *BIM* mRNA) is shown. B, *BIM* mRNA exon 3/exon 4 ratio. *$P < .05$ vs baseline. C, Change in *BIM* mRNA exon 3/exon 4 ratio before and 2 d after treatment with vorinostat and gefitinib. Bars indicate mean ± SD

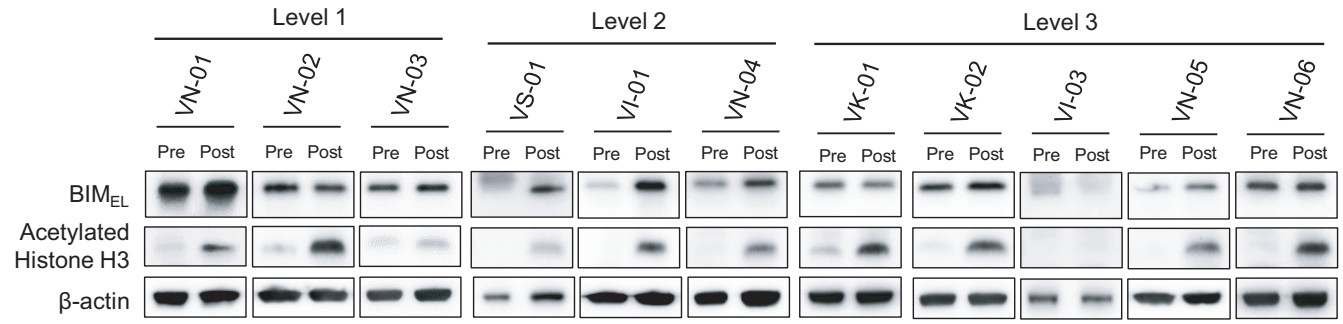

FIGURE 3 Pharmacodynamic analysis of vorinostat through protein expression of acetylated histone H3 and pro-apoptotic BIM$_{EL}$ in PBMC. PBMC were harvested at baseline (Pre) and 4 h after dose (Post) of gefitinib and 200 mg (A: n = 3), 300 mg (B: n = 3), and 400 mg (C: n = 5) vorinostat on day 2 of cycle 1. Protein expression in PBMC was determined by western blotting

It is well recognized that a small population of cells adapts to initial treatment with EGFR-TKI as persisters and becomes the base of acquired resistant lesions.[29] *BIM* deletion polymorphism which prevents tumor cell apoptosis is insufficient for tumor cell growth. The persisters need to acquire additional resistance factors which allow tumor cell growth. The persisters need to acquire additional resistance factors which allow tumor cell growth. *EGFR*-T790M may be one of such additional resistance factors, because we detected T790M in *EGFR*-mutated NSCLC cell line PC-9, which had been genetically edited to have homozygous *BIM* deletion polymorphism,

after the induction of gefitinib resistance in the in vitro condition.[26] Although we did not examine additional resistance mechanisms in specimens from patients enrolled in the VICTROY-J study, resistant factors other than EGFR-T790M might also be detectable.

Prognosis of patients with T790M-negative, *EGFR*-mutated NSCLC who are refractory to EGFR-TKI is reported to be very poor.[30-33] In the present study, we excluded patients with *EGFR*-T790M-positive NSCLC because our previous in vitro results showed that vorinostat did not sensitize T790M-positive *EGFR*-mutated NSCLC cells to gefitinib.[19] Therefore, we recruited patients with T790M-negative *EGFR*-mutated NSCLC who were refractory to EGFR-TKI, and who had been treated with at least one regimen of cytotoxic chemotherapy. As a result, our study comprised heavily pretreated patients. Although the limited number of patients in our study precludes firm conclusions about clinical benefit, the DCR of 83.3% and median PFS of 5.2 months suggests that the combination should be evaluated in phase II studies.

In addition to the gefitinib/vorinostat combination we evaluated, the concept of combining EGFR-TKI and HDAC inhibitors can be applied by using new-generation EGFR-TKI, as well as novel HDAC inhibitors that have recently been described. Recently, the third-generation EGFR-TKI, osimertinib, has been recognized as one of the standard drugs for first-line treatment of *EGFR*-mutated NSCLC.[5] However, a population of patients show intrinsic resistance, even to osimertinib.[5] In these patients, resistance may be mediated by the *BIM* deletion polymorphism, as we found that *BIM* deletion polymorphism-positive *EGFR*-mutated NSCLC cell lines (PC-3) were resistant to osimertinib-induced apoptosis[19,26]; importantly, the addition of vorinostat could resensitize these cells to EGFR-TKI. Moreover, we reported that HDAC3 was an important regulator of *BIM* pre-mRNA splicing and that the activity of vorinostat was likely to require inhibition of HDAC3.[26] Therefore, the combination of osimertinib and new-generation HDAC inhibitors, including HDAC3-selective inhibitors and others (eg, panobinostat),[34] might be a promising first-line treatment for *BIM* deletion polymorphism-positive *EGFR*-mutated NSCLC.

In conclusion, we have determined vorinostat at 400 mg/day biweekly combined with gefitinib 250 mg daily as the recommended dose for phase II studies in patients with NSCLC who are double-positive for *BIM* deletion polymorphisms and *EGFR* mutations. Further, it is warranted to evaluate our approach for exploration of the efficacy of combination EGFR-TKI/HDAC therapy in larger cohorts.

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DISCLOSURE

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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