



Original Article

Reciprocal regulation between B lymphoma Mo-MLV insertion region 1 homolog and type I insulin-like growth factor receptor in pemetrexed-resistant lung cancer cells

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ABSTRACT

Objectives: The objective of this study was to investigate the role of type I insulin-like growth factor receptor (IGF-1R) in pemetrexed-resistant lung cancer cells and its interaction with B lymphoma Mo-MLV insertion region 1 homolog (BMI1), previously identified as a key resistance gene. **Materials and Methods:** The study started with the analysis of the activation of IGF-1R in pemetrexed-resistant A549 (A400) lung cancer cells by Western blot analysis of its form of phosphorylation. Cancer stem cell (CSC) activity was assessed by tumor sphere culture. IGF-1R inhibition was performed by picropodophyllin (PPP), an IGF-1R inhibitor, or by shRNA-mediated RNA silencing. A Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse xenograft model was used to access *in vivo* pemetrexed sensitivity. To further understand the relationship between IGF-1R and BMI1, both BMI1 knockdown and overexpression experiments were performed to assess IGF-1R phosphorylation by western blot. **Results:** Increased IGF-1R phosphorylation was found in A400 cells, and subsequent IGF-1R inhibition resulted in a reduction in CSC activity in these resistant cells. In the *in vivo* studies, PPP treatment effectively suppressed tumor growth and reduced BMI1 expression in A400 tumor tissue. Further investigation showed that BMI1 knockdown in A400 cells resulted in decreased IGF-1R phosphorylation, whereas BMI1 overexpression in A549 cells resulted in increased IGF-1R phosphorylation, indicating an interaction between these two proteins. **Conclusion:** A novel reciprocal regulatory relationship between IGF-1R and BMI1 has been identified in lung cancer cells, suggesting potential therapeutic strategies to combat pemetrexed resistance in lung cancer patients.

KEYWORDS: *B lymphoma Mo-MLV insertion region 1 homolog, Cancer stem cells, Lung cancer, Pemetrexed resistance, Type I insulin-like growth factor receptor*

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths globally and in Taiwan. In Taiwan, approximately 10,000 new cases are diagnosed annually. Despite significant advances in lung cancer treatment, the overall prognosis remains poor. According to our previous report, the 5-year survival rate of lung cancer patients in Taiwan was 15.9% between 2002 and 2008, with a median overall survival of 13.2 months [1]. While this improved to 25% between 2010 and 2016 [2], the outlook for lung cancer patients is still dismal. Contributing factors include the advanced stage (IIIb or IV) at diagnosis for most patients and the aggressive biological nature of lung cancer, characterized by rapid growth and a high metastatic potential [3]. While early-stage patients may undergo surgery and those with

specific genetic mutations can benefit from targeted therapies, chemotherapy remains a critical treatment option. The response rate to the folic acid antagonist Pemetrexed (Alimta) as a monotherapy ranges from 16% to 23% [4]. Understanding the mechanisms of resistance to Alimta and discovering more effective therapeutic alternatives is thus an urgent priority.

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Pemetrexed is a folic acid analog that inhibits purine and pyrimidine synthesis by suppressing thymidylate synthase (TS), dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase, thereby blocking cell proliferation [5]. Increased TS gene expression in lung cancer cells has been linked to pemetrexed resistance [6]. In our previous study, we found that the upregulation of B lymphoma Mo-MLV insertion region 1 homolog (BMI1) in A549 lung cancer cells contributed to pemetrexed resistance. Furthermore, PTC-209, a small-molecule inhibitor of BMI1, has been shown to sensitize pemetrexed-resistant A400 lung cancer cells to pemetrexed treatment *in vivo* [7], suggesting an additional mechanism of resistance to pemetrexed other than the alteration of TS gene expression in lung cancer. BMI1 is known to enhance cancer stem cell (CSC) activity in various cancers [8]. CSCs represent a subpopulation of cancer cells characterized by their ability to initiate tumors, resist treatments, and promote metastasis [9]. Targeting CSCs is, therefore, considered a promising strategy for effective cancer therapy. In the context of primary lung cancer tissues, BMI1 has been observed to exhibit a positive correlation with the expression levels of CSC markers, including CD133 and ALDH1A1, across both mRNA and protein levels [10]. Furthermore, the upregulation of BMI1 in nonsmall cell lung cancer (NSCLC) has been demonstrated to be associated with an increase in tumor size and distant metastasis, thus serving as an effective prognostic factor in NSCLC [11]. Biologically, BMI1 has been shown to enhance the invasive capacity of NSCLC cells [12]. In addition, our research, in conjunction with that of other groups, has elucidated the role of BMI1 in chemoresistance [7,12,13]. Consequently, targeting BMI1 has been proposed as a novel therapeutic strategy for NSCLC [14].

The type I insulin-like growth factor receptor (IGF-1R) is a cell surface receptor tyrosine kinase that regulates cell growth, survival, differentiation, and metabolism [15]. In nonsmall cell lung cancer (NSCLC) tissues, the positive expression rate of IGF-1R ranges from 40% to over 75% [16,17], and elevated IGF-1R levels are associated with poor disease-free survival in NSCLC [18]. In addition, high IGF-1R expression predicts postoperative recurrence in lung adenocarcinoma (LUAD) patients [19]. IGF-1R is also implicated in the development of cancer cell chemoresistance. For example, in glioblastoma multiforme (GBM), increased IGF-1R expression was observed in cells resistant to EGFR inhibitors, while the intracellular PI3K pathway remained active. Dual targeting of IGF-1R and EGFR pathways resulted in significant apoptosis of EGFR-resistant GBM cells [20], and IGF-1R inhibition sensitized GBM cells to gefitinib, an EGFR inhibitor [21]. In NSCLC, IGF-1R expression has been linked to resistance to vorinostat, a histone deacetylase inhibitor, and blocking the IGF-1R pathway with an IGF-1R antibody sensitized vorinostat-resistant NSCLC cells to treatment [22]. Importantly, recent studies have shown that IGF-1R signaling can enhance CSC properties [23,24], similar to BMI1's role in maintaining stemness [10,25]. Given that our previous work demonstrated BMI1's involvement in pemetrexed resistance and its known function in CSC maintenance, we hypothesized that IGF-1R might interact with or regulate BMI1 to promote pemetrexed resistance in lung cancer cells.

In this study, we focus on the role of IGF-1R in pemetrexed resistance in lung cancer cells. We also investigate the link between BMI1 and IGF-1R. These studies may provide new insights into managing pemetrexed-resistant lung cancer patients using IGF-1R inhibitors in combination therapies.

MATERIALS AND METHODS

Ethical statement

The animal experiment protocol used in this study was approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University (Taichung, Taiwan) under approval No. 2017.

Cell lines and cultivation

The human lung cancer cell line A549 was obtained from the Bioresource Collection and Research Centre (Hsinchu, Taiwan), and its authentication was confirmed by short tandem repeat profiling conducted at the Center for Genomic Medicine, National Cheng Kung University (Tainan, Taiwan). A549 cells were cultured in dulbecco's modified eagle medium (DMEM) medium supplemented with 10% fetal bovine serum and 1X penicillin/streptomycin. The pemetrexed-resistant lung cancer cell line A400, derived from A549 cells, was cultured under the same conditions as A549 cells, with the addition of 400 nM pemetrexed (purchased from Tocris Bioscience, Bristol, UK). BMI1-overexpressing A549 cells (A549-BS1) were established as described in our previous report [7].

Cell viability assay

Cells were seeded into 96-well plates at a density of 5×10^3 cells per well. After drug treatment, the cells were cultured for 96 h in a 37°C incubator with 5% CO₂. To assess cell viability, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Cat. No. M2003) was added to the wells. Absorbance was measured at 570 nm to determine MTT reduction, and the viability of untreated cells was used as a baseline for comparison. The IGF-1R inhibitor, picropodophyllin (PPP), was purchased from Tocris Bioscience.

Tumorsphere assay

Lung cancer cells (2×10^4) were seeded in 2 ml of DMEM/F12 medium containing 0.4% bovine serum albumin (Sigma-Aldrich, St Saint Louis, MO, USA), 10 ng/mL EGF (PeproTech, Cranbury, NJ, USA), 10 ng/mL basic fibroblast growth factor (PeproTech), 4 µg/mL heparin (Sigma-Aldrich), 0.5X B27 supplement (Gibco™, Waltham, MA, USA), and 1 µM hydrocortisone (Sigma-Aldrich). The cells were cultured in ultra low attachment 6-well plates (Corning Life Science, Corning, NY, USA) for 7 days. During the incubation period, 0.5 ml of fresh medium was added to each well every 3 days. Tumorspheres were counted and imaged 10 days after seeding.

RNA interference

RNA interference was conducted using lentiviral vectors carrying shRNA and prepared as described in our previous study [26]. During lentiviral transduction, cells were treated with polybrene at a concentration of 8 µg/ml. After 24 h, cells were subjected to selection with 2 µg/mL puromycin,

and surviving cells were collected for subsequent experiments. LacZ, IGF-1R-, and BMI1-specific shRNA clones were obtained from the RNA Technology Platform and Gene Manipulation Core (Taipei, Taiwan). The target sequences for the shRNA constructs are listed below:

- sh-LacZ: 5'-CGCGATCGTAATCACCCGAGT-3'
- sh-IGF-1R#1: 5'-GCGGTGTCCAATAACTACATT-3'
- sh-IGF-1R#2: 5'-GCCGAAGATTTACAGTCAAA-3'
- sh-BMI1#1: 5'-CAGATTGGATCGGAAAGTAAA-3'
- sh-BMI1#2: 5'-ATTGATGCCACAACCATAATA-3'.

Overexpression of B lymphoma Mo-MLV insertion region 1 homolog in A549 cells

Lentiviruses carrying the tRFP or BMI1 gene were transduced into A549 cells and selected with 2 µg of puromycin as described in our previous report [7,13]. Successful overexpression of transgenes was confirmed by western blot.

Western blot

Cells were lysed using RIPA buffer, and the total protein concentration was determined with a BCA protein quantification kit (Pierce™, Thermo Scientific, Waltham, MA, USA). A total of 20 µg of protein was loaded onto a 10% acrylamide SDS-PAGE gel, separated by electrophoresis, and then transferred to a PVDF membrane (BioTrace™ PVDF Transfer Membrane, Pall Corporation, Port Washington, NY, USA). The membranes were blocked with 5% skimmed milk in TBST and incubated with primary antibodies, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein signals were developed using a chemiluminescence substrate (Immobilon® ECL Ultra Western HRP Substrate, Millipore, Burlington, MA, USA). Chemiluminescence signals were captured using the Amersham Imager 680 (GE Healthcare Bio-Sciences Corp., Marlborough, MA, USA) and quantified with ImageJ software. The antibodies used in this study were IGF1R (GeneTex, Cat. No. PA5-29819), p-IGF1R Tyr1165/Tyr1166 (Genetex, Cat. No. GTX50154) and BMI1 (Novus Biologicals, Cat. No. NBP1-33748), and GAPDH (GeneTex, Cat. No. GTX100118).

NOD/SCID xenograftment model

In this study, NOD/SCID immunodeficient mice were used as a xenograft tumor model for lung cancer to evaluate the therapeutic efficacy of PPP. NOD/SCID mice were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). Pemetrexed-resistant A400 cells were suspended in 2.5 mg/mL Matrigel and injected subcutaneously into the dorsal region of the mice using a 30G needle, with a dose of 1×10^6 cells/50 µL per injection site. Once the tumors reached a size of 50 mm³, PPP was administered intraperitoneally at a dose of 10 mg/kg, twice a week, for a duration of 2 months. Tumor size was measured weekly until the tumors in the vehicle-treated group reached 1000 mm³. Tumor volume was calculated using the formula: $(D \times d^2)/2$, where D represents the longest tumor dimension and d represents the widest dimension.

Immunohistochemistry

Following dewaxing and antigen retrieval, tissue sections from A400 xenograft tumors were incubated with either

anti-BMI1 or anti-p-IGF1R^{Tyr1165/1166} antibodies. The sections were then processed using the avidin-biotin-peroxidase system (LSAB2 System-HRP, DAKO, Carpinteria, CA), and 3,3'-diaminobenzidine (DAKO) was applied for color development. Staining results were quantitatively analyzed using the TissueFAX Plus cytometer (TissueGnostics GmbH, Vienna, Austria).

Statistical analysis

Statistical analysis was performed with Prism (version 5.0, GraphPad Software, La Jolla, CA, USA). Student's *t*-test was used to compare two groups, and one-way analysis of variance followed by *post hoc* Tukey honestly significant difference (HSD) test was used for more than two groups. A significant difference was defined as $P < 0.05$.

RESULTS

Increased type I insulin-like growth factor receptor activation in pemetrexed-resistant A400 cells

We established pemetrexed-resistant lung cancer cells from A549 cells and designated as A400 cells [27]. To investigate the potential involvement of IGF-1R in pemetrexed resistance, we examined the phosphorylation of IGF-1R at tyrosine 1165/1166 (p-IGF-1R^{Tyr1165/1166}) using western blot analysis. The results showed a significant increase in p-IGF-1R^{Tyr1165/1166} levels in pemetrexed-resistant A400 cells compared to the parental A549 cells [Figure 1a]. In addition, treatment with PPP, a small-molecule IGF-1R inhibitor, at a concentration of 0.5 µM reduced p-IGF-1R^{Tyr1165/1166} levels [Figure 1a]. Notably, PPP exhibited a stronger proliferation inhibition effect on A400 cells compared to parental A549 cells at concentrations of 0.1 and 0.2 µM [Figure 1b]. These findings suggest that IGF-1R activation may play a role in the development of pemetrexed resistance in lung cancer cells.

Inhibition of type I insulin-like growth factor receptor activation by picropodophyllin reduces cancer stem cell activity in A400 cells

CSCs are considered a key mechanism underlying chemoresistance in cancer [28]. To further investigate the role of IGF-1R in CSC activity in pemetrexed-resistant A400 cells, we performed a tumorsphere formation assay. Treatment with PPP at a concentration of 0.5 µM significantly reduced tumorsphere formation in pemetrexed-resistant A400 cells [Figure 2a]. In addition, IGF-1R knockdown via RNA interference [Figure 2b] also resulted in a marked reduction in tumorsphere formation in A400 cells [Figure 2c], similar to the effect of PPP treatment as shown in Figure 2a. These findings suggest that IGF-1R promotes pemetrexed resistance in lung cancer cells by enhancing CSC activity.

Picropodophyllin suppresses tumor growth of pemetrexed-resistant A400 cells *in vivo*

We next evaluated the *in vivo* anti-tumor effect of PPP in pemetrexed-resistant A400 cells. After subcutaneous injection of A400 cells into NOD/SCID mice, PPP treatment was initiated when the tumors reached 50 mm³. PPP was administered at a dose of 10 mg/kg, twice per week, for a total of 2 months. At the end of the treatment period, tumor weights were significantly reduced in the PPP-treated group compared to the

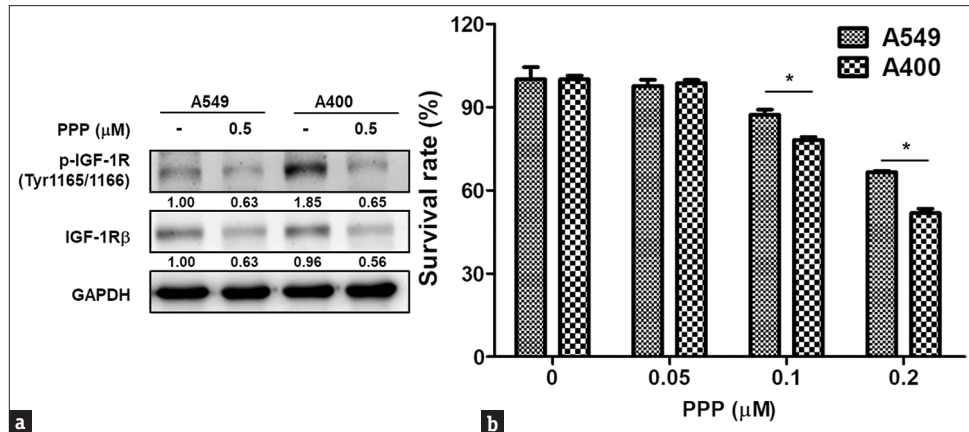


Figure 1: Type I insulin-like growth factor receptor (IGF-1R) inhibition enhances pemetrexed sensitivity in resistant lung cancer cells. (a) Western blot analysis of IGF-1R and phosphorylated IGF-1R (p-IGF-1R^{Tyr1165/1166}) expression in A549 and pemetrexed-resistant A400 cells. Cells were treated with or without 0.5 μM picropodophyllin (PPP) for 48 h. GAPDH served as a loading control. (b) Cell viability assessment using 3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide assay. A549 and A400 cells were exposed to various concentrations of PPP for 72 h. Data are presented as mean ± SD; *, *P* < 0.05

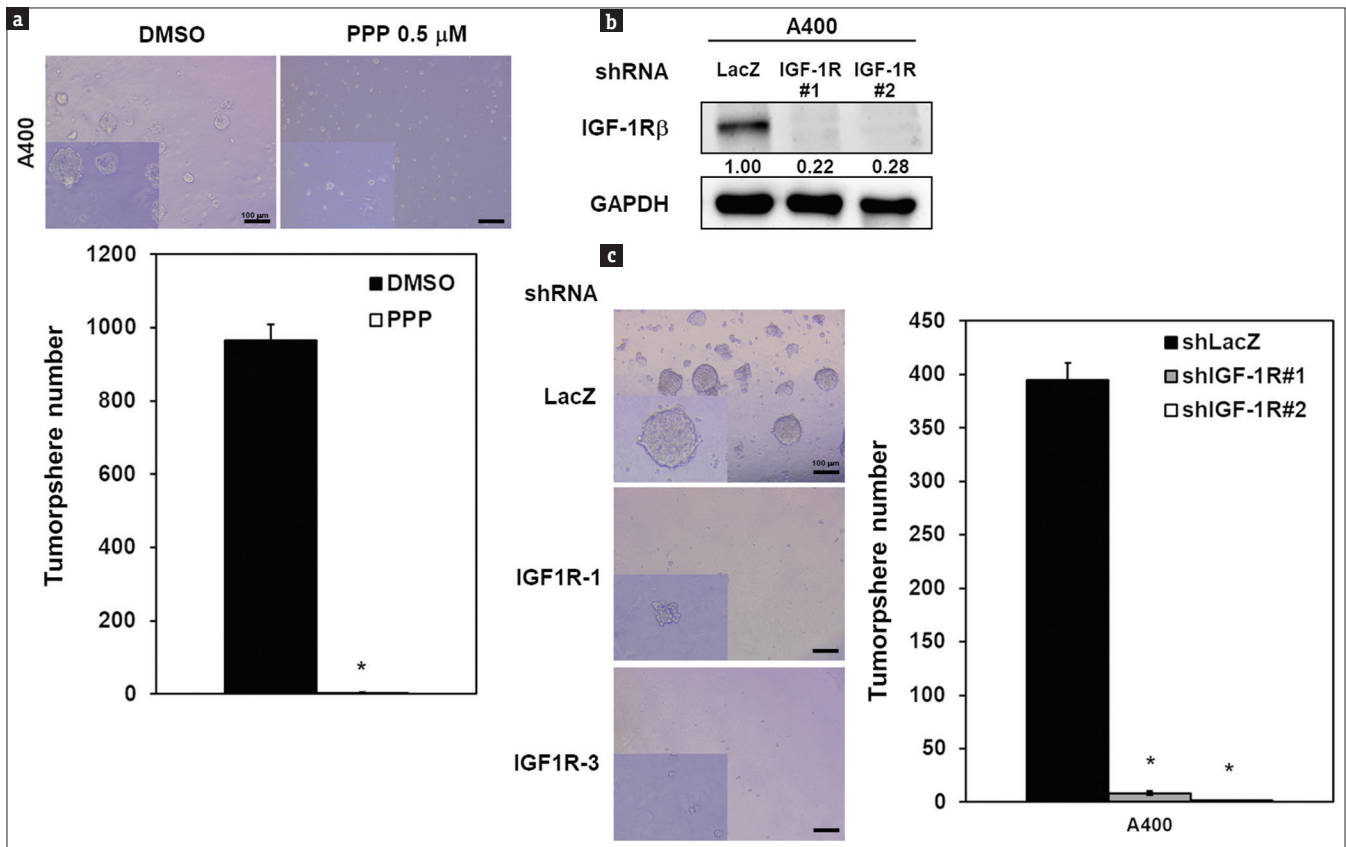


Figure 2: Type I insulin-like growth factor receptor (IGF-1R) inhibition reduces cancer stem cell (CSC) activity. (a) Tumorsphere formation in A400 cells treated with 0.5 μM picropodophyllin or 0.1% (v/v) DMSO (vehicle control) for 10 days. Tumorsphere numbers were quantified under an inverted microscope. *, *P* < 0.05. Scale bars: 100 μm. (b and c) Effects of IGF-1R knockdown on CSC activity. A400 cells were transduced with lentiviral vectors expressing IGF-1R-specific shRNA (IGF-1R#1 or IGF-1R#2) or control shRNA (shLacZ). (b) Western blot analysis confirming IGF-1R knockdown efficiency. GAPDH was used as a protein loading control. The numbers indicated relative expression levels compared to sh-LacZ transduced cells. (c) Assessment of CSC activity by tumorsphere formation assay. *, *P* < 0.05 compared to shLacZ control. Scale bars: 100 μm

control [Figure 3a]. Immunohistochemical analysis revealed that PPP treatment effectively suppressed the protein level of p-IGF-1R^{Tyr1165/1166} [Figure 3b]. Interestingly, PPP treatment also reduced the expression of BMI1 in xenograft tumors [Figure 3c]. Furthermore, analysis of the LUAD dataset from the Cancer Genome Atlas (TCGA) using the GEPIA_2 web tool showed

a significant positive correlation between IGF-1R and BMI1 mRNA levels [Figure 3d, Spearman *r* = 0.62, *P* < 0.001]. These findings suggest that inhibition of IGF-1R may be a viable strategy for managing pemetrexed resistance in lung cancer and that IGF-1R activation may upregulate BMI1 expression.

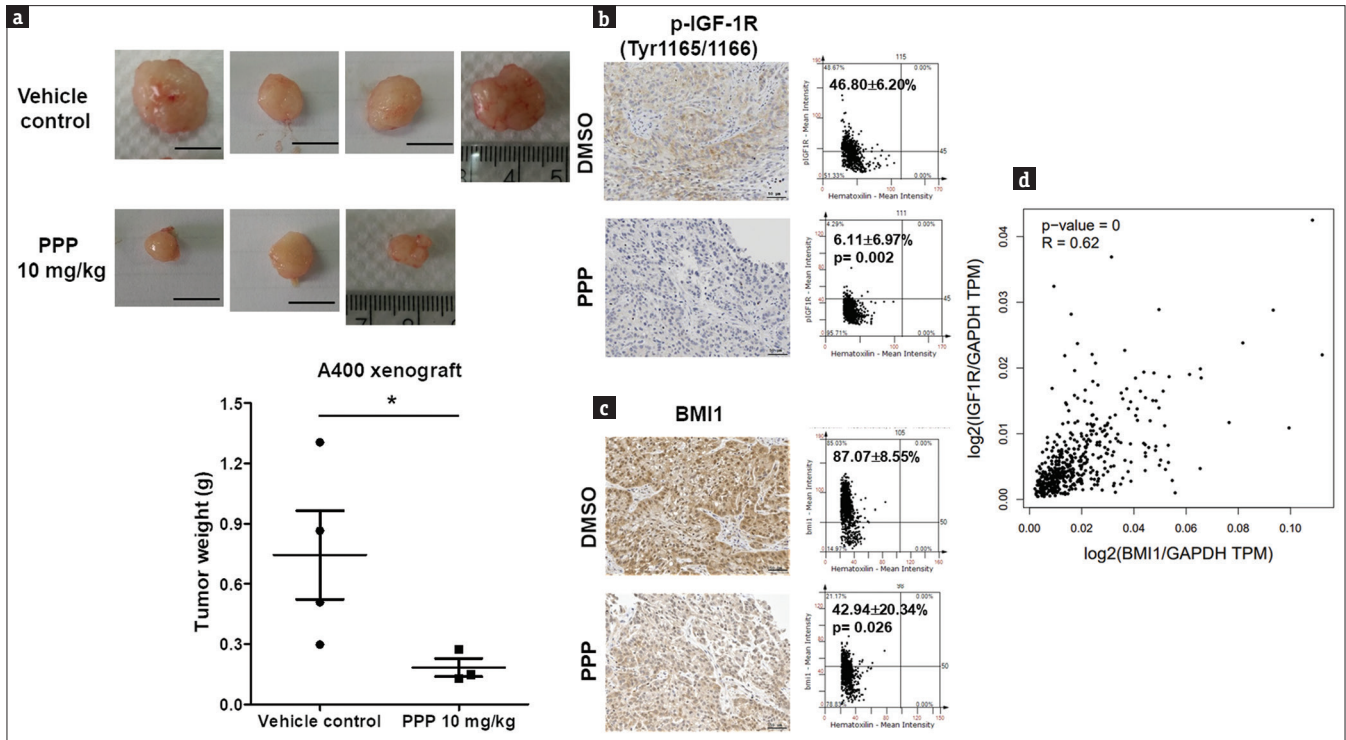


Figure 3: Type I insulin-like growth factor receptor (IGF-1R) inhibition enhances pemetrexed efficacy in A400 xenograft model. (a-c) NOD/SCID mice bearing A400 cell xenografts (tumor volume $\sim 50 \text{ mm}^3$) were treated with picropodophyllin (10 mg/kg, intraperitoneally) twice weekly for two months. (a) Tumor weights at sacrifice. *, $P < 0.05$. Scale bars: 1 cm. (b and c) Immunohistochemical (IHC) analysis of tumor tissues showing protein levels of phosphorylated IGF-1R (p-IGF-1R^{Tyr1165/1166}) (b) and B lymphoma Mo-MLV insertion region 1 homolog (BMI1) (c). Cellular color signals (left panels) were converted into pixel intensities by TissueFAX software and displayed as single-cell dot plots (right panels). The numbers inserted in dot plots indicated the percentage of positively stained cells. (d) Correlation analysis of IGF-1R and BMI1 mRNA expression levels in lung adenocarcinoma patients from The Cancer Genome Atlas database, obtained through GEPIA_2 platform. All expression values were \log_2 -transformed transcripts per million (TPM) normalized to GAPDH. Correlations were analyzed using the Pearson correlation coefficient

Reciprocal regulation between B lymphoma Mo-MLV insertion region 1 homolog and type I insulin-like growth factor receptor in lung cancer cells

Our previous research has shown that BMI1 contributes to pemetrexed resistance in lung cancer cells [7,13]. In Figure 3c, we observed a reduction in BMI1 protein levels in A400 tumors after PPP treatment. To further investigate the relationship between IGF-1R and BMI1, we first treated A400 cells with PPP [Figure 4a]. The results revealed that inhibition of IGF1R by a small molecule inhibitor led to the downregulation of BMI1. The suppressive effect of BMI1 protein expression under IGF1R inhibition was also confirmed by knocked down the IGF-1R using two independent shRNA sequences [Figure 4b]. In addition, we found that the knockdown of the BMI1 protein level in A400 cells also led to the downregulation of the p-IGF-1R^{Tyr1165/1166} [Figure 5a]. Conversely, overexpressing BMI1 in A549 cells upregulated p-IGF-1R^{Tyr1165/1166}, which could also be reduced by PPP treatment [Figure 5b]. These findings suggest a reciprocal regulatory mechanism between IGF-1R activation and BMI1 expression in lung cancer cells.

DISCUSSION

Our findings reveal a novel mechanism underlying pemetrexed resistance in lung cancer, which involves the activation of IGF-1R. Similarly, recent research by Sun *et al.* demonstrated elevated levels of IGF-1R in pemetrexed-resistant

malignant pleural mesothelioma (MPM/PEM) cells [29]. In that study, treatment with PPP disrupted cell cycle progression by inhibiting microtubule formation in MPM/PEM cells [29]. In support of these findings, analysis of the LUAD dataset from the TCGA database, using the median level of IGF-1R mRNA as a cutoff, identified an enrichment of the Mitotic_Spindle gene set [Supplementary Figure 1a]. Among the enriched genes, the mRNA level of microtubule-affinity regulating kinase 4—a gene critical for microtubule assembly and cell cycle progression – was significantly higher in subjects with elevated IGF-1R levels [Supplementary Figure 1b]. These data reinforce the role of IGF-1R as a positive regulator of pemetrexed resistance in lung cancer.

Zhang *et al.* previously reported a significant correlation between IGF-1R and BMI1 in LUAD tissues, where 66.9% of IGF-1R-positive LUAD tissues also exhibited positive BMI1 expression [30]. Our study further corroborates this positive correlation using data from the TCGA dataset [Figure 3d]. To date, our research is the first to experimentally demonstrate the reciprocal regulation between IGF-1R and BMI1. Recently, Kohyanagi *et al.* found that inhibiting Akt with a small-molecule inhibitor downregulated BMI1 in human osteosarcoma HOS cells [31]. Since IGF-1R activation is known to drive dysregulated PI3K/Akt signaling [32], it is possible that IGF-1R positively regulates BMI1 expression through Akt activation, potentially stabilizing BMI1 protein by preventing its ubiquitin-proteasomal degradation [33].

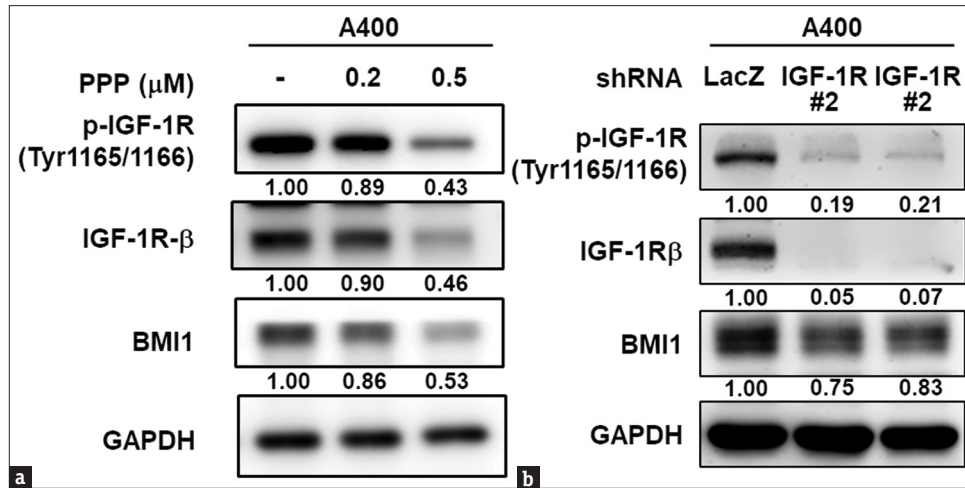


Figure 4: Type I insulin-like growth factor receptor (IGF-1R) inhibition decreases B lymphoma Mo-MLV insertion region 1 homolog (BMI1) expression in pemetrexed-resistant lung cancer cells. (a) Western blot analysis of phosphorylated IGF-1R (p-IGF-1R^{Tyr1165/1166}), total IGF-1R, and BMI1 levels after treatment with pemetrexed at 0.2 or 0.5 μM for 48 h. The numbers indicated relative expression levels compared to nontreated control. (b) Western blot analysis of p-IGF-1R^{Tyr1165/1166}, total IGF-1R, and BMI1 protein levels after lentiviral transduction with IGF-1R-specific shRNAs (IGF-1R#1 or IGF-1R#2) for 48 h. GAPDH was used as a protein loading control. The numbers indicated relative expression levels compared to sh-LacZ transduced cells

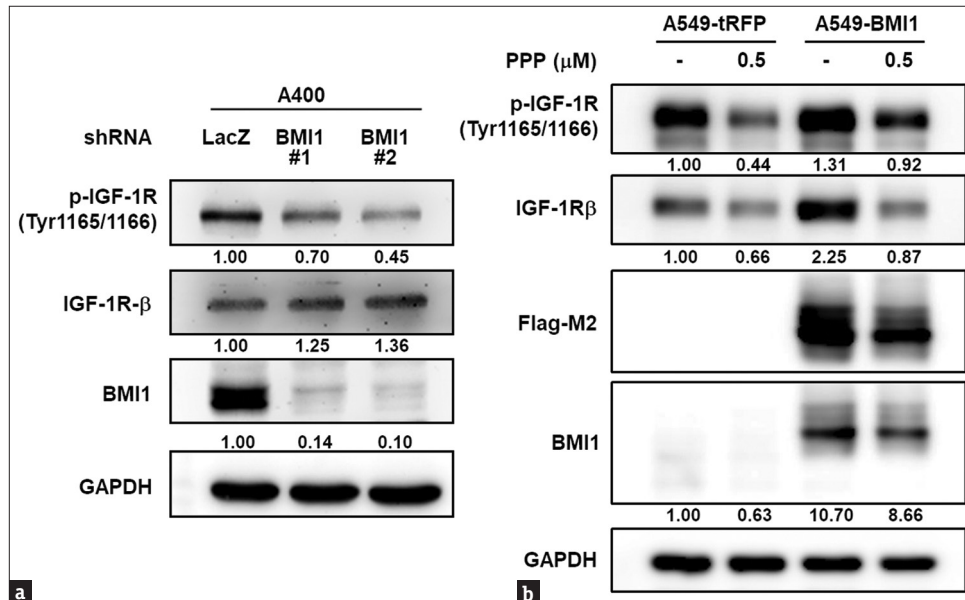


Figure 5: B lymphoma Mo-MLV insertion region 1 homolog (BMI1) regulates type I insulin-like growth factor receptor (IGF-1R) phosphorylation in lung cancer cells. (a) Western blot analysis of phosphorylated IGF-1R (p-IGF-1R^{Tyr1165/1166}), total IGF-1R, and BMI1 levels after BMI1 knockdown using lentiviral transduction of BMI1-specific shRNAs (BMI1#1 or BMI1#2) for 48 h. The numbers indicated relative expression levels compared to shLacZ transduced cells. (b) A549 cells were transduced with lentiviral vectors expressing flag-tagged BMI1 cDNA (A549-BMI1) or Turbo RFP (tRFP, control) and treated with pemetrexed for 48 h. DMSO (0.1%) served as vehicle control. Protein levels of p-IGF-1R^{Tyr1165/1166}, total IGF-1R, and BMI1 were measured by western blot. Flag-tagged BMI1 protein levels were determined by using a monoclonal mouse anti-flag antibody (Flag-M2). GAPDH was used as a protein loading control. The numbers indicated relative expression levels compared to vehicle control

Beyond Akt, casein kinase 2α (CK2α) has been shown to phosphorylate BMI1 at serine 110, protecting it from degradation in ovarian cancer cells [34]. Investigating the phosphorylation status of BMI1 following IGF-1R inhibition could shed light on whether IGF-1R regulates BMI1 expression through phosphorylation-mediated stabilization.

While our study establishes a significant correlation between IGF-1R and BMI1, the exact molecular mechanisms underlying their interaction remain to be fully elucidated. One potential pathway involves Neurogenic locus notch homolog protein 1 (NOTCH1) signaling, as our analysis of the

TCGA dataset revealed strong positive correlations between BMI1 and NOTCH1 [Supplementary Figure 2a, Spearman $r = 0.65$, $P < 0.001$], hairy and enhancer of split 1 (HES1) [Supplementary Figure 2b, Spearman $r = 0.45$, $P < 0.001$], and Myelocytomatosis oncogene (MYC) [Supplementary Figure 2c, Spearman $r = 0.4$, $P < 0.001$]. These findings align with previous studies showing that BMI1 inhibition downregulates NOTCH signaling proteins in leukemia cells [35] and that NOTCH activation regulates IGF-1R expression in leukemia-initiating cells [36]. These observations suggest a potential feedback loop in which BMI1 regulates IGF-1R

through NOTCH1 signaling, although further experimental validation is needed to confirm this hypothesis.

To explore the role of NOTCH1 in the reciprocal regulation of BMI1 and IGF-1R, future studies should investigate whether NOTCH1 activation is increased in pemetrexed-resistant cells compared to their parental counterparts. This could be achieved by measuring the expression of the NOTCH1 intracellular domain and downstream targets such as HES1 and MYC. In addition, the effects of NOTCH1 inhibition—using γ -secretase inhibitors or siRNA—on BMI1 expression and IGF-1R phosphorylation should be examined to determine whether NOTCH1 is a critical mediator of the BMI1-IGF-1R axis. These mechanistic studies could be further validated using clinical samples from pemetrexed-resistant lung cancer patients, including immunohistochemical analysis of NOTCH1, BMI1, and IGF-1R expression, as well as correlation studies to assess their clinical relevance.

It is acknowledged that the present study has certain limitations. First, the findings are primarily based on the A549 cell line and its resistant derivative A400. Although this approach provides a clean experimental system, it may not fully capture the heterogeneity of lung cancer in patients. To enhance the generalizability of our findings, additional lung cancer cell lines should be included in future studies. Second, we were unable to validate our results using clinical samples due to challenges in obtaining pemetrexed-treated lung cancer tissues. However, our analysis of the TCGA dataset provides supportive evidence for the clinical relevance of IGF-1R and BMI1 in lung cancer. To address these limitations, future work will involve to obtain relevant tissue samples and expansion of experimental models to include more diverse cell lines and *in vivo* systems.

The therapeutic potential of targeting the IGF-1R-BMI1 axis in pemetrexed-resistant lung cancer is supported by the growing body of evidence on the efficacy of BMI1 and IGF-1R inhibitors in preclinical and clinical studies. For example, PTC-209, a small-molecule inhibitor of BMI1, has shown promising anti-tumor effects in various cancer models, including breast, colon, and lung cancers [37]. Similarly, IGF-1R inhibitors such as linsitinib [38] and figitumumab [39] have been evaluated in clinical trials for solid tumors, including NSCLC. Although the clinical efficacy of single-agent IGF-1R inhibitors has been modest, their combination with other targeted therapies or chemotherapy has shown to be tolerable and enhanced anti-tumor activity in some studies [40-42]. Our findings suggest that simultaneous inhibition of IGF-1R and BMI1 could be a promising strategy to overcome pemetrexed resistance in lung cancer. This approach is supported by the reciprocal regulation between IGF-1R and BMI1 observed in our study.

However, several challenges need to be addressed before this strategy can be translated into clinical practice. First, the potential toxicity of combined IGF-1R and BMI1 inhibition needs to be carefully evaluated, as both pathways play important roles in normal tissue homeostasis. Second, biomarkers for patient selection should be developed to identify those who are most likely to benefit from this therapeutic approach. Finally, the optimal dosing and

scheduling of combination therapy need to be determined through rigorous preclinical and clinical studies.

CONCLUSIONS

Our study shows that IGF-1R activation plays a critical role in pemetrexed resistance in lung cancer cells. In pemetrexed-resistant A400 cells compared to parental A549 cells, we found significantly increased levels of p-IGF-1R^{Tyr1165/1166}. The IGF-1R inhibitor PPP effectively suppressed both IGF-1R activation and CSC activity *in vitro*. It also showed significant anti-tumor activity *in vivo*. Importantly, we uncovered a novel reciprocal regulatory mechanism between IGF-1R and BMI1, whereby IGF-1R inhibition led to a reduction in BMI1 expression and BMI1 modulation affected IGF-1R phosphorylation status. The positive correlation between IGF-1R and BMI1 expression in the TCGA LUAD dataset further supports this relationship. These findings suggest that targeting the IGF-1R-BMI1 axis may be a promising therapeutic strategy to overcome pemetrexed resistance in lung cancer patients.

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Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Financial support and sponsorship

Nil.

Conflicts of interest

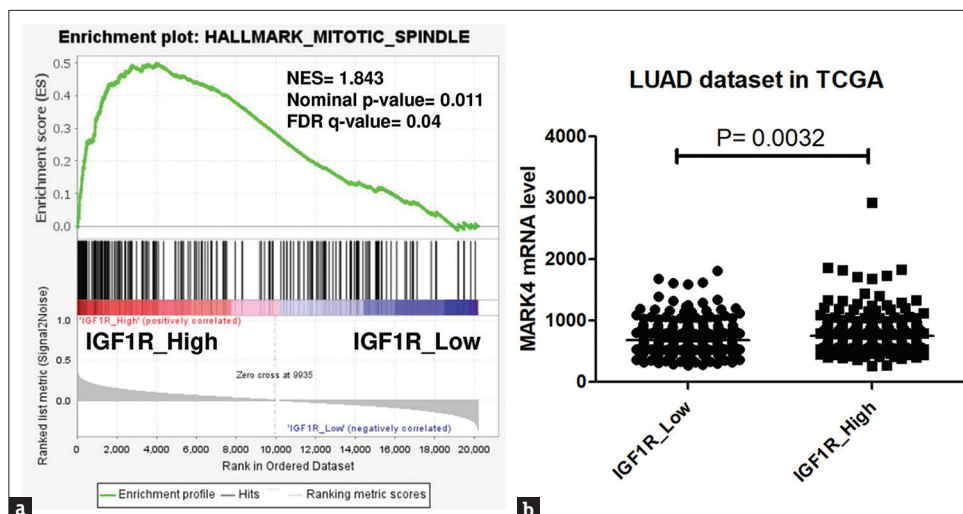
There are no conflicts of interest.

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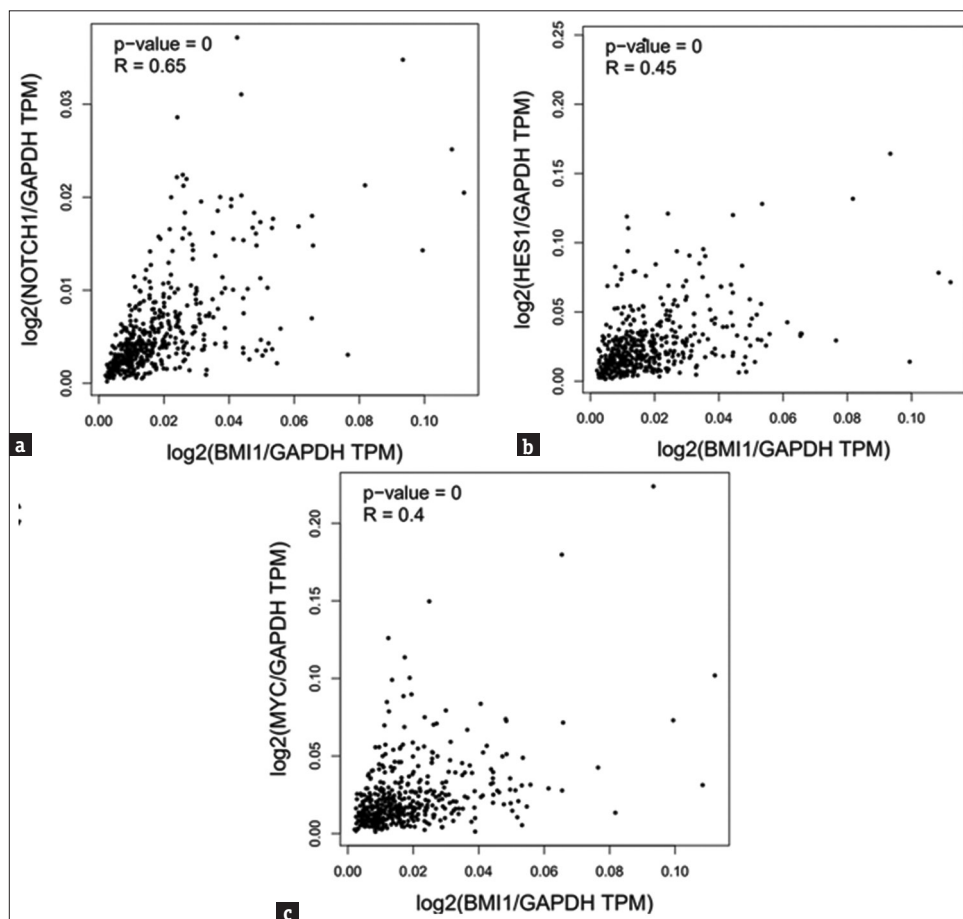
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SUPPLEMENTARY MATERIAL



Supplementary Figure 1: Type I insulin-like growth factor receptor (IGF-1R) expression correlates with mitotic spindle pathway and microtubule-affinity regulating kinase 4 (MARK4) expression in lung adenocarcinoma (LUAD). (a) Gene Set Enrichment Analysis (GSEA) of the HALLMARK_MITOTIC_SPINDLE gene set in LUAD patients from The Cancer Genome Atlas (TCGA) database. Patients were stratified into IGF-1R_Low and IGF-1R_High groups using median IGF-1R expression as the cutoff. NES: Normalized Enrichment Score; FDR: false discovery rate. (b) Comparison of MARK4 mRNA expression levels between IGF-1R_Low and IGF-1R_High groups in LUAD patients from TCGA database



Supplementary Figure 2: B lymphoma Mo-MLV insertion region 1 homolog (BMI1) mRNA expression positively correlates with NOTCH1, HES1, and MYC in lung adenocarcinoma (LUAD). Correlation analyses of gene expression from The Cancer Genome Atlas LUAD dataset using the GEPIA_2 platform. (a) Correlation between BMI1 and NOTCH1 expression ($R = 0.65$). (b) Correlation between BMI1 and HES1 expression ($R = 0.45$). (c) Correlation between BMI1 and MYC expression ($R = 0.40$). All expression values were log₂-transformed transcripts per million (TPM) normalized to GAPDH