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

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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.

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1 | INTRODUCTION

Lung cancer is the most lethal type of cancer, and is categorized into non-small (NSCLC) and small cell lung cancer. Of patients with NSCLC, approximately 33% have locally advanced NSCLC, the standard treatment for which is concurrent chemoradiotherapy (CRT). 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. Therefore, CRT followed by durvalumab treatment is used as the standard treatment for patients with locally advanced NSCLC. 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. 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. In addition, the cytotoxicity of PTX and VNR used in CRT, is also induced through apoptosis. Apoptosis is characterized by extrinsic and intrinsic pathways. The extrinsic pathway is induced by death receptors (e.g., 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.

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 II. 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.

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. Several studies have shown that BIM affects the induction of apoptosis by EGFR-TKI treatment. 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. Therefore, in patients with BIM polymorphism, intrinsic resistance to EGFR-TKI is observed. In fact, the median PFS duration associated with EGFR-TKI treatment is significantly shorter in the BIM polymorphism than in the wild-type group.

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. 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% CO2 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: 5′-CCACCAATGGAAAGGTTCA-30; reverse: 5′-CTGTCATTCTCCACCCACAC-30) and deletion alleles (forward: 5′-CCACCAATGGAAAGGTTCA-30; reverse: 5′-GGCACAGGCTCTATGGGAGAA30) (Thermo Fisher Scientific, Waltham, MA, USA), according to a previous study. The presence or absence of the wild-type BIM allele and the BIM polymorphism allele was confirmed by melt curve analysis (Figure S2).

2.5 Knockdown of BIM

Small interfering RNA duplexes targeting BIM (BIM-siRNA) (forward: 5′-CCUUCCUGUAUGUUCUGtt-3′; reverse: 5′-CAGAACUUGAUCAGGAAGGtt-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 0.25 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.
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

![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.](image)

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

| Patient characteristics | CRT group | Chemotherapy group | P value | P value |
|-------------------------|-----------|--------------------|---------|---------|
|                         | BIM deletion (-) n = 73 | BIM deletion (+) n = 15 | 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. |
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).

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

| Patient characteristics                  | PFS Hazard ratio [95% CI] | P value | OS Hazard ratio [95% CI] | P value |
|-----------------------------------------|---------------------------|---------|--------------------------|---------|
| **Univariate analysis**                 |                           |         |                          |         |
| Age, years                              |                           |         |                          |         |
| \( \geq 75 \)                            | 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              |                           |         |                          |         |
| \( \geq 40 \)                            | 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.

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 isofrom with the BIM polymorphism in humans. 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).

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-FITC 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).
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/PD-1 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...
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

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**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
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. 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. Conversely, BIM depletion studies using siRNA showed that BIM is not associated with cytotoxicity of PTX in breast cancer cells, similar to our results. In addition, an in vivo study showed that BIM was not associated with PTX-induced apoptosis. 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. In that study, when PD-1 or PD-L1 reactions occurred

| 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 (+) | BIM deletion (-) | P value |
|--------------------------|------------------|------------------|--------|
| Age (years)              | n = 61           | n = 13           |        |
| Median (range)           | 71 (40-90)       | 68 (42-83)       | .135   |
| Sex                      | Male/Female      | 42/19            | .252   |
| Smoking amount, Pack-years | Median (range) | 45 (0-140) | 50 (13.5-80) | .404 |
| ECOG PS                  | 0/1/2            | 23/31/5          | .422   |
| Histology                | Ad/Sq/Others     | 45/8/8           | .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      | .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.
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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DISCLOSURE

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REFERENCES

1. [1] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin. 2019; 69: 7-34.
2. Chen VW, Ruiz BA, Hsieh MC, Wu XC, Ries LA, Lewis DR. Analysis of stage and clinical/prognostic factors for lung cancer from SEER registries: AJCC staging and collaborative stage data collection system. Cancer. 2014;120(Suppl 23):3781-3792.
3. NCCN.org. Non-Small Cell Lung Cancer. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) 2020; Version 6.2020. https://www.nccn.org/professionals/physician_gls/pdf/nscl.pdf. Accessed June, 26 2020.
4. Antonia S, Villegas A, Daniel D, et al. Durvalumab after chemotherapy in stage III non-small-cell lung cancer. N Engl J Med. 2017;377:1919-1929.
5. Galluzzi L, Vitale I, Warren S, et al. Consensus guidelines for the definition, detection and interpretation of immunogenic cell death. J Immunother Cancer. 2020;8:e000337.
6. Meng X, Feng R, Yang L, Xing L, Yu J. The role of radiation oncology in immuno-oncology. Oncologist. 2019;24:542-552.
7. Gudkov AV, Komarova EA. The role of p53 in determining sensitivity to radiotherapy. Nat Rev Cancer. 2003;3:117-129.
8. Huang L, Snyder AR, Morgan WF. Radiation-induced genomic instability and its implications for radiation carcinogenesis. Oncogene. 2003;22:5848-5854.
9. Sunters A, Fernández de Mattos S, Stahl M, et al. FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. J Biol Chem. 2003;278:49795-49805.
10. Li R, Moudgil T, Ross HJ, Hu HM. Apoptosis of non-small-cell lung cancer cell lines after paclitaxel treatment involves the BH3-only proapoptotic protein Bim. Cell Death Differ. 2005;12:292-303.
11. Tan TT, Degenhardt K, Nelson DA, et al. Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. Cancer Cell. 2005;7:227-238.
12. Klotz DM, Nelson SA, Krobeth K, et al. The microtubule poison vinorelbine kills cells independently of mitotic arrest and targets cells lacking the APC tumour suppressor more effectively. J Cell Sci. 2012;125:887-895.
13. Miller AV, Hicks MA, Nakajima W, Richardson AC, Windle JJ, Harada H. Paclitaxel-induced apoptosis is BAK-dependent, but BAX and BIM-independent in breast tumor. PLoS One. 2013;8:e60685.
14. Singh R, Letai A, Sarosiek K. Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins. Nat Rev Mol Cell Biol. 2019;20:175-193.
15. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol. 2008;9:47-59.
16. Ng KP, Hillmer AM, Chuah CT, et al. A common BIM deletion polymorphism mediates intrinsic resistance and inferior responses to tyrosine kinase inhibitors in cancer. Nat Med. 2012;18:521-528.
17. Costa DB, Halmos B, Kumar A, et al. BIM mediates EGFR tyrosine kinase inhibitor-induced apoptosis in lung cancers with oncogenic EGFR mutations. PLoS Medicine. 2007;4:1669-1679. discussion 1680.
18. Cragg MS, Kuroda J, Puthalakath H, Kumar A, Strasser A. Gefitinib-induced killing of NSCLC cell lines expressing mutant EGFR requires BIM and can be enhanced by BH3 mimetics. PLoS Medicine. 2007;4:1681-1689. discussion 1690.
19. Gong Y, Somwar R, Politii K, et al. Induction of BIM is essential for apoptosis triggered by EGFR kinase inhibitors in mutant EGFR-dependent lung adenocarcinomas. PLoS Medicine. 2007;4:e294.
20. Soh SX, Siddiqui FJ, Allen JC, et al. A systematic review and meta-analysis of individual patient data on the impact of the BIM deletion polymorphism on treatment outcomes in epidermal growth factor receptor mutant lung cancer. Oncotarget. 2017;8:41474-41486.
21. Goldstraw P, Crowley J, Chansky K, et al. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumours. J Thorac Oncol. 2007;2:706-714.
22. Tanimoto A, Takeuchi S, Arai S, et al. Histone deacetylase 3 inhibition overcomes BIM deletion polymorphism-mediated osimertinib resistance in EGFR-mutant lung cancer. Clin Cancer Res. 2017;23:3139-3149.
23. Kawada K, Yonei T, Ueoka H, et al. Comparison of chemosensitivity tests: clonogenic assay versus MTT assay. *Acta Med Okayama*. 2002;56:129-134.

24. Savry A, Carre M, Berges R, et al. Bcl-2-enhanced efficacy of microtubule-targeting chemotherapy through Bim overexpression: implications for cancer treatment. *Neoplasia (New York, NY)*. 2013;15:49-60.

25. Yanagihara N, Kobayashi D, Kuribayashi K, Tanaka M, Hasegawa T, Watanabe N. Significance of SALL4 as a drug-resistant factor in lung cancer. *Int J Oncol*. 2015;46:1527-1534.

26. Izumi Y, Nakashima T, Masuda T, et al. Suplatast tosilate reduces radiation-induced lung injury in mice through suppression of oxidative stress. *Free Rad Biol Med*. 2019;136:52-59.

27. O’Connor L, Strasser A, O’Reilly LA, et al. Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J*. 1998;17:384-395.

28. Yang JY, Xia W, Hu MC. Ionizing radiation activates expression of FOXO3a, Fas ligand, and Bim, and induces cell apoptosis. *Int J Oncol*. 2006;29:643-648.

29. Nakagawa T, Takeuchi S, Yamada T, et al. EGFR-TKI resistance due to BIM polymorphism can be circumvented in combination with HDAC inhibition. *Cancer Res*. 2013;73:2428-2434.

30. Li Z, Zhang J, Liu Z, Woo CW, Thiele CJ. Downregulation of Bim by brain-derived neurotrophic factor activation of TrkB protects neuroblastoma cells from paclitaxel but not etoposide or cisplatin-induced cell death. *Cell Death Differ*. 2007;14:318-326.

31. Czernick M, Rieger A, Goping IS. Bim is reversibly phosphorylated but plays a limited role in paclitaxel cytotoxicity of breast cancer cell lines. *Biochem Biophys Res Commun*. 2009;379:145-150.

32. Dronca RS, Liu X, Harrington SM, et al. T cell Bim levels reflect responses to anti-PD-1 cancer therapy. *JCI Insight*. 2016;1:e86014.

**SUPPORTING INFORMATION**

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

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