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Clinical significance of *BIM* deletion polymorphism in chemoradiotherapy for non-small cell lung cancer

Yu Wakabayashi[1] | Takeshi Masuda[2] | Kazunori Fujitaka[1] | Taku Nakashima[2] | Joe Okumoto[1] | Kiyofumi Shimoji[1] | Yoshifumi Nishimura[3] | Kakuhiro Yamaguchi[1] | Shinjiro Sakamoto[2] | Yasushi Horimasu[2] | Shintaro Miyamoto[2] | Hiroshi Iwamoto[1] | Shinichiro Ohshimo[4] | Hironobu Hamada[5] | Noboru Hattori[1]

[1]Department of Molecular and Internal Medicine, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan

[2]Department of Respiratory Internal Medicine, Hiroshima University Hospital, Hiroshima, Japan

[3]Department of Respiratory Internal Medicine, Higashihiroshima Medical Center, Higashihiroshima, Japan

[4]Department of Emergency and Critical Care Medicine, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan

[5]Department of Physical Analysis and Therapeutic Sciences, Hiroshima University, Hiroshima, Japan

Correspondence
Takeshi Masuda, Department of Respiratory Internal Medicine, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
Email: ta-masuda@hiroshima-u.ac.jp

Abstract

The standard treatment for locally advanced non-small cell lung cancer (NSCLC) is chemoradiotherapy (CRT) followed by anti-programmed cell death-ligand 1 (anti-PD-L1) treatment. *BIM* deletion polymorphism induces the suppression of apoptosis resulting from epidermal growth factor (EGFR)-tyrosine kinase inhibitors in *EGFR*-mutated NSCLC patients. We aimed to examine the effects of *BIM* polymorphism on CRT and anti-PD-L1/PD-1 treatment in NSCLC patients. In this retrospective study of 1312 patients with unresectable NSCLC treated at Higashi-Hiroshima Medical Center and Hiroshima University Hospital between April 1994 and October 2019, we enrolled those who underwent CRT or chemotherapy using carboplatin + paclitaxel or cisplatin + vinorelbine, or anti-PD-L1/PD-1 treatment. Of 1312 patients, 88, 80, and 74 underwent CRT, chemotherapy, and anti-PD-L1/PD-1 treatment, respectively, and 17.0%, 15.2% and 17.6% of these patients showed *BIM* polymorphism. Among patients receiving CRT, the progression-free survival was significantly shorter in those with *BIM* deletion than in those without. In the multivariate analyses, *BIM* polymorphism was an independent factor of poor anti-tumor effects. These results were not observed in the chemotherapy and anti-PD-L1/PD-1 treatment groups. In in vitro experiments, *BIM* expression suppression using small interfering RNA in NSCLC cell lines showed a significantly suppressed anti-tumor effect and apoptosis after irradiation but not chemotherapy. In conclusion, we showed that *BIM* polymorphism was a poor-predictive factor for anti-tumor effects in NSCLC patients who underwent CRT, specifically radiotherapy. In the implementation of CRT in patients with *BIM* polymorphism, we should consider subsequent treatment, keeping in mind that CRT may be insufficient.

KEYWORDS

BIM, chemoradiotherapy, chemotherapy, non-small cell lung cancer, radiotherapy

Abbreviations: BIM-siRNA, small interfering RNA duplexes targeting BIM; CRT, chemoradiotherapy; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; ICI, immune check-point inhibitor; NC-siRNA, negative control siRNA; NSCLC, non-small cell lung cancer; OS, overall survival; PD-L1, programmed cell death-ligand 1; PFS, progression-free survival; PTX, paclitaxel; qRT-PCR, quantitative real-time PCR; VNR, vinorelbine.

1 | INTRODUCTION

Lung cancer is the most lethal type of cancer,[1] and is categorized into non-small (NSCLC) and small cell lung cancer. Of patients with NSCLC, approximately 33% have locally advanced NSCLC,[1] the standard treatment for which is concurrent chemoradiotherapy (CRT).[2] A randomized phase III trial (the PACIFIC study) showed that the progression-free survival (PFS) duration was significantly longer in locally advanced NSCLC patients who underwent CRT followed by consolidation treatment with durvalumab (anti-PD-L1 antibodies) than in those who underwent CRT followed by placebo treatment.[3] Therefore, CRT followed by durvalumab treatment is used as the standard treatment for patients with locally advanced NSCLC.[2] The anti-tumor effects of ICI, including anti-PD-L1/PD-1 antibody, are enhanced by immune induction resulting from immunogenic cancer cell death due to irradiation or chemotherapy.[4,5] Therefore, anti-tumor CRT effects could play an important role in the effect of anti-PD-L1 treatment as consolidation therapy.

The mechanism behind the anti-tumor effect exerted by radiation is DNA damage followed by cancer cell apoptosis.[6,7] In addition, the cytotoxicity of PTX and VNR used in CRT, is also induced through apoptosis.[8-12] Apoptosis is characterized by extrinsic and intrinsic pathways. The extrinsic pathway is induced by death receptors (eg, Fas and tumor necrosis factor receptors) whereas the intrinsic pathway, which shows mitochondrial involvement, is induced under conditions of cellular stress by radiation and chemotherapy.[13]

BIM, a molecule belonging to the Bcl-2 family, is involved in the intrinsic pathway of apoptosis, and is also known as Bcl-2-like protein ll. It is present in the cytoplasm and it emigrates to the mitochondria according to cell death signaling, promoting leakage of cytochrome *c* by the activation of apoptosis-promoting factors, such as BAX and BAK, or inactivating apoptosis inhibitors, such as Bcl-2. Cytochrome *c* leakage together with ATP and Apaf-1 activate caspase 9, which is an initiator caspase of apoptosis. Activated caspase 9 upregulates caspase 3/7, which is an effector caspase, and apoptosis finally occurs.[6,14]

The presence of *BIM* deletion polymorphism in intron 2 has been detected only among east Asians, as germ cell mutations at a frequency of 12.3%. In individuals with this polymorphism, *BIM* isoform protein deletes Bcl2-homology domain 3 that activates apoptosis and the rate of apoptosis is suppressed.[15] Several studies have shown that *BIM* affects the induction of apoptosis by EGFR-TKI treatment.[16-18] MEK-ERK signaling promotes BIM protein decomposition in the EGFR pathway, although MEK-ERK signaling inhibition by EGFR-TKI increases the BIM protein expression level, resulting in an anti-tumor effect.[17] Therefore, in patients with *BIM* polymorphism, intrinsic resistance to EGFR-TKI is observed.[15] In fact, the median PFS duration associated with EGFR-TKI treatment is significantly shorter in the *BIM* polymorphism than in the wild-type group.[19]

However, there has not been a study to examine the effects of *BIM* deletion on CRT. Therefore, we investigated the anti-tumor effect of *BIM* polymorphism on CRT in patients with locally advanced NSCLC. Furthermore, we also evaluated how the presence of this polymorphism affects the efficacy of ICI treatment that is used as consolidation treatment after CRT.

2 | MATERIALS AND METHODS

2.1 | Patients and study design

We retrospectively reviewed the medical records of patients with unresectable NSCLC patients treated at the Higashi-Hiroshima Medical Center or Hiroshima University Hospital between April 1994 and April 2018. The participants provided written informed consent for the use of their specimens in the study. We enrolled patients who received carboplatin + PTX or cisplatin + VNR as CRT or chemotherapy; we excluded those with a performance status ≥ 3, those with sarcomatoid carcinoma, and those without clinical data required for investigating the anti-tumor effect of the treatment. In addition, patients with NSCLC treated with anti-PD-L1 or PD-1 antibodies at Hiroshima University Hospital between February 2016 and October 2019 who gave their written informed consent for the study participation were enrolled (Figure S1). The patients were staged according to the UICC TNM Classification of Malignant Tumors 7th edition.[20] This study was approved by the Ethics Committee of Hiroshima University Hospital (No. M33-19) and Higashi-Hiroshima Medical Center (No. 2020-8), and conducted in accordance with the ethical standards of the 1975 Declaration of Helsinki.

2.2 | Materials

A549 human lung adenocarcinoma and EBC-1 human lung squamous carcinoma were purchased and authenticated from ATCC (Manassas, VA, USA) and Japanese Collection of Research Bioresources (Tokyo, Japan), respectively. Carboplatin, PTX, cisplatin, and VNR were purchased from Wako Junyaku Kogyo Co. (Osaka, Japan).

2.3 | Cell culture and treatment

Cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were incubated at 37°C in a 5% CO_2 incubator and used within 6 months after resuscitation.

2.4 | Detection of BIM polymorphism

DNA was extracted from the peripheral blood of patients at the time of diagnosis and amplified by PCR using the Phenol Chloroform method and DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). The genomic DNAs were amplified using ABI 7500 Fast (Applied Biosystems, Foster City, CA, USA). PCR conditions were as follows: initial denaturation at 98°C for 2 minutes and 40 cycles of 98°C for 10 seconds, 57°C for 10 seconds, 68°C for 30 seconds, and a final melting dissociation curve analysis

at 95°C for 0.15 minute, 60°C for 1 minute, and 99°C for 0.15 minute. PCR reactions were conducted using the discriminating primers for the wild-type alleles (forward: 50-CCACCAATGGAAAAGGTTCA-30; reverse: 50-CTGTCATTTCTCCCCACCAC-30) and deletion alleles (forward: 50-CCACCAATGGAAAAGGTTCA-30; reverse: 50-GGCACAGCCTCTATGGAGAA30) (Thermo Fisher Scientific, Waltham, MA, USA), according to a previous study.[21] The presence or absence of the wild-type *BIM* allele and the *BIM* polymorphism allele was confirmed by melting curve analysis (Figure S2).

2.5 | Knockdown of BIM

Small interfering RNA duplexes targeting *BIM* (BIM-siRNA) (forward: 5′-CCUUCUGAUGUAAGUUCUGtt-3′; reverse: 5′-CAGAACUUAC AUCAGAAGGtt-3′) and a negative control siRNA (NC-siRNA) duplex were chemically synthesized by Thermo Fisher/Ambion. Cells were seeded at a density of 5×10^3 cells/well in 24-well plates. After 24 hours, transfection was carried out using lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) for the suppression of *BIM* gene expression, according to the manufacturer's instructions. For verification of the knockdown effect of BIM-siRNA (10 or 50 nM), the level of BIM mRNA expression was investigated using qRT-PCR, as follows. Total RNA was isolated with RNeasy Mini Kits (Qiagen). The isolated total RNA was reverse-transcribed into cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems) following the manufacturer's instructions. qRT-PCR was conducted on an ABI 7500 Fast (Applied Biosystems) for BIM alleles using beta-actin, as a control housekeeping gene.

2.6 | Cell count assay

We prepared 24-well assay plates containing 5.0×10^3 cells in medium/well and after incubation for 24 hours at 37°C, the cells were transfected with BIM-siRNA (10 nM) or NC-siRNA (10 nM). Then, 24 hours after the transfection, the cells were irradiated with 30 Gy or treated with carboplatin (20 μmol/L in A549, 40 μmol/L in EBC-1) + PTX (1.5 μmol/L in A549, 90 μmol/L in EBC-1) or cisplatin (0.25 μmol/L in A549, 6.3 μmol/L in EBC-1) + VNR (8 μmol/L in A549, 6 μmol/L in EBC-1). Irradiation dose and concentration of these drugs were determined based on the results of previous studies.[22-25] Number of cells was measured at 48 hours after irradiation or at administration of chemotherapy drugs.

2.7 | Apoptosis assay

We prepared 96-well assay plates containing 1.5×10^4 cells in medium/well. After incubation for 24 hours at 37°C, the cells were transfected with 10 nM BIM-siRNA and NC-siRNA. Then, 24 hours after the transfection, cells were irradiated with 30 Gy or treated with the chemotherapy drugs. Concentrations of drugs were described in the cell count assay paragraph above. Degree of caspase activity was investigated at 48 hours after irradiation or the administration of chemotherapy using ApoLive-Glo Multiplex Assay (Promega) following the manufacturer's instructions. Similarly, the degree of apoptosis was evaluated by the annexin V expression level using Apoptotic/Necrotic/Healthy Cells Detection Kit (PromoKine) following the manufacturer's instructions. Apoptotic cells were identified with FITC-Annexin V, and necrotic or dead cells were detected with Ethidium Homodimer III. All flow cytometry experiments were carried out on BD FACSVerse (BD Biosciences). The cells in four different quadrants were analyzed and interpreted as follows: upper left: necrosis (AnnexinV -/Ethidium Homodimer III +); upper right: late apoptosis (AnnexinV +/Ethidium Homodimer III +); lower right: early apoptosis (AnnexinV +/Ethidium Homodimer III -) and lower left: viable cells (AnnexinV -/Ethidium Homodimer III -).

2.8 | Statistical analysis

All the results are expressed as medians (ranges) or means ± standard deviations. The Mann-Whitney *U* or Student's *t* test was used for the evaluation of statistical differences between the groups. Cox regression analyses of PFS and OS were carried out for the determination of prognostic factors. Factors with a *P*-value <.05 in the univariate analysis were selected for inclusion in the multivariable analysis. In addition, multivariate analysis included the following factors: gender, ECOG PS status, and clinical stage in the chemotherapy or anti-PD-L1/PD-1 treatment group. Survival curves were estimated by Kaplan-Meier analysis and a log-rank test was used for the examination of the significance of the differences between

TABLE 1 Characteristics of patients in the CRT and chemotherapy groups

Patient characteristics	CRT group $n = 88$	Chemotherapy group $n = 99$
Age (years)		
Median (range)	65 (38-85)	67 (36-84)
Gender		
Male/Female	72/16	70/29
ECOG PS		
0/1/2	61/26/1	34/64/1
Smoking amount, Pack-years		
<40/≥40	35/53	53/46
Histology		
Ad/Sq/Others	57/25/6	79/17/3
Clinical stage		
IIIA/IIIB/IV, recurrence	38/50/0	1/31/67
EGFR mutation		
Positive/negative/not tested	10/45/33	17/55/27
BIM deletion polymorphism		
Positive/negative	15/73	15/84

Abbreviations: Ad, adenocarcinoma; BIM, B-cell chronic lymphocytic leukemia (CLL)/lymphoma 2-like 11; CRT, chemoradiotherapy; EGFR, epidermal growth factor receptor; Sq, squamous carcinoma.

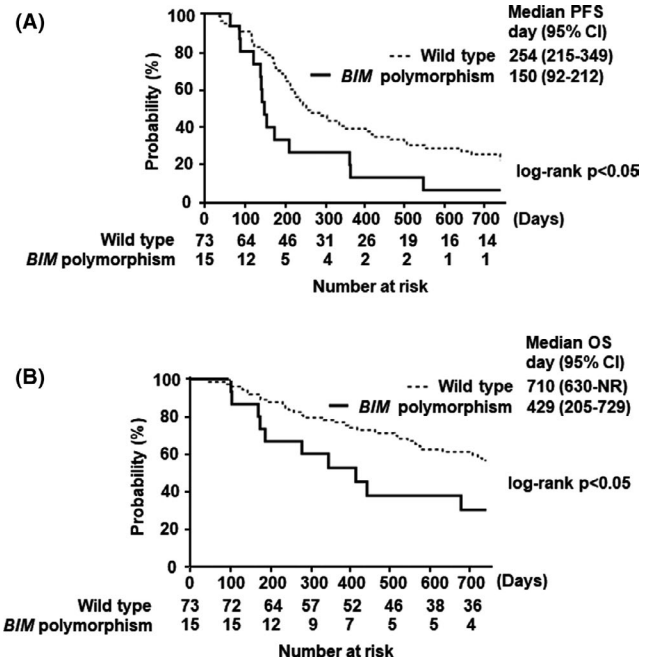

FIGURE 1 Kaplan-Meier survival curves for (A) progression-free survival and (B) overall survival between*BIM*polymorphism-positive and -negative patients who underwent chemoradiotherapy. CI, confidence interval; OS, overall survival; PFS, progression-free survival; NR, not reached

the groups. *P*-value <.05 was considered statistically significant. Analyses were done using JMP pro 14 software (SAS Institute).

3 | RESULTS

3.1 | Baseline characteristics and incidence of BIM polymorphism in patients with CRT

In total, 88 patients underwent CRT; their clinical characteristics are shown in Table 1. Median patients' age was 65 years (38-85 years). The predominance of male gender (81.8%) and those with an ECOG PS score of 0 (69.3%) was observed. The histological classification was adenocarcinoma and squamous cell carcinoma in 57 (64.8%) and 25 patients (28.4%), respectively. The proportions of those with clinical stage IIIA and IIIB disease were 38 (43.2%) and 50 (56.8%), respectively, while there were 15 patients (17.0%) with *BIM* polymorphism. The patients' backgrounds with and without *BIM* polymorphism were not significantly different (Table 2).

3.2 | Survival analysis of patients with and without BIM polymorphism who underwent CRT

The Kaplan-Meier curves of PFS and OS in patients who underwent CRT are shown in Figure 1. PFS and OS were significantly shorter in

TABLE 2 Comparison of characteristics between *BIM* deletion polymorphism-positive and -negative patients who underwent CRT or chemotherapy

	CRT group			Chemotherapy group		
Patient characteristics	*BIM* deletion (-) *n* = 73	*BIM* deletion (+) *n* = 15	*P* value	*BIM* deletion (-) *n* = 84	*BIM* deletion (+) *n* = 15	*P* value
Age (years)						
Median (range)	66 (38-85)	64 (53-77)	.639	66.5 (36-84)	68 (42-80)	.990
Gender						
Male/Female	61/12	11/4	.350	61/23	9/6	.334
Smoking amount, Pack-years						
Median (range)	40 (0-160)	43.75 (0-96)	.646	34.5 (0-121)	40 (0-100)	.720
ECOG PS						
0/1/2	48/24/1	13/2/0	.217	30/53/1	4/11/0	.653
Histology						
Ad/Sq/Others	48/20/5	9/5/1	.897	68/14/2	11/3/1	.608
Clinical stage						
IIIA/IIIB/IV, recurrence	31/42/0	7/8/0	.765	1/29/54	0/2/13	.229
EGFR mutation						
Positive/negative/not tested	10/38/25	0/7/8	.189	16/44/24	1/11/3	.289
EGFR-TKI after CRT or chemotherapy						
±	6/67	0/15	.250	13/71	0/15	.102

Abbreviations: Ad, adenocarcinoma; CRT, chemoradiotherapy; EGFR, epidermal growth factor receptor; BIM, B-cell chronic lymphocytic leukemia (CLL)/lymphoma 2-like 11; Sq, squamous carcinoma.

TABLE 3 Univariate and multivariate Cox analyses of PFS or OS in patients who underwent CRT

	PFS		OS	
Patient characteristics	**Hazard ratio [95% CI]**	***P* value**	**Hazard ratio [95% CI]**	***P* value**
Univariate analysis				
Age, years				
≥75	1.214 [0.363-3.031]	.719	1.153 [0.345-2.878]	.791
Gender				
Male	1.778 [0.763-5.183]	.197	1.756 [0.755-5.113]	.206
Smoking amount, Pack-years				
≥40	1.479 [0.787-2.906]	.228	1.528 [0.814-2.300]	.190
ECOG PS				
Continuous variable	0.697 [0.407-1.128]	.146	0.767 [0.380-1.412]	.411
Histological type				
Ad	0.553 [0.298-1.044]	.067	0.567 [0.311-1.041]	.067
Clinical stage				
IIIB	1.694 [1.049-2.782]	.031	2.181 [1.139-4.446]	.018
BIM deletion polymorphism				
Positive	2.038 [1.109-3.519]	.023	2.378 [1.141-4.593]	.022
EGFR mutation				
Positive	0.717 [0.343-1.501]	.378	0.311 [0.075-1.288]	.107
EGFR-TKI after CRT				
+	0.553 [0.201-1.521]	.251	0.257 [0.035-1.866]	.179
Multivariate analysis				
Clinical stage				
IIIB	1.678 [1.039-2.756]	.034	2.206 [1.152-4.498]	.016
BIM deletion polymorphism				
Positive	1.948 [1.037-3.418]	.039	2.206 [1.024-4.356]	.044

Abbreviations: Ad, adenocarcinoma; BIM, B-cell chronic lymphocytic leukemia (CLL)/lymphoma 2-like 11; CI, confidence interval; CRT, chemoradiotherapy; OS, overall survival; PFS, progression-free survival.

the *BIM* polymorphism than in the wild-type group ($P = .018$, $P = .03$, respectively).

3.3 | Univariate and multivariate Cox regression analyses for PFS and OS in patients who underwent CRT

Univariate and multivariate Cox regression analysis results are shown in Table 3. In the univariate Cox regression models, clinical stage IIIB disease and *BIM* polymorphism-positivity were shown to be significant predictors (hazard ratio [95% confidence interval {CI}]: 1.694 [1.049-2.782], $P = .031$; 2.038 [1.109-3.519], $P = .023$, respectively). In the multivariate analysis, clinical stage IIIB disease and *BIM* polymorphism-positivity were shown to be independent predictors (1.678 [1.039-2.756], $P = .034$; 1.948 [1.037-3.418], $P = .039$, respectively) (Table 3). Similar results were also observed in the univariate and multivariate Cox hazard regression analyses for OS (Table 3).

3.4 | Effect of the presence or absence of BIM polymorphism on the efficacy of chemotherapy

Subsequently, we sought to examine whether *BIM* polymorphism influences the efficacy of radiotherapy or chemotherapy. However, the number of patients treated with curative radiotherapy alone was very small. Thus, we only examined whether *BIM* polymorphism affected chemotherapy efficacy. Clinical characteristics of the patients who underwent chemotherapy are shown in Table 1. The proportion of those with *BIM* polymorphism was 15 (15.2%). The backgrounds of those with and without *BIM* polymorphism were not significantly different (Table 2). In the 13 patients who were treated with EGFR-TKI, EGFR-TKI was given after chemotherapy. Results of the univariate and multivariate Cox regression analyses are shown in Table S1. In the univariate Cox regression models of OS, gender, poor PS and administration of EGFR-TKI were significant predictors of OS (hazard ratio [95% CI]: 1.990 [1.017-3.896], $P = .045$; 1.925 [1.193-3.167], $P = .008$; 0.374 [0.161-0.866], $P = .022$, respectively). In the multivariate analysis, poor PS and administration of EGFR-TKI were also

shown to be independent predictors (1.769 [1.097-2.900], $P = .021$, 0.333 [0.140-0.791], $P = .013$, respectively). In contrast, *BIM* polymorphism did not have a significant effect nor was a prognostic factor. In agreement with this result, the PFS and OS values were not significantly different between the *BIM* polymorphism and the wild-type group (Figure 2).

3.5 | Degree of BIM expression in cancer cells affects the anti-tumor effect of radiotherapy in vitro

Our results indicated that the presence of *BIM* polymorphism influenced the anti-tumor effect and prognoses in patients with CRT, especially radiotherapy. Therefore, we clarified these results in vitro. The expression of *BIM* in A549 and EBC-1 cells with wild-type *BIM* was suppressed using siRNA (Figure 3). RT-PCR showed that the expression level of *BIM* was suppressed to approximately 1/5 in these cells. The inhibited *BIM* expression degree was similar to the expression level of an isoform with the *BIM* polymorphism in humans.[15] First, we confirmed that the *BIM* expression level in the cancer cells increased at 48 hours after irradiation (Figure S3A,B). Subsequently, we showed that the number of 30 Gy-irradiated cancer cells was significantly higher in the BIM-siRNA group than in the control group (Figure 4A,B). These results suggested that the anti-tumor effect of radiation was limited by the inhibition of *BIM* expression. Conversely, the number of chemotherapy-treated cells was not significantly different between the BIM-siRNA and the control groups (Figure S4A–D).

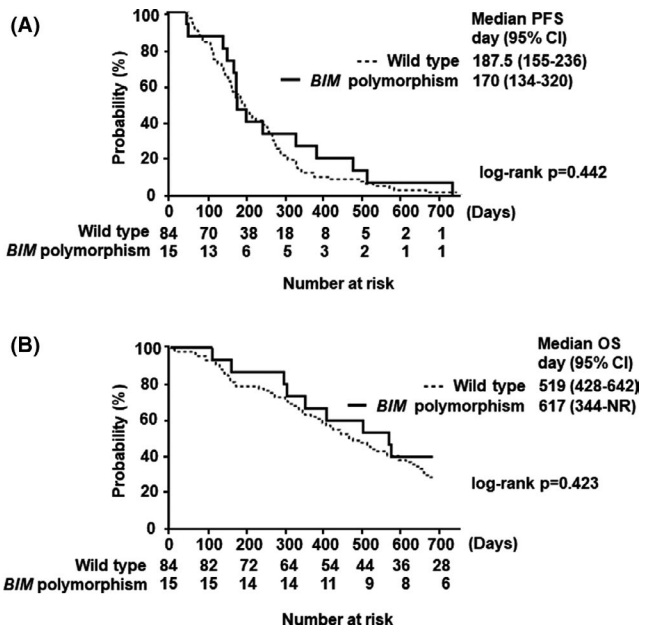

FIGURE 2 Kaplan-Meier survival curves for (A) progression-free survival and (B) overall survival between*BIM*polymorphism-positive and -negative patients who underwent chemotherapy. CI, confidence interval; OS, overall survival; PFS, progression-free survival; NR, not reached

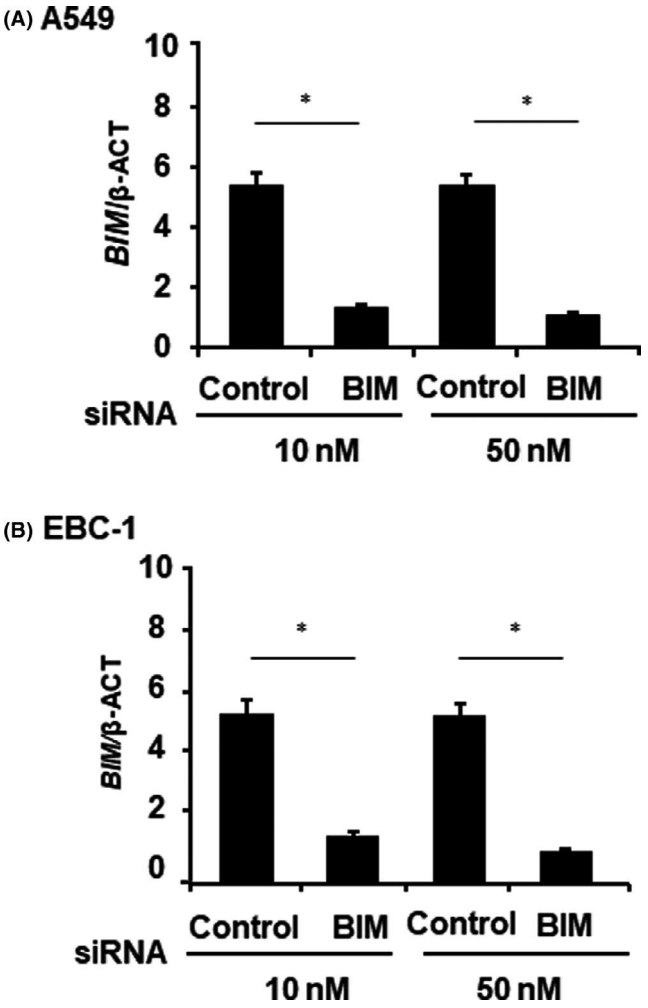

FIGURE 3 Evaluation of the knock-down efficiency of siRNA against BIM. Expression levels of *BIM* mRNA in siRNA treated (A) A549 or (B) EBC-1 cells were evaluated by quantitative real-time polymerase chain reaction. Data represent the mean values of four samples (± standard deviation) and were analyzed with Student's *t* test. $^*P < .01$

3.6 | Suppressed expression of BIM in lung cancer cells attenuates the degree of apoptosis induced by irradiation

To investigate whether the inhibition of the radiation-induced anti-tumor effect in cancer cells is associated with apoptosis, we compared the caspase 3/7 activity or annexin-V expression levels between the BIM-siRNA and control groups after irradiation. We showed that the degree of caspase activity was significantly lower in the BIM-siRNA group than in the control group (Figure 4C,D). In addition, we carried out Annexin V-FIITC and Ethidium Homodimer III double staining to observe the early apoptotic effect. In A549 and EBC-1, the number of cells showing early apoptosis was significantly lower in the BIM-siRNA than in the control group (Figure 5). Conversely, the degree of apoptosis in the chemotherapy-treated cells was not significantly different between the BIM-siRNA and control groups (Figures S5A–D and S6A–D).

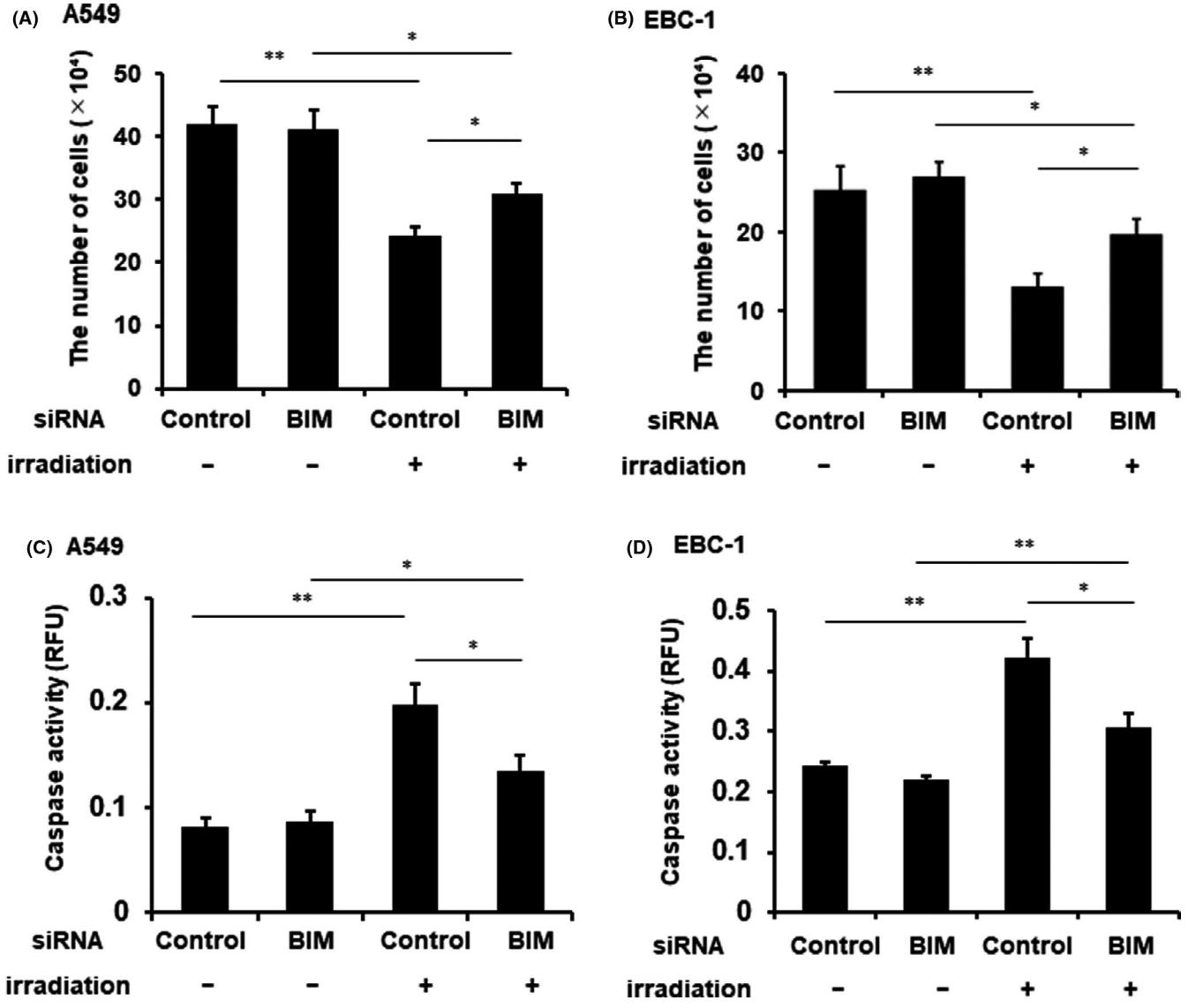

FIGURE 4 Number of (A) A549 or (B) EBC-1 cells knocked down by siRNA or control after 30-Gy irradiation. Assessment of apoptosis using caspase activity in (C) A549 or (D) EBC-1 cells after irradiation or control were examined as described in Materials and Methods. The data presented in Figures A-D represent the mean values of four samples (± standard deviation) and were analyzed with Student's *t* test. RFU, relative fluorescent unit, $^{*}P < .05$, $^{**}P < .01$

3.7 | Effect of the presence or absence of BIM polymorphism on anti-PD-L1 or PD-1 treatment

As anti-PD-L1 antibody is used in the standard treatment of locally advanced NSCLC,[2] we examined the effect of *BIM* polymorphism on the treatment with ICI including anti-PD-L1/PD1 antibodies. There were 13 (17.6%) patients with *BIM* polymorphism who underwent anti-PD-L1 or PD-1 treatment. Patients' backgrounds with and without *BIM* polymorphism were not significantly different (Table 4). Univariate and multivariate Cox regression analyses are shown in Table S2. In the univariate Cox regression model of survival time, EGFR mutation-positivity was a significant predictor of survival time after anti-PD-L1/PD-1 treatment (hazard ratio [95% CI]: 2.297 [1.007-5.238], $P = .048$). In the multivariate analysis, EGFR mutation-positivity was a significant independent predictor (2.413 [1.008-5.777], $P = .048$). Additionally, *BIM* polymorphism was not a significant prognostic factor. PFS and survival time after anti-PD-L1/PD-1 were not significantly different between the *BIM* polymorphism and wild-type groups (Figure 6).

4 | DISCUSSION

In the present study, we showed that PFS and OS durations were significantly shorter in CRT-treated patients with NSCLC with *BIM* polymorphism than in those without. In addition, the multivariate analyses showed that *BIM* polymorphism was an independent predictor of poor anti-tumor effect and prognoses in relation to CRT. Our findings also indicated that *BIM* polymorphism was associated with radiotherapy efficacy, but not with chemotherapy, in patients

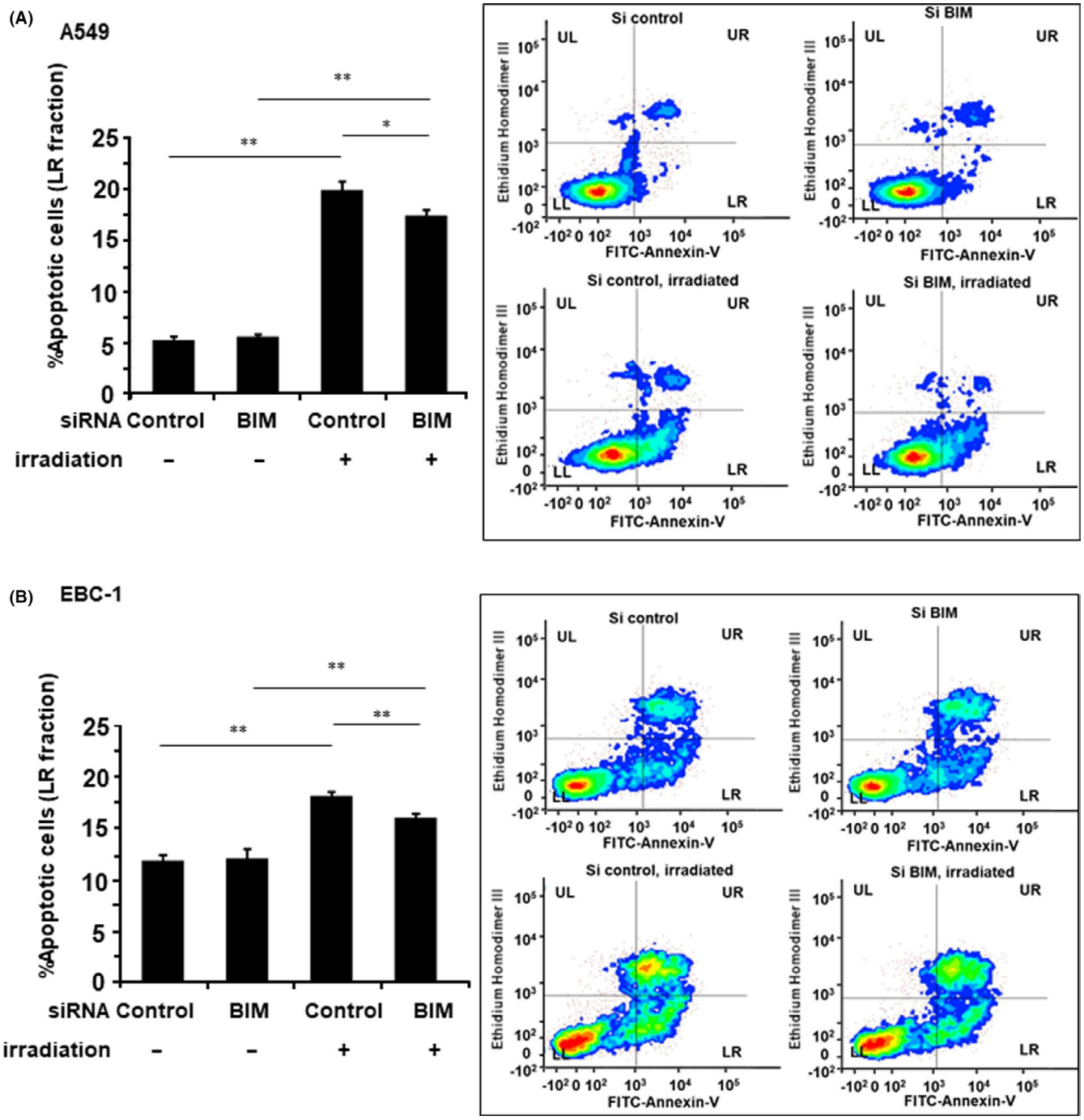

FIGURE 5 Left; assessment of apoptosis using annexin-V expression level in (A) A549 or (B) EBC-1 cells knocked down by siRNA or control after irradiation or control were examined. Right: representative flow cytometric plots. The data presented in Figures represent the mean values of four samples (± standard deviation) and were analyzed with Student's *t* test. UL, upper left: necrosis (Annexin V -/Ethidium Homodimer III +); UR, upper right: late apoptosis (Annexin V +/Ethidium Homodimer III +); LR, lower right: early apoptosis (Annexin V +/Ethidium Homodimer III -) and LL, lower left: viable cells (Annexin V -/Ethidium Homodimer III -), $^{*}P < .05$, $^{**}P < .01$

with CRT. This result was confirmed through in vitro experiments. In addition, this study showed that the effect of anti-PD-L1 (or PD-1) treatment, which is used as consolidation treatment after CRT, is not dependent on the presence of *BIM* polymorphism.

To the best of our knowledge, this study is the first to clarify that *BIM* polymorphism is associated with the efficacy of CRT in patients with NSCLC. Several previous studies have shown that the effect of EGFR-TKI was attenuated to a greater degree in patients with NSCLC with *EGFR* mutation and *BIM* polymorphism than in those without.[19] Other works have focused on the way in which *BIM* is involved in the apoptosis induced by radiotherapy in vitro using cell lines.[26,27] Our study showed that the effect of CRT, particularly the effect of radiotherapy, decreased in patients with *BIM* polymorphism. Therefore, it should be considered that the anti-tumor effect

TABLE 4 Comparison of characteristics of patients who underwent anti-PD-L1/PD-1 treatment between the BIM gene deletion polymorphism-positive and -negative groups

Patient characteristics	*BIM* deletion (-) *n* = 61	*BIM* deletion (+) *n* = 13	*P* value
Age (years)			
Median (range)	71 (40-90)	68 (42-83)	.135
Sex			
Male/Female	42/19	11/2	.252
Smoking amount, Pack-years			
Median (range)	45 (0-140)	50 (13.5-80)	.404
ECOG PS			
0/1/2	23/31/5	3/8/2	.422
Histology			
Ad/Sq/Others	45/8/8	9/3/1	.605
Stage			
III/IV, recurrence	16/45	4/9	.6051
EGFR mutation			
Positive/negative/not tested	7/52/2	0/13/0	.336
EGFR-TKI before or after anti-PD-L1/PD-1 treatment			
±	8/53	0/13	.167
Prior line of therapy			
0/1/2/≥3	12/19/16/14	2/3/3/5	.708

Abbreviations: Ad, adenocarcinoma; BIM, B-cell chronic lymphocytic leukemia (CLL)/lymphoma 2-like 11; EGFR, epidermal growth factor receptor; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand-1; Sq, squamous carcinoma.

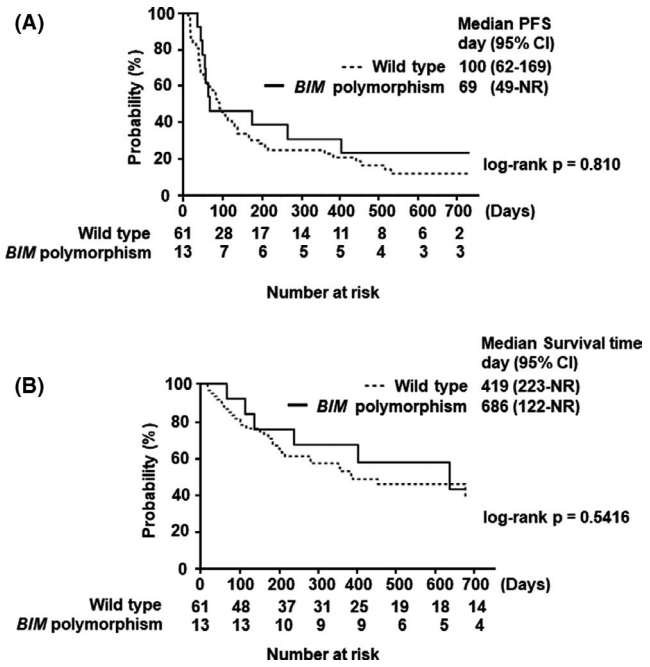

FIGURE 6 Kaplan-Meier survival curves for (A) progression-free survival, (B) survival time after anti-PD-L1/PD-1 treatment between*BIM*polymorphism-positive and -negative patients. CI, confidence interval; PD-L1, programmed death ligand-1; PFS, progression-free survival; NR, not reached

of CRT may be insufficient in the subsequent choice of treatment method. In contrast, it has been reported that a histone deacetylase inhibitor, vorinostat, can epigenetically restore BIM function and susceptibility of EGFR-TKI in *EGFR*-mutated NSCLC cells with *BIM* polymorphism.[21,28] Therefore, in a future study, we hope to investigate whether vorinostat could lead to improving the anti-tumor effect of CRT in patients with NSCLC with *BIM* polymorphism.

In the present study, there was a lack of an association between *BIM* polymorphism or depletion and chemotherapy efficacy in patients with locally advanced NSCLC or the experiments using cell lines. Several studies using cell culture models have shown that *BIM* plays a role in the apoptosis of cancer cells by PTX or VNR.[8-11,23,29] Conversely, *BIM* depletion studies using siRNA showed that BIM is not associated with cytotoxicity of PTX in breast cancer cells,[30] similar to our results. In addition, an in vivo study showed that *BIM* was not associated with PTX-induced apoptosis.[12] Separately, in our study, carboplatin or cisplatin in addition to PTX or VNR was given to the patients or cancer cells. The aforementioned facts could explain why *BIM* polymorphism or depletion was not associated with the effect of chemotherapy in our study.

Furthermore, we showed that *BIM* polymorphism was not associated with the anti-tumor effect exerted by ICI treatment. A previous study on patients with melanoma showed that the presence of *BIM* in CD8-positive T cells was involved in the anti-tumor effect of ICI treatment.[31] In that study, when PD-1 or PD-L1 reactions occurred

in tumor-reactive CD8-positive T cells, the degree of *BIM* expression was enhanced; the CD8-positive T cells caused apoptosis, resulting in an impossibility in eliminating the melanoma cells. Here, when the PD-1 or PD-L1 pathway was blocked by ICI, the BIM expression level in the T cells remained low. Thus, the degree of apoptosis of the T cells was suppressed and an anti-tumor effect was exerted. However, the *BIM* expression level in patients with *BIM* polymorphism would be low regardless of their ICI administration status. This may explain why the effect of ICI treatment between those with and without *BIM* polymorphism was not different. However, this finding requires further clarification through a further study to investigate the underlying mechanism.

The present study had some limitations. First, it had a retrospective design and a small sample size. Therefore, a prospective multicenter study with a larger number of participants is warranted for verification of our findings. Next, the number of patients treated with curative radiotherapy alone was very small. Therefore, we could not directly investigate the relationship between the presence of *BIM* polymorphism and the anti-tumor effect exerted by radiotherapy.

In conclusion, we showed that *BIM* polymorphism was independently predictive of poor anti-tumor effects and prognoses in locally advanced NSCLC patients treated with CRT, specifically radiotherapy. In the implementation of CRT in patients with NSCLC with *BIM* polymorphism, it is necessary to consider the subsequent treatment methods, keeping in mind that the effect of CRT may not be sufficient. Future studies are warranted to investigate whether histone deacetylase inhibitors have the ability to improve the anti-tumor effects of CRT in patients with NSCLC with *BIM* polymorphism.

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ORCID

Takeshi Masuda https://orcid.org/0000-0003-3557-0049
Hiroshi Iwamoto https://orcid.org/0000-0003-4922-5334

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SUPPORTING INFORMATION

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

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