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RESEARCH

The role of BIM gene deletion in ALK-mutated Non-small cell lung cancer treated with alectinib

Shuang Hou[1] · Weijun Zhang[2] · Wei Pang[1] · Haiqun Xia[1] · Jinyun Tan[1] · Qingfang Huang[1] · Ping Yang[1]

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Abstract

Alectinib, as a first-line therapeutic option for advanced ALK mutation-positive non-small-cell lung cancer (NSCLC), is now widely used in the clinic. However, the associated mechanisms of resistance are unknown. The first documented case of ALK-mutated NSCLC's resistance to alectinib is herein reported in relation to BIM gene deletion status. In particular, cell inhibition assay (CCK8 assay), cell transfection, fluorescence microscopy, RT-PCR, cell proliferation assay, cell migration assay and western blotting were undertaken for exploring the link between BIM status and alectinib resistance. Clinical cases showed that the BIM gene was absent in alectinib-resistant tumor tissues. Further experimental validation yielded that NSCLC with deleted BIM genes were less sensitive to aleitinib. BIM gene deletion can increase resistance to alectinib, and the potential efficacy of a combination of BIM sensitizer and alectinib to overcome alectinib resistance can be explored.

Keywords Non-small lung cancer · Drug resistance · ALK-mutated · B-cell lymphoma 2 (BCL-2)-like 11 (BIM) gene · Alectinib

Introduction

Lung cancer represents one of the most prevalent and lethal types of cancer. Among its various subtypes, a subgroup of NSCLC, which accounts for 2–7% of cases, harbor the EML4-ALK fusion-type protein tyrosine kinase [1, 2]. For patients with advanced ALK-positive NSCLC, alectinib is recognized as a superior option over crizotinib due to its enhanced efficacy and tolerability, median Progression Free Survival (PFS) 42.3 months in patients with brain metastases at baseline in the ALESIA study, with the NCCN guidelines also recommending it for first-line treatment [3, 4]. However, despite initial responses, eventual tumor relapse can be a challenge, especially since the underlying mechanisms of alectinib resistance remain unclear.

B-cell lymphoma 2 (BCL-2)-like 11 (BIM) protein, a member of the Bcl-2 family, was discovered by O'Connor et al. in 1998 [5]. Being a critical mediator of the apoptotic response to anticancer therapy, BIM's upregulation can induce tumor cell apoptosis, thereby highlighting its antitumor effects [6, 7]. For instance, in EGFR-mutated NSCLC patients, BIM gene deletion polymorphisms have been associated with lower Overall Survival (OS), PFS and Objective Response Rate (ORR) in comparison with those without this polymorphism [8]. Similarly, in ALK fusion-positive NSCLC patients, such polymorphisms have been associated with poor clinical response to crizotinib [9].

Herein, the case of a patient with BIM gene deletion polymorphism as well as the ALK fusion-protein is presented, and in this case, only eight months of PFS was achieved following alectinib treatment, much lower than the PFS data from the ALESIA study. To the best of the current authors' knowledge, this is the first documented instance of an ALK fusion-positive NSCLC patient that showcases how alectinib efficacy, and eventually disease prognosis, is impacted by BIM gene deletion polymorphism. In fact, in vitro cell experiments further underscored the influence

Shuang Hou and Weijun Zhang have contributed equally to this work and share first authorship.

✉ Ping Yang
150077677@qq.com

[1] Department of Radiation Oncology, DongGuan SongShan Lake Tungwah Hospital, DongGuan, China

[2] Departments of Radiation Oncology, Cancer Center of Guangzhou Medical University, Guangzhou, Guangdong, China

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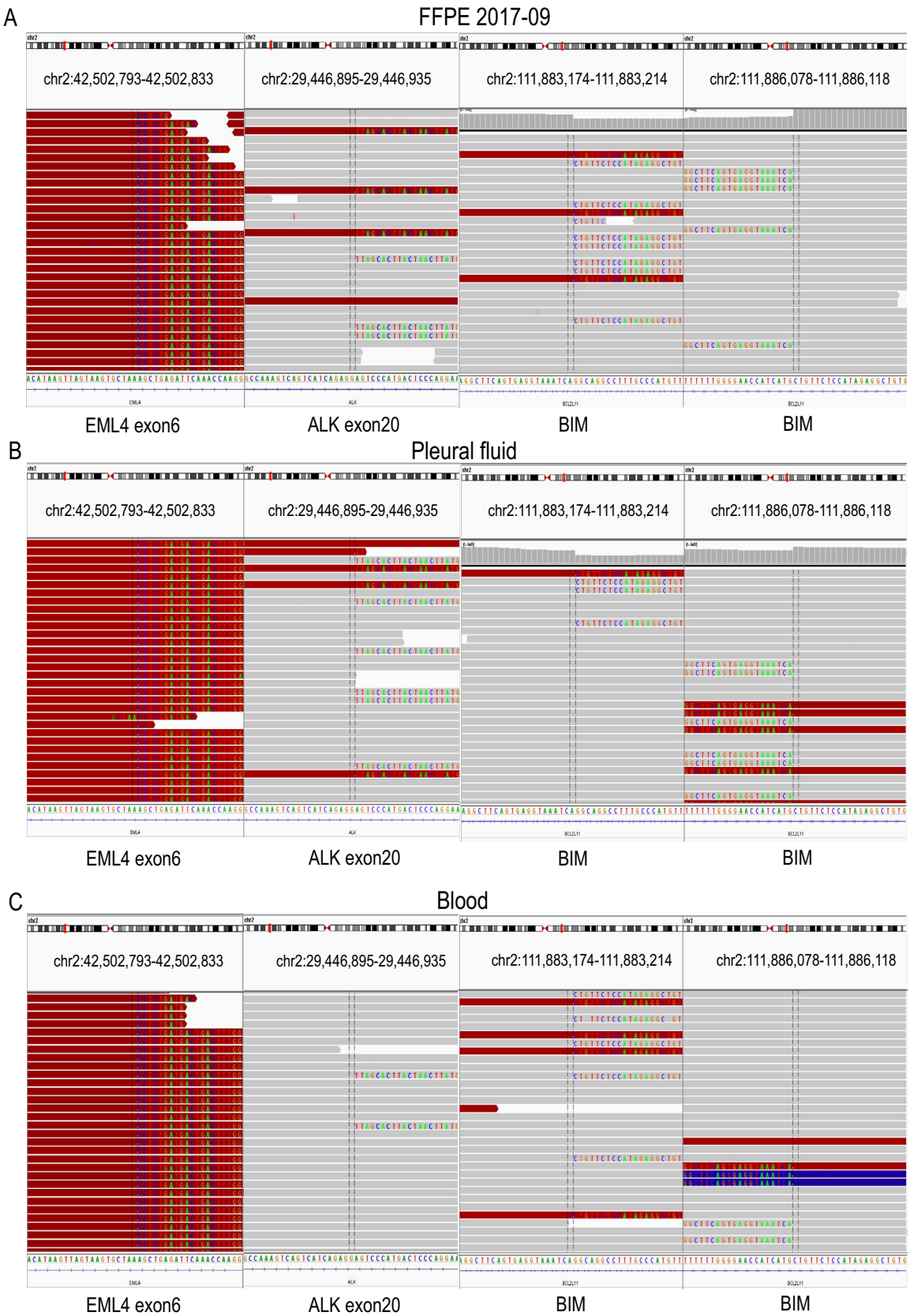
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◂**Fig. 1** **A** Tissue samples were taken on September 2017, **B** pleural fluid samples were collected on September 2019, and **C** blood was taken on September 2019. EML4: exon 6 ~ ALK: exon 20 fusion and BIM heterozygous deletion polymorphism mutations detected were found in all samples

of such polymorphism on alectinib's efficacy in ALK fusion-positive NSCLC cell lines.

Case presentation

The case involved a 45-year-old nonsmoking Chinese male diagnosed with stage IIIA (pT1N2M0) lung adenocarcinoma harboring both EML4-ALK gene fusion and BIM gene deletion polymorphism after resection of the left lower lobe in September 2017 (Fig. 1A). The patient then underwent chest radiation (56 Gy/28 fractions) alongside four cycles of pemetrexed plus cisplatin (PP) from late October to early January of the following year. This was followed by regular follow-ups every three months.

Taking into account the above, alectinib treatment was discontinued. With a Performance Status (PS) score of three, the patient subsequently underwent four cycles of docetaxel along with intermittent pleural effusion drainage, resulting in stable disease (SD) in lung lesions for three months (from September to December) (Fig. 2). Unfortunately, he succumbed to respiratory failure in March 2020. The patient had provided written informed consent for the anonymous use of his data for educational and research purposes.

Materials and methods

Cell culture

Human non-small cell lung adenocarcinoma (H2228) cell lines that were positive for the EML4-ALK fusion gene were acquired from the American type culture collection (ATCC, USA). Cell cultures were established in RPMI 1640 medium (Gibco) containing 1% penicillin and streptomycin (Invitrogen, USA) as well as 10% fetal bovine serum (FBS, Gibco). They were then incubated in a humidified incubator at 37 °C and under 5% CO_2.

Materials

Alectinib was obtained from Baiao Biomedical Technology (Beijing, China), while RPMI-1640 and FBS were acquired from Gibco (MD, USA). The CCK8 Kit was purchased from Dojindo Laboratories (Kyushu, Japan), with Transwell chambers and Annexin V-FITC/PI obtained from Beyotime Biotechnology (Shanghai, China). Primary antibodies against BIM, GAPDH and secondary antibodies were purchased from Abcam (Cambridge, UK).

Methods

CCK8 assay

After seeding H2228 cells (3.0×10^4 cells/mL) into a 96-well plate, they were allowed to attach prior to a 48-h of treatment with different concentrations of alectinib (0, 0.01, 0.1 and 1 μM). This was followed by the addition of 10 μl of CCK8 solution (Sigma, MO, USA) to each well before another 2-h incubation. A microplate reader was eventually used to record absorbance readings at 450 nm (Molecular Devices, CA, USA).

Cell transfection

To construct stable transgenic plants with silenced BIM gene, cells were transfected with shBIM. The resulting cell line was named H2228/BIM cell line to refer to the H2228 cell line with deleted BIM gene.

Fluorescence microscopy

Following transfection, 2×10^5 cells were seeded into each well of a six-well plate. They were kept in a 37 °C incubator for 48 h, after which an inverted fluorescence microscope was used to visualize the cells.

RT-PCR

BIM expression was assessed by RT-PCR, and for this purpose, RNA was first extracted from the cells using Trizol (Invitrogen) as required by the available instructions. After reverse transcription into cDNA with a high-capacity cDNA Synthesis kit (Applied Biosystems, CA, USA), qPCR was performed on an Applied Biosystems 7500 Real-Time PCR instrument (Thermo Fisher Scientific Inc., UK) using a SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd.). The BIM primers were 5′- GGGTA CTTCATTGA TGCCACAA-3′ and 5′-GCTCAGTGTAGCCCAGGAT-3′ PCR product. β-actin acted as the internal control, with the primers being 5′-GTCCACCGCAAATGCTTCTA-3′ and 5′- TGCTGTCACCTTCACCGTTC- 3′.

Cell proliferation assay

The CCK8 assay was used to determine cell proliferation. After transfection using different lentiviruses or control constructs, the resulting cells were added to 96-well plates (10×10^3 /ml) for a 48-h incubation. CCK8 solution (10 μl; Sigma, MO, USA) was then added to each well and after

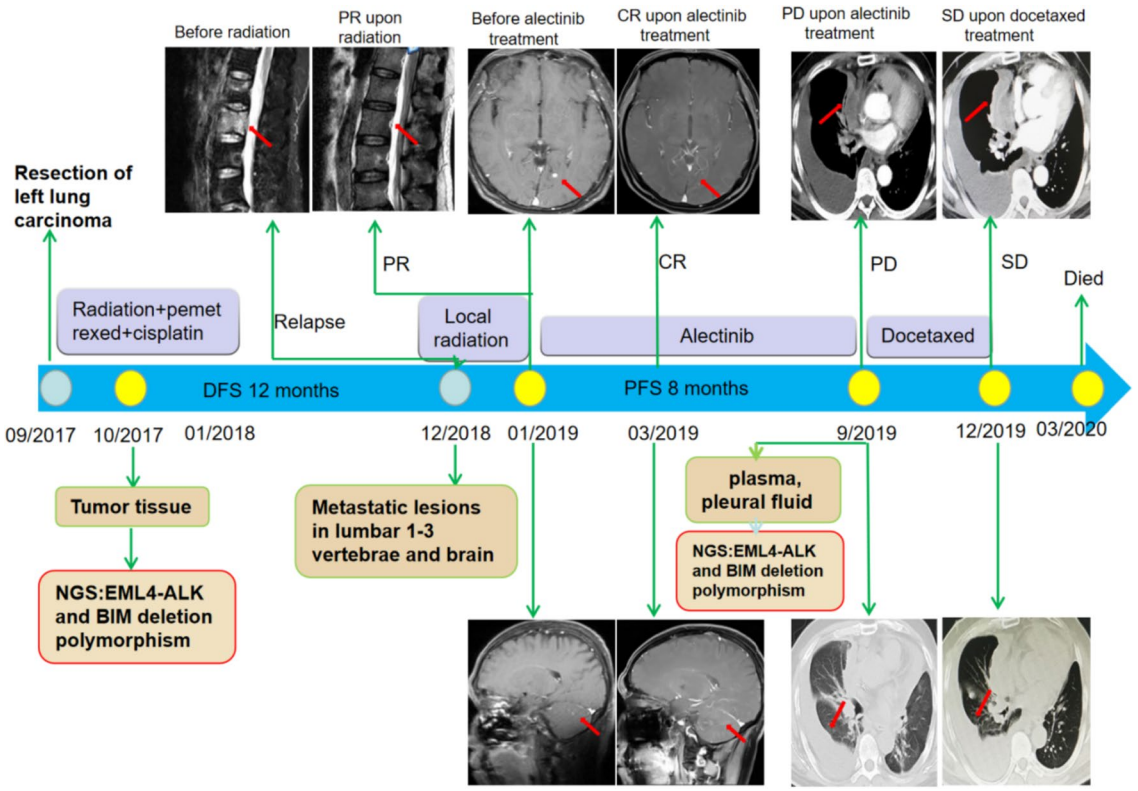

Fig. 2 Timeline summarizing the therapeutic history for a non-small-cell lung cancer patient. Brain and lumbar 1–3 vertebrae lesions (red arrows) as observed on MRI scans. Massive right pleural effusion as well as multiple pleural and right lung metastasis as obtained by CT images (red arrows). Abbreviations: NGS: next-generation sequencing; PFS: progression-free survival; DFS: disease-free survival; SD: stable disease; PD: progressive disease; PR: partial response; CR: complete response (Color figure online)

incubation for 2 h, absorbance readings were recorded at 450 nm using a microplate reader (Molecular Devices, CA, USA).

Cell migration assay

Following transfection, H2228 cells were harvested and resuspended in serum-free media at a concentration of 3.0×10^4 cells/mL. In a Transwell insert, the harvested cells and culture media were added to the upper and lower chambers respectively. This was followed by a 24-h incubation, after which the insert was rinsed thrice with PBS, fixed for 15 min with methanol, and eventually used to analyze cellular migration by counting the cells in five random fields of view per insert.

Flow cytometry

After seeding H2228 cells into six-well plates, cell transfection was carried out prior to a 48-h treatment with alectinib. This was followed by staining using Annexin V-FITC and PI for 15 min, with flow cytometry eventually performed on a flow meter (FACSVerse, Becton, Dickinson and Company, US) for cell cycle analysis.

Western blotting

After cell lysis in RIPA buffer (Sigma Aldrich), the concentration of the resulting proteins was determined using a spectrophotometer (Thermo Fisher Scientific, USA). This was followed by electrophoresis to separate the proteins prior to their transfer to a PVDF membrane (Millipore). The latter was then blocked with a quick sealant, after which a 4-h incubation was performed at 25 ± 1 °C with rabbit primary antibodies. The protein bands were eventually developed using a ChemiDoc XRS imaging system (Upland, USA) for visualization.

NGS

RNA was isolated from FFPE tissue using Ambion RecoverAll Total Nucleic Acid Isolation Kit for FFPE. Ten nanograms of RNA for each sample was processed by using the Ion AmpliSeq RNA Library Kit and the Ion AmpliSeq RNA Lung Fusion Panel. This panel targets, in a single assay, nine

acceptor driver genes (EGFR, ALK, ROS1, KRAS, BRAF, RET, HER2, NRAS and PIK3CA) in addition to many donor genes and includes 5′ and 3′ ALK, ROS1, and RET gene expression assays, as an indicator of a translocation at these genes, and five expression control genes. Quantified libraries were sequenced on Ion 316 chip. Data were analyzed with integrated workflows in Ion Reporter Software 4.2.

Statistical analysis

Each experiment involved a minimum of three replicates, with the results subsequently provided as mean values ± SD. Half maximal inhibitory concentration(IC50) of alectinib for the H2228 cell line was calculated using logistic model and Pearson's fit test. Multiple group comparisons were then analyzed by one-way ANOVA, while treatments were compared with the control using Student's t test. Results were considered significant for $p < 0.05$.

Results

Alectinib's IC50 and construction of BIM knockdown H2228 cell line

Based on the CCK8 assay, the IC50 of alectinib for H2228 calculated by Pearson's fit test and logistic regression based on the number of cells at different alectinib concentrations, was found to be around 0.13 μM (Fig. 3A). BIM was knocked down in H2228 via shBIM lentivirus transfection, with immunofluorescence test as well as RT-PCR further confirming successful transfection (Fig. 3B). BIM expression was significantly down-regulated in H2228/BIM (Fig. 3C) compared with the regular H2228 cell line,.

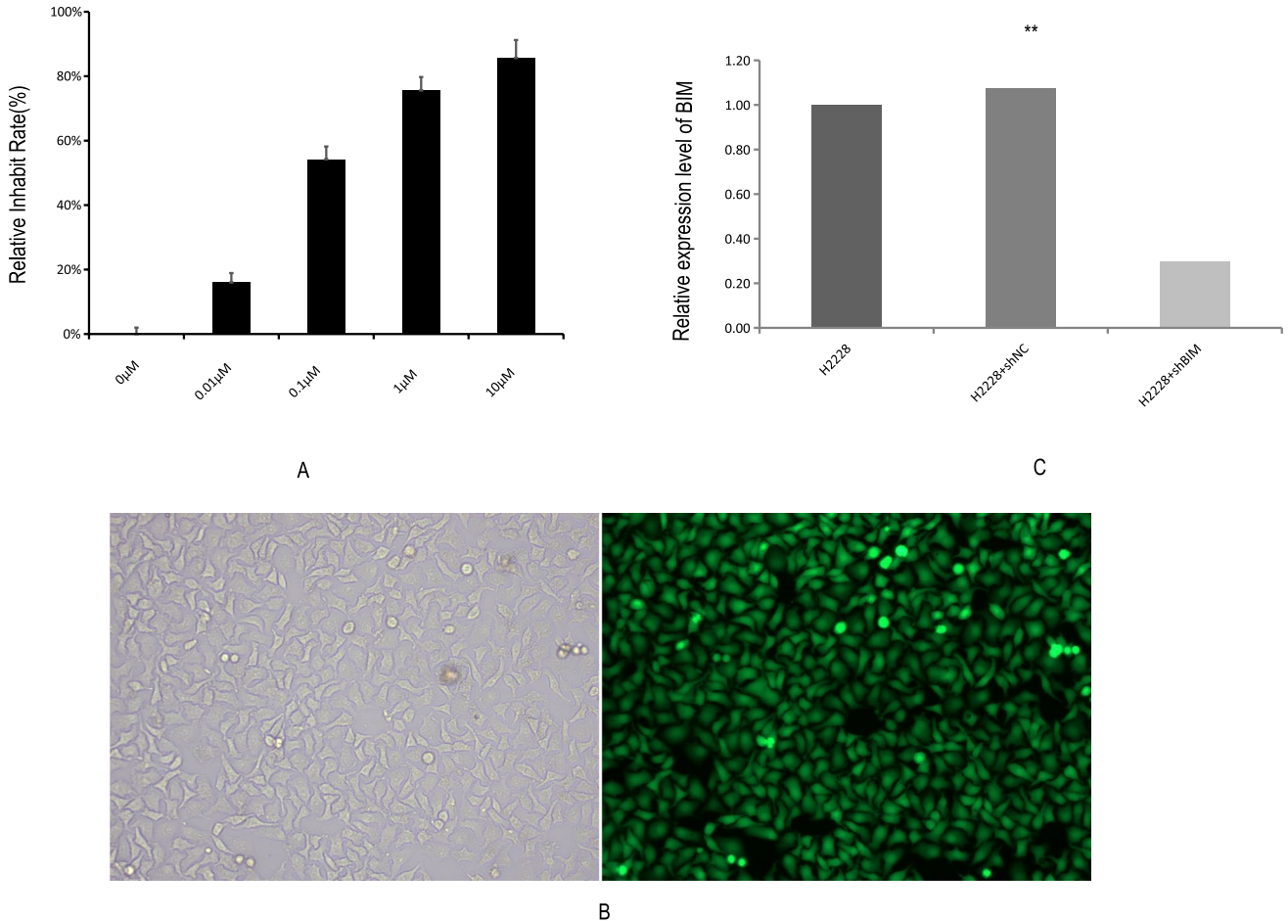

Fig. 3 **A** Extent of inhibition of H228 cell line by different concentrations of alectinib. The *X*-axis is the different concentrations of alectinib and the *Y*-axis is the relative degree of inhibition in the H228 line of cells. The inhibition rate was 15.99% at 0.01 uM concentration, 54.22% at 0.1 uM concentration, 75.6% at 1 uM concentration and 85.65% at 10 uM concentration. The IC50 value of alectinib against H228 cell line was further measured by logsitic modeling to be about 0.13 uM. **B** H2228 cell line and its fluorescence photo figure. **C** BIM expression levels in the H2228 cell line and the H2228/BIM cell line in RT-PCR experiments

BIM knockdown increased the chemoresistance of H2228 cells to alectinib in terms of proliferation and metastasis.

To investigate the relationship between BIM knockdown and alectinib chemoresistance, H2228 and H2228/BIM cells were treated with 0.13 μM of the compound for 48 h. Subsequent CCK-8 assays revealed that the knockdown enhanced the proliferative activity of H2228 relative to control cells (Fig. 4A). In addition, H2228 cell migration was also markedly impaired after BIM knockdown (Fig. 4B). Therefore, knocking down the BIM can potentially block alectinib's inhibitory effects on the proliferation and metastasis of H2228 cells.

BIM knockdown increased the chemoresistance of H2228 cells to alectinib in terms of apoptosis.

To confirm the functional relevance of BIM and alectinib interactions in H2228 cell line, the apoptosis rate of H2228 and H2228/BIM cells was assessed by flow cytometry after a 48-h treatment with alectinib (0.13 μM). The results demonstrated that, compared with the control group, H2228/BIM cells was less responsive to alectinib as reflected in the relatively low apoptosis rate (Fig. 5A). The results of western blotting further showed increased BIM expression after alectinib treatment in both cell lines, although the expression in H2228/BIM was still lower in comparison with that of H2228 (Fig. 5B). The results highlight alectinib's potential to inhibit the malignant progression of H2228 cell lines is dependent on the function of BIM, especially since BIM knockdown can increase the chemoresistance of H2228 cells to alectinib.

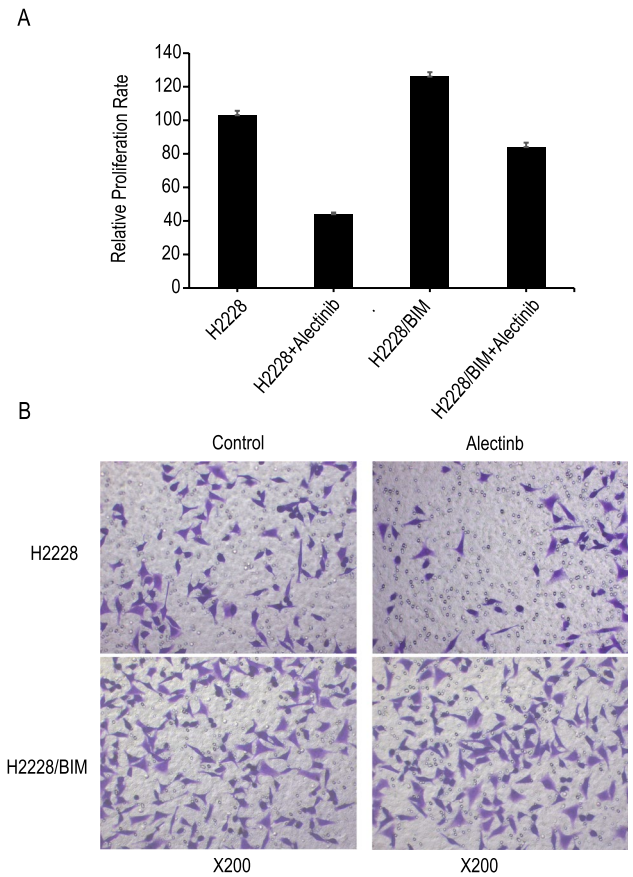

Fig. 4 Proliferative activity of H2228 cell line versus H2228/BIM cell line with/without alectinib. **A** Histogram of cell proliferation in different cell lines. **B** Cell proliferation was observed under the microscope. Magnification ratio: 200x

Discussion

BIM, an oncogene made up of six exons, is located on the human chromosome 2q12-q13 [10], and its proteins which are known to be proapoptotic, have three isoforms, namely Bim.S, Bim.L, and Bim.exL. As a member of the BCL-2 family, BIM is widely present in various normal cells, where it can be activated by certain apoptosis-stimulating signals. At this point, the activated BIM is released from the microtubule protein complex into the cytoplasm before being translocated to the mitochondrial membrane, where it induces the release of Bak and Bax through direct activation of Bak and Bax molecules or indirect binding to anti-apoptotic proteins in the Bcl-2 family (e.g., Mcl-1, Bcl-2, Bcl-xL, etc.). These proteins, in turn, bind to the outer mitochondrial membrane, causing mitochondrial outer membrane permeabilization that subsequently leads to the release of cytochrome C and other soluble proteins into the cytoplasm. In particular, the cytochrome C can bind to apoptotic protease activating factor-1 (Apaff-1), forming apoptotic vesicles which further activate the caspases cascade reaction to eventually induce apoptosis [11, 12]. BIM deletion polymorphism refers to the deletion of a 2903-bp segment between intron 2 and exon 3 which leads to splicing errors in exons 3 and 4. Therefore, the resulting BIM isoforms are not spliced correctly, causing variations on their levels of expression. At the same time, these altered BIM isoforms lack the proapoptotic BH3 structural domain, thereby preventing their proapoptotic functions [13].

BIM deletion polymorphisms tend to be prevalent in Asian populations, accounting for approximately 12.3–24.3% in NSCLC patients, but are less observed in European and African populations [14]. A number of multifactorial analyses have shown that BIM deletion polymorphisms are independent predictors of short OS and PFS in NSCLC patients receiving EGFR-TKIs-based treatment [15–17]. Furthermore, Li et al. reported that BIM deletion polymorphisms also reduced the effectiveness osimertinib-based therapy, a third-generation EGFR-TK [18]. Similarly,

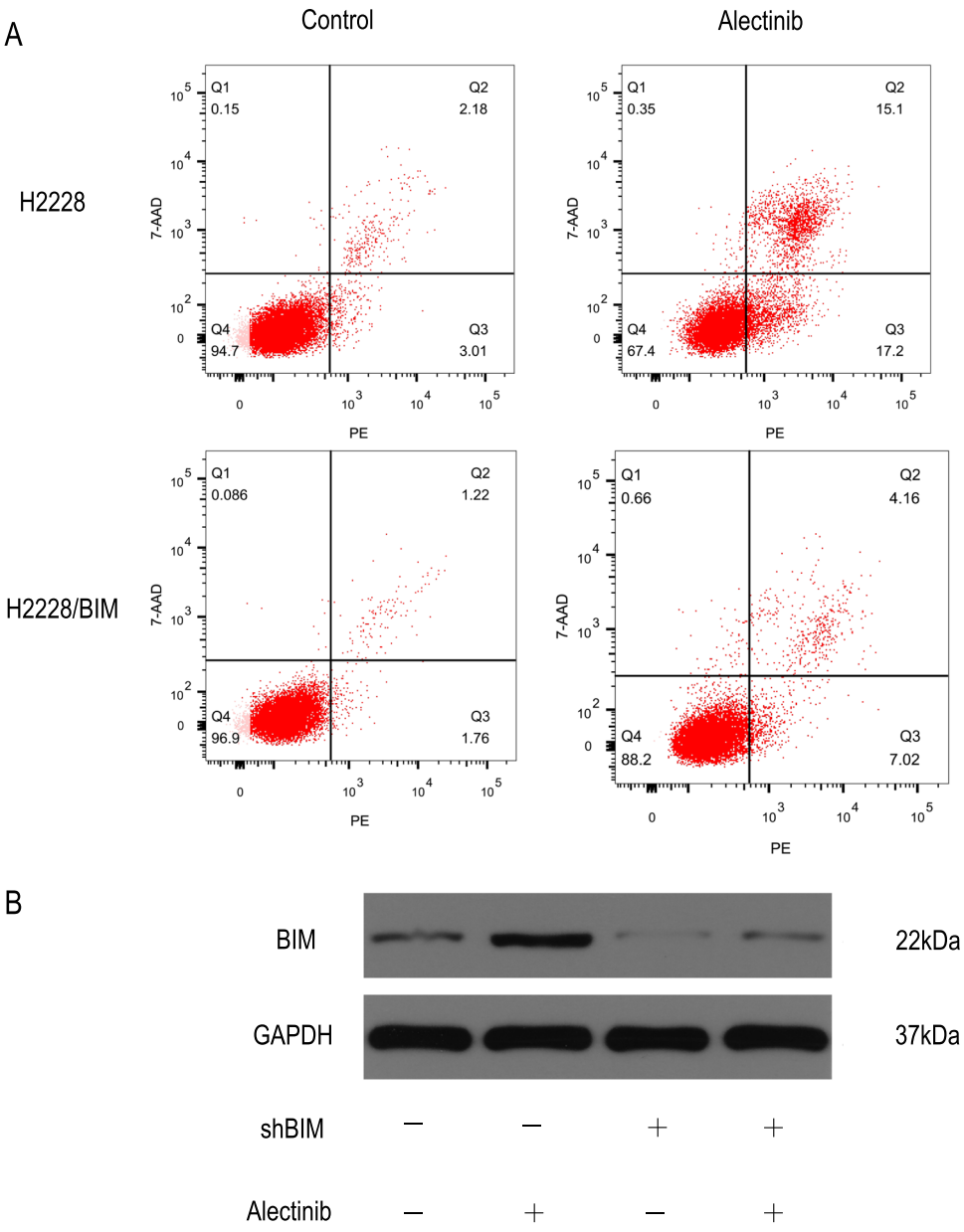

Fig. 5 Apoptosis of H2228 cells and H2228/BIM cells after 48 h of treatment with alectinib and control. **A** Survival of cells treated with alectinib or control in flow cell experiments. **B** Protein expression after treatment with alectinib or control in WB cells

when retrospectively investigating 69 NSCLC patients with ALK/ROS1 gene fusion who were on crizotinib treatment, Zhang et al. found the incidence of BIM deletion polymorphisms to be 13% (9/69). In particular, patients harboring these polymorphisms had lower PFS and ORR than those without the polymorphisms (median PFS 182 days vs. 377 days, $P=0.008$) (ORR 44.4% vs. 81.7%, $P=0.41$). In addition, multifactorial analysis of variance indicated that BIM deletion polymorphisms were independent predictors of crizotinib's efficacy in ALK-positive NSCLC patients [9]. In a similar way to the above, a number of other studies have also confirmed that such BIM polymorphisms were correlated to the efficacy of targeted therapy in NSCLC, with the polymorphisms also reducing the therapeutic efficacy of TKIs, thereby suggesting this could be a potential intrinsic resistance mechanism. However, it is yet to be reported whether BIM deletion polymorphisms also influence alectinib's therapeutic effectiveness in ALK-positive NSCLC patients. As such, this is the first report that highlights a PFS of only eight months for an advanced NSCLC patient (positive for EML4-ALK and BIM deletion polymorphism) after first-line oral treatment with alectinib. This value was, in fact, much less than the 34.8 months reported in the ALEX study [19].

There are various mechanisms of resistance to targeted therapy in driver gene-positive NSCLC, and these include secondary mutations, bypass activation, pathotype conversion and other EGFR resistance [20–22]. However, in

ALK-positive NSCLC, there are only few reports of BIM deletion polymorphisms leading to intrinsic resistance to TKIs, with the underlying mechanisms even less investigated, especially in the case of alectinib. The current study demonstrated that BIM was involved in the resistance of the malignant progression to alectinib in BIM-deficient NSCLC cell lines. Specifically, downregulating BIM increased the resistance of NSCLC to alectinib-induced apoptosis. Scholars have also reported that, in cases of EGFR-mutated NSCLC, where patients were sensitive to treatments with targeted agents such as gefitinib, EGFR and EGFR-TKIs-induced apoptosis was dependent on the upregulation of BIM [23]. Moreover, it has further been shown that a combination of erlotinib and the BH3 mimetic ABT-737 could up-regulate BIM expression to overcome resistance to erlotinib in epidermal growth factor receptor mutant NSCLC cells harboring BIM deletion polymorphisms [24]. In other studies, histone deacetylase (HDAC) inhibitors were shown to influence BIM in a deletion allele from mRNA alternative splicing, thereby increasing the expression of active BIM protein. Furthermore, in combination with ositinib, this HDAC-based approach also resulted in tumor regression in NSCLC cells homozygous for the BIM deletion polymorphism despite harboring an epidermal growth factor receptor mutation [25]. Aurora B inhibition stabilizes BIM protein expression by decreasing Ser87 phosphorylation and reducing EGFR-mutant lung cancer acquired drug resistance [26]. Combining the HDAC inhibitor resminostat and epidermal growth factor receptor inhibitor have been shown to preferentially induce the expression of proapoptotic BIM transcripts containing exon 4 rather than exon 3, hence increasing the level of pro-apoptotic BIM protein (BIMEL) and stimulating apoptosis in vitro. As a result, it was possible to circumvent the epidermal growth factor receptor inhibitor associated with BIM deletion polymorphisms and tolerance [27]. A retrospective analysis further indicated that EGFR-TKIs, combined with bevacizumab, as first-line treatment for patients with advanced NSCLC with epidermal growth factor receptor mutations and BIM gene deletion had significantly higher ORR and PFS compared with those treated with EGFR-TKIs alone [28]. The above studies provide valuable insight into the mechanism of drug resistance in BIM-deficient NSCLC. Nevertheless, the role they play in the onset and progression of resistance to alectinib in ALK-mutated non-small cell lung cancer is yet to be established. Additional cases would therefore be required to further investigate the associated resistance mechanisms.

This report is the first documented case of drug resistance to alectinib in ALK-mutated NSCLC as a result of BIM gene mutation. Exploring whether a combination of BIM sensitizers and alectinib could help to overcome alectinib resistance could be the subject of subsequent research, and it is expected that such findings would provide insights to guide the switch to a new line of therapy for patients who exhibit alectinib resistance in clinical settings. However, this study was not without limitations, one of which was that the genetic testing was far from being comprehensive. At the same time, the lack of NGS results at the different stages of the disease makes it unclear whether other mutation loci could have also influenced the therapeutic efficacy. Currently, genetic testing is primarily performed on tumor biopsy samples, in addition to which advances in circulating tumor cell detection methods have provided more options for confirming the diagnosis of tumors [29]. Through the improvement of detection methods, more clinical potential patients can be screened. In addition, there was a relatively small sample size, but these conclusions can be further assessed by supplementing current research with future ones. Finally, exploring the mechanism between the BIM deletion and alectinib resistance would also required.

Conclusion

BIM gene deletion can increase resistance to alectinib, and the potential efficacy of a combination of BIM sensitizer and alectinib to overcome alectinib resistance can be explored. It is expected that such findings would provide insights that guide the switch to a different line of treatment for alectinib-resistant patients.

Author contributions Conception was performed by Shuang Hou. Operation of the experiment was performed by Wei Pang, Haiqun Xia, Jinyun Tan, Qingfang Huang. Preparation of the manuscript was performed by Weijun Zhang and Shuang Hou. Revision for important intellectual content was performed by Ping Yang. All authors read and approved the manuscript, and to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Data availability The data that support the findings of this study are available on request from the corresponding author, upon reasonable request.

Declarations

Competing interests The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Consent for publication Informed consent to publish the case report was provided in writing by the patient.

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