Article

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Wnt/β-Catenin Pathway-Mediated *PD-L1* Overexpression Facilitates the Resistance of Non-Small Cell Lung Cancer Cells to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors

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Background: Epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) is considered highly effective treatment for advanced non-small cell lung cancer (NSCLC), who often develop drug resistance after 10 months of treatment. Herein, the aim was to unravel the mechanism behind the resistance to icotinib in NSCLC.
Methods: Establishment of icotinib-resistant PC-9 cells (PC-9R) was achieved through repeated exposure to increasing concentrations of icotinib for more than 12 months. PC-9R cells were transfected with programmed cell death ligand 1 (*PD-L1*) knockdown plasmid (*PD-L1*-KD)/overexpression plasmid (*PD-L1*-OE), and treated with Wnt pathway agonist CHIR99021 or β-catenin antagonist ICG-001. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium assay was employed for detecting cell sensitivity to icotinib. The invasion and migration abilities of the cells were evaluated using Transwell and scratch assays. Quantification of *PD-L1*, matrix metalloproteinase (MMP)-2, MMP-9 and Wnt/β-catenin pathway-related proteins was conducted by means of quantitative real-time polymerase chain reaction or Western blotting.
Results: Half-maximal inhibitory concentrations (IC_{50}) of PC-9 and PC-9R cells to icotinib were 1.73 µM and 25.18 µM, respectively. The expression of *PD-L1*, Wnt family member 1 (Wnt1) and β-catenin was higher in PC-9R cells than in PC-9 cells ($p < 0.05$). The transfection of *PD-L1*-OE resulted in elevated IC_{50}, migration, invasion, and MMP-2 and MMP-9 expression in PC-9R cells ($p < 0.05$), while transfection with *PD-L1*-KD had the opposite effect ($p < 0.05$). The expression of *PD-L1*, β-catenin, MMP-2 and MMP-9, and IC_{50}, migration and invasion was increased following PC-9R cells treatment with CHIR99021 ($p < 0.05$). These impacts were observed to be in direct contrast in the case of ICG-001 treatment ($p < 0.05$).
Conclusion: Activation of the Wnt/β-catenin pathway mediates the high expression of *PD-L1* to promote the resistance of NSCLC cells to icotinib. Thus, targeted inhibition of *PD-L1* expression is of benefit for the treatment of NSCLC.

Keywords: non-small cell lung cancer; *PD-L1*; EGFR-TKI; Wnt/β-catenin pathway; icotinib

Introduction

Non-small cell lung cancer (NSCLC) is traditionally treated using platinum or other cytotoxic chemicals, but some advanced NSCLC patients do not benefit from these medicines in terms of survival rate [1,2]. In the past decades, around 30% of NSCLC patients were found to be positive for epidermal growth factor receptor (EGFR) activating mutations [3]. EGFR is a receptor tyrosine kinase (RTK), and EGFR-tyrosine kinase inhibitor (EGFR-TKI), such as icotinib, is touted for its high-level efficacy in NSCLC treatment and thus recommended for application in the initial course of treatment for individuals bearing *EGFR* mutations [4]. Unfortunately, the NSCLC patients would develop resistance to EGFR-TKI after receiving the treatment for 10 months [5]. To boost the therapeutic efficacy of EGFR-TKI, it is of great significance to unravel the mechanism underlying NSCLC cell resistance to this drug.

Reportedly, the mechanism of EGFR-TKI resistance in NSCLC cells is pertinent to the upregulation of programmed cell death ligand 1 (*PD-L1*) [6–8]. *PD-L1*, which encodes the ligand of programmed cell death protein 1 (PD-1), is expressed in activated T cells. PD-1/*PD-L1* axis inhibits T cells activation and proliferation in cancer, thus avoiding the anti-tumor immune response of T cells [9]. A retrospective study also proved that EGFR-TKI presents worse treatment efficacy in patients with *PD-L1* overexpression [10]. In addition, NSCLC samples with EGFR-TKI resistance have high *PD-L1* expression [11]. Therefore, targeting *PD-L1* might be a useful avenue to ameliorate the EGFR-TKI resistance in NSCLC.

Interestingly, β-catenin can induce *PD-L1* transcription and expression, contributing to the immunological escape of cancer cells [12]. β-catenin, a vital downstream functional effector of the Wnt signaling pathway, is im-

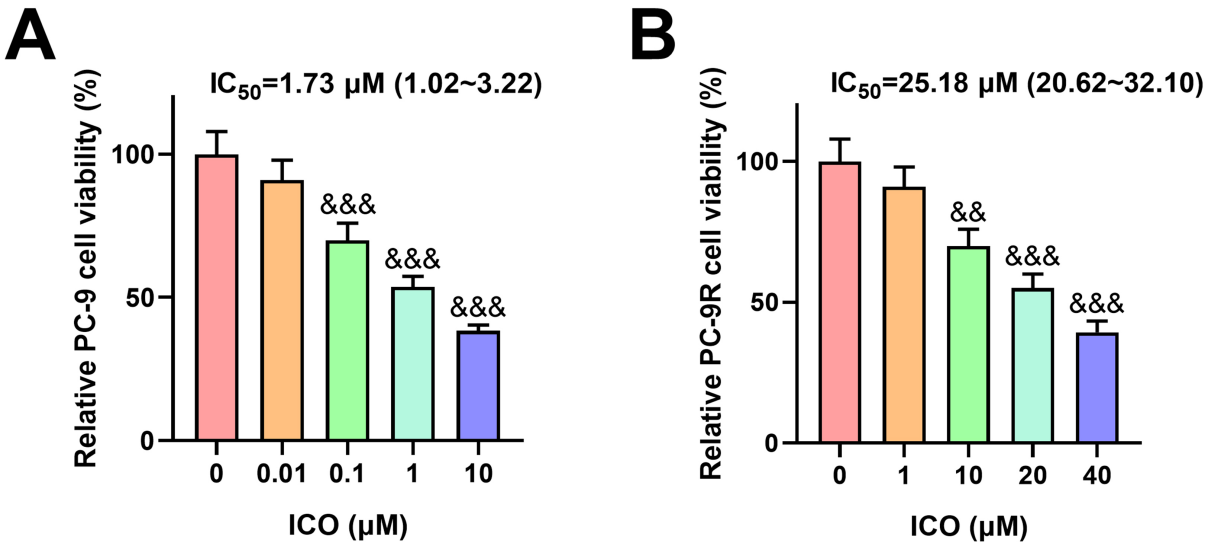

Fig. 1. Sensitivity of PC-9 and PC-9R cells to icotinib. (A,B) The sensitivity of PC-9 and PC-9R cells to icotinib was detected by MTS assay. $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. 0 μM icotinib. n = 3. Abbreviations: PC-9R, icotinib-resistant PC-9 cells; MTS, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium; ICO, icotinib; IC_{50}, half-maximal inhibitory concentrations.

Table 1. Primers used in this study.

Genes (Human)	5′ → 3′
PD-L1	F: GGCATTTGCTGAACGCATTTAC
PD-L1	R: TGCTTTCGCCAGGTTCCATT
Wnt1	F: TGCGCTTCCTCATGAACCTT
Wnt1	R: TGCTAGCGAGTCTGTTTGGG
β-catenin	F: TGGAACATGAGATGGGTGGC
β-catenin	R: GTGTTCTACACCATTACTCAATTCT
GAPDH	F: AGGGTGGTGGACCTCAT
GAPDH	R: TGAGTGTGGCAGGGACT

Abbreviations: *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *PD-L1*, programmed cell death 1 ligand 1; F, forward; R, reverse; *Wnt1*, Wnt family member 1.

Table 2. Antibodies used in this study.

Name	Catalog no.	Molecular weight (kDa)	Dilution
PD-L1	ab205921	33	1/1000
Wnt1	ab15251	41	1/1000
β-catenin	ab224803	86	1/400
MMP-2	ab92536	74	1/1000
MMP-9	ab76003	78	1/1000
GAPDH	ab8245	37	1/1000
Goat anti-rabbit	ab205718	—	1/2000
Goat anti-mouse	ab205719	—	1/2000

Abbreviations: MMP, matrix metalloproteinase.

plicated in tissue homeostasis and tumorigenesis [13]. Of note, blocking the Wnt/β-catenin pathway can suppress the EGFR-TKI resistance of NSCLC cells [14]. Moreover, β-catenin was identified to be abundantly expressed in NSCLC cells with EGFR-TKI resistance, and knocking down β-catenin can attenuate EGFR-TKI resistance of NSCLC cells [15]. Therefore, we hypothesize that Wnt/β-catenin pathway activation-induced upregulation of *PD-L1* might contribute to resistance of NSCLC cells to EGFR-TKI.

Materials and Methods

Cell Culture

Human NSCLC cell line PC-9 (AW-CELLS-H0295, Anweisci, Shanghai, China) was incubated in 1640 medium (SNM-001B, Sunncell, Wuhan, China) supplemented with 10% fetal bovine serum (FBS; F0601, Anweisci, Shanghai, China) and 1% penicillin-streptomycin (SNA-001, Sunncell, Wuhan, China) at 37 °C under the condition of 5% CO_2. Icotinib-resistant PC-9 cells (PC-9R) were prepared as previously described [16]. In short, PC-9 cells were repeatedly exposed to icotinib (HY-15164, MedChemExpress, Shanghai, China) at increasing concentrations for more than 12 months. PC-9 clones that remained viable after the prolonged icotinib exposure were selected (named PC-9R cells), followed by further incubation executed in the same way as PC-9 cells. All cell lines were identified via STR analysis, and mycoplasma detection was performed, and all cells were free of mycoplasma.

Plasmid Transfection

Overexpression plasmid (*PD-L1*-OE, G125526, Youbio, Changsha, China) and knockdown plasmid (*PD-L1*-KD, sc-39699-SH, Santa Cruz, CA, USA) were applied to manipulate *PD-L1* expression in PC-9R cells, while

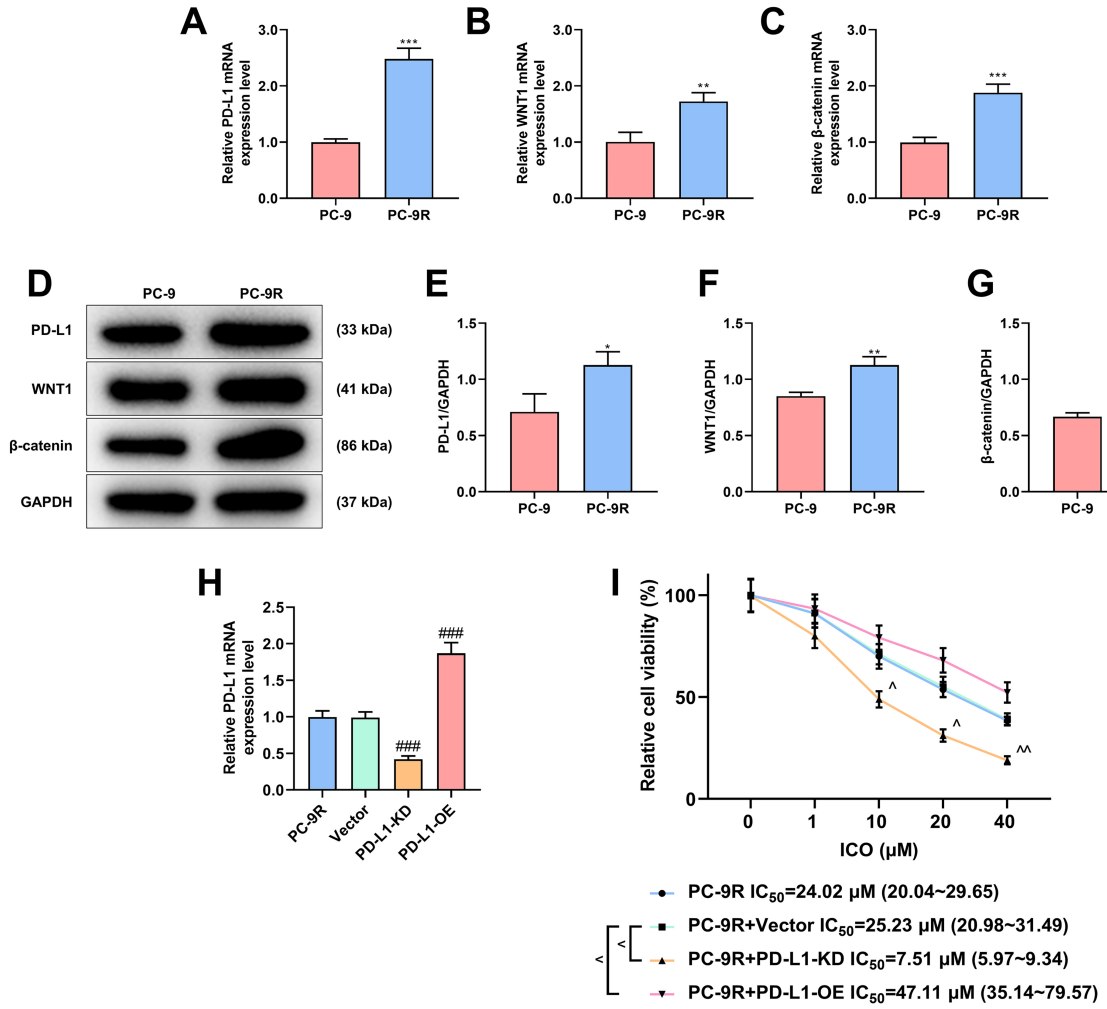

Fig. 2. Expression of *PD-L1*, *Wnt1* and *β*-catenin in PC-9 and PC-9R cells and the influence of PD-L1 on cell sensitivity to icotinib. (A–C) mRNA expression of *PD-L1*, *Wnt1* and *β-catenin* in PC-9 and PC-9R cells was measured by qRT-PCR, with *GAPDH* being the internal control. (D–G) Protein expression of PD-L1, Wnt1 and *β*-catenin in PC-9 and PC-9R cells was detected by Western blotting, with GAPDH being internal control. For (H,I), PC-9R cells were transfected with empty vector, *PD-L1*-KD or *PD-L1*-OE. (H) Transfection efficiency of *PD-L1* was measured in PC-9R cells by qRT-PCR, with *GAPDH* being the internal control. (I) Sensitivity of transfected PC-9R cells to icotinib (MTS experiment). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. PC-9. $^{\#\#\#}p < 0.001$ vs. vector. $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ vs. PC-9R+vector. n = 3. Abbreviations: KD, knockdown plasmid; OE, overexpression plasmid.

empty vector was used as negative control (NC). The coding sequence of PD-L1 is shown in **Supplementary File 1**. DNA transfection reagent (CBMD250, Abace-biology, Beijing, China) was prepared in serum-free medium and then mixed with the above-mentioned plasmids. The mixture was cultivated for 48 h with cells pre-inoculated in a 6-well plate. Transfection efficiency was then evaluated using quantitative real-time polymerase chain reaction (qRT-PCR).

Cell Treatment

In the first part of the experiment, PC-9R cells were transfected with NC, *PD-L1*-KD or *PD-L1*-OE for 48 h, respectively. In the second part, the cells were treated with Wnt pathway agonist CHIR99021 (6 μM; GC16702, GLPBIO, Shanghai, China) for 24 h [17] or *β*-catenin antagonist ICG-001 (20 μM; GC16893, GLPBIO, Shanghai, China) for 24 h [18].

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H Tetrazolium (MTS)

Transfected or treated PC-9 or PC-9R cells were them incubated with icotinib at different concentrations for 48 h, and then with MTS reagent (ST1009, Saint-Bio, Shanghai, China) at 37 °C for 3 h. Absorbance was measured at 490 nm using a microplate reader (SpectraMax Id5, Molecular Devices, San Jose, CA, USA). Relative cell viability (%) = (experimental absorbance/control absorbance) × 100%.

qRT-PCR

After isolating total RNA by utilizing Trizol reagent (AN51L758, Life-iLab, Shanghai, China), cDNA synthesis was conducted using a reverse transcription kit (E3010, NEB, Beverly, MA, USA). cDNA samples combined with qPCR Mix (M3003, New England BioLabs, Ipswich, MA, USA) and specific primers (Table 1) were subjected to qRT-PCR on the fluorescence quantitative analyzer (Fascan 48E, Tianlong, Xi'an, China). Gene quantification was performed using the $2^{-\Delta\Delta CT}$ method, and the gene expression level was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) [19].

Western Blotting

Total proteins obtained from lysed PC-9/PC-9R cells (RIPA Lysis Buffer, AP01L013, Life-iLab, Shanghai, China) were electrophoretically separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (80648470, Cytiva, Shanghai, China). Protein concentration was measured using a BCA kit (BB-CAPCK500, Bio-swamp, Wuhan, China). Next, the separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (MF452, Mei5bio, Beijing, China). The membranes were blocked in solution of 5% bovine serum albumin (BSA, AP36L014, Life-iLab, Shanghai, China). After being probed with primary and secondary antibodies (Table 2, batch number, abcam, Cambridge, UK), the blot was developed using electrochemiluminescence (ECL) kit (26100, Biolite, Xi'an, China) and visualized on an imaging system (SynGene, Frederick, MD, USA). Relative protein expression = gray value of the target protein band/gray value of GAPDH.

Transwell Assay

Transwell inserts pre-coated with Matrigel (354262, Corning Inc., Corning, NY, USA) were employed to measure NSCLC cell invasiveness. Subsequently, PC-9/PC-9R cells in serum-deprived medium were transferred into upper inserts. The basolateral chamber was filled with the medium containing 20% FBS for cell invasion detection. Twenty-four hours later, only the invading cells were subjected to fixation with 4% paraformaldehyde (AC28L112, Life-iLab, Shanghai, China) and color development with 0.1% crystal violet (BL802A, Biosharp, Beijing, China). The cells were viewed under an inverted microscope (N300M, Yongxin, Ningbo, China). Relative invasion rate (%) = (the number of invading cells in the experimental group/the number of invading cells in the control group) $\times$ 100%.

Wound Healing Assay

Cells were cultivated in a 6-well plate. To generate wounds, cultured cell layer was struck through by a sterile pipette tip. The debris was washed away with phosphate-buffered saline (PBS; BL302A, Biosharp, Beijing, China), after which the cells were incubated at 5% CO_2 and 37 °C for 24 h. Following incubation, the cells were photographed under an inverted microscope (N300M, Yongxin, Ningbo, China).

Statistical Analysis

The experiments of this study were repeated thrice. Data were analyzed by SPSS 21.0 system (SPSS Inc., Chicago, IL, USA) and are expressed as mean $\pm$ standard deviation (SD). Two-group and multi-group comparisons were conducted using independent samples *t*-test and one-way analysis of variance and follwed by Tukey post hoc tests, respectively. Results with $p < 0.05$ were perceived as statistically significant.

Results

Icotinib Resistance Raises IC_{50} and Expression of PD-L1, Wnt Family Member 1 (Wnt1) and β-Catenin

Sensitivity of PC-9 and PC-9R cells to icotinib was tested in MTS experiments. The results showed that icotinib could reduce cell viability in a dose dependent manner, the half-maximal inhibitory concentrations (IC_{50}) of the two cells were 1.73 µM and 25.18 µM, respectively, indicating a decrease in the sensitivity of PC-9R cells to icotinib (Fig. 1A,B, $p < 0.01$). Additionally, the mRNA levels of *PD-L1*, *Wnt1* and β-catenin were higher in PC-9R cells than in PC-9 cells (Fig. 2A–C, $p < 0.01$). The protein levels of PD-L1, Wnt1 and β-catenin were also higher in PC-9R cells than in PC-9 cells (Fig. 2D–G, $p < 0.05$).

PD-L1 Overexpression Raises IC_{50} and Promotes Migration and Invasion of PC-9R Cells, but PD-L1 Knockdown Exerts the Opposite Effects

To investigate how *PD-L1* influences PC-9R cells, we manipulated the *PD-L1* expression in PC-9R cells through transfection of *PD-L1*-KD and *PD-L1*-OE. The results showed that *PD-L1* expression was decreased in *PD-L1*-KD group and increased in *PD-L1*-OE group (Fig. 2H, $p < 0.001$). Interestingly, the transfection of *PD-L1*-KD diminished the IC_{50} of icotinib in PC-9R cells (IC_{50} = 7.51 µM), whereas an opposite effect was observed in the case of *PD-L1*-OE transfection (IC_{50} = 47.11 µM) (Fig. 2I, $p < 0.05$). Furthermore, PC-9R cells exhibited higher invasion (Fig. 3A,B, $p < 0.01$) and migration rates (Fig. 3C,D, $p < 0.01$), as well as higher levels of matrix metalloproteinase (MMP)-2 and MMP-9 (Fig. 3E–G, $p < 0.05$), relative to PC-9 cells; however, the above indexes were diminished by *PD-L1*-KD, yet augmented by *PD-L1*-OE (Fig. 3A–G, $p < 0.05$).

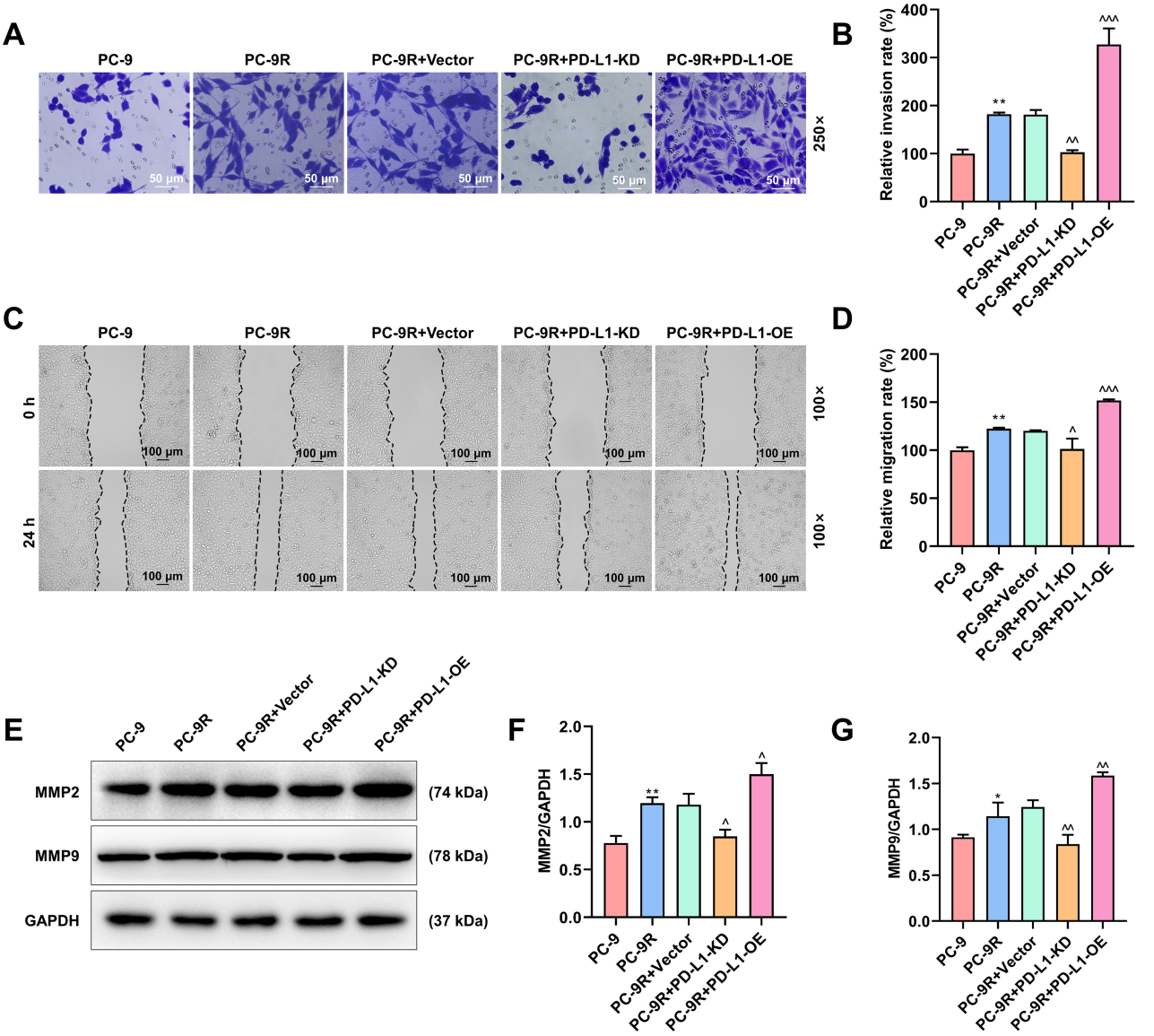

Fig. 3. Influences of *PD-L1*-KD and *PD-L1*-OE transfection on migration, invasion, and expression of MMP-2 and MMP-9 in PC-9 and PC-9R cells. (A,B) Cell invasion was assessed by Transwell assay (magnification: 250×; scale bar = 50 μm). (C,D) Cell migration was assessed by wound healing assay (magnification: 100×; scale bar = 100 μm). (E–G) MMP-2 and MMP-9 protein levels were quantitated by Western blotting, with GAPDH being the internal control. *$p < 0.05$, **$p < 0.01$ vs. PC-9. ^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$ vs. PC-9R+vector. n = 3.

Wnt Pathway Agonist Increases IC_{50}, Migration and Invasion of PC-9R Cells, while β-Catenin Antagonist Exerts the Opposite Effects

To understand how Wnt/β-catenin pathway impacts PC-9R cells, Wnt pathway agonist CHIR99021 and β-catenin antagonist ICG-001 were used to treat PC-9R cells. In PC-9R cells, CHIR99021 augmented *β-catenin* and *PD-L1* mRNA and protein levels, an impact that was opposite to that after ICG-001 treatment (Fig. 4A–E, $p < 0.05$). Moreover, the IC_{50} of icotinib in PC-9R cells was diminished by ICG-001 (IC_{50} = 7.54 μM) but raised by CHIR99021 (IC_{50} = 46.60 μM) (Fig. 4F, $p < 0.05$). In a similar pattern, ICG-001 suppressed the migration and invasion abilities, as well as MMP-2 and MMP-9 expression, in PC-9R cells, which were enhanced by CHIR99021 (Fig. 5A–G, $p < 0.05$).

Discussion

The present study dissected the intricacies surrounding icotinib resistance by exploring the differences between PC-9R and PC-9 cells at the molecular level. Firstly, the IC_{50} values of icotinib for the two categories of cells were different from each other. In a previous study, the IC_{50} value of icotinib for PC-9 cells, after a 48-hour exposure, was 2.80 μM [20], which was within the range from 1.02 to 3.22 μM based on our calculation. The IC_{50} value of ico-

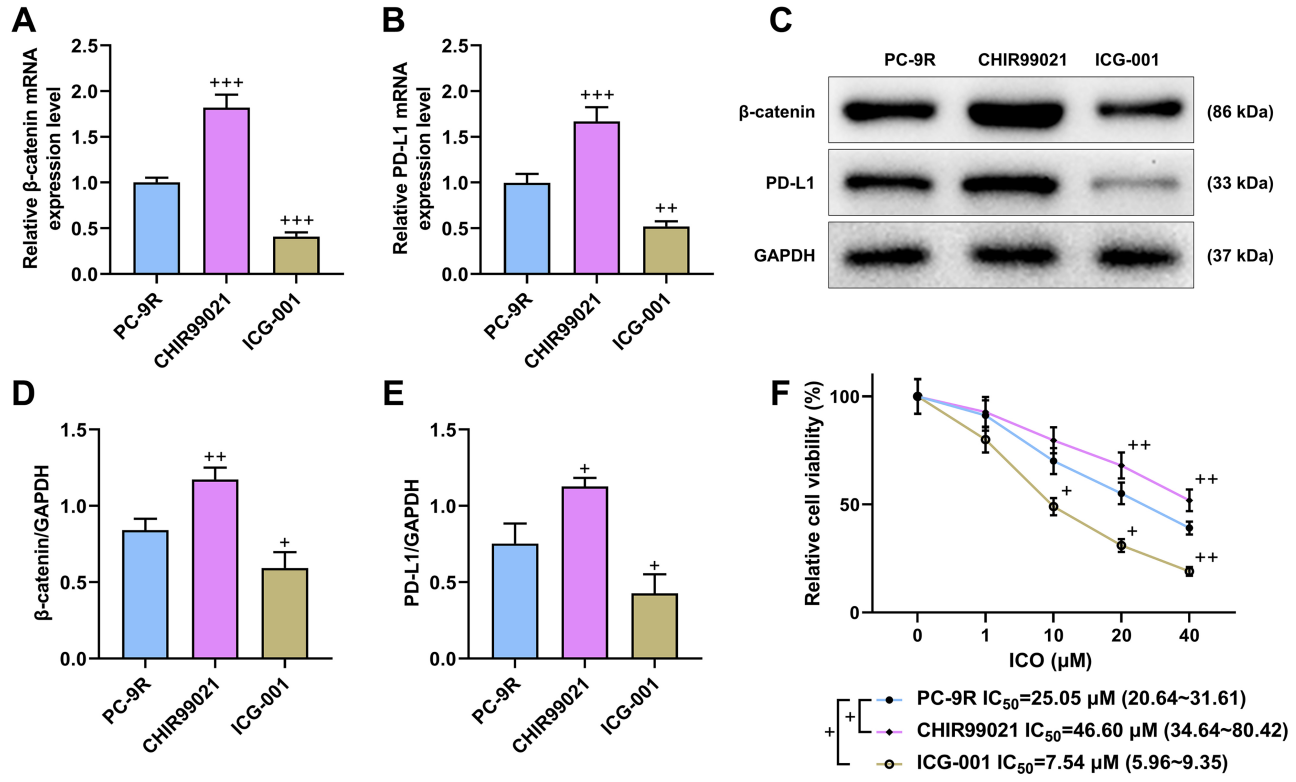

Fig. 4. Influences of CHIR99021 or ICG-001 on *β-catenin* and *PD-L1* expression as well as icotinib sensitivity in PC-9R cells. PC-9R cells were exposed to Wnt pathway agonist CHIR99021 (6 μM) or β-catenin antagonist ICG-001 (20 μM). (A,B) mRNA expression of *β-catenin* and *PD-L1* in PC-9R cells was measured by qRT-PCR, with *GAPDH* being the internal control. (C–E) Protein expression of β-catenin and PD-L1 in PC-9R cells was measured by Western blotting, with GAPDH being the internal control. (F) Treated PC-9R cell sensitivity to icotinib was assessed by MTS experiment. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. PC-9R. n = 3.

tinib for PC-9R cells being 10 times higher than that for PC-9 cells was a clear indication that an icotinib-resistant cellular model was successfully established. A prior study has shown that the migration and invasion abilities of erlotinib-resistant PC-9 cells were stronger than those of PC-9 cells [21]. Consistently, we found that icotinib-resistant PC-9 cells exhibited higher MMP-2 and MMP-9 expression and displayed stronger migration and invasion abilities than PC-9 cells. The type IV collagenases MMP-2 and MMP-9 are reliable indicators of metastasis and prognosis for NSCLC patients, as evidenced by their higher expressions in NSCLC tissues than in adjacent tissues [22]. Furthermore, oral squamous cell carcinoma cells that are resistant to icotinib have been shown to express higher levels of MMP-2 and MMP-9, predisposing the extracellular matrix to destruction, thereby facilitating cancer cell metastasis [23]. PC-9R cells displayed a higher *PD-L1* expression in comparison to PC-9 cells, an observation that has long been found in NSCLC patients with drug resistance, who often have a worse prognosis [24].

On these bases, we further probed into the effect of *PD-L1* on the malignant phenotype of PC-9R cells. PD-L1 depletion can reduce IC_{50} of cisplatin in PC-9 cells [24]. Similarly, we found that *PD-L1*-KD can diminish the IC_{50} of icotinib in PC-9 cells, while *PD-L1*-OE exerted the opposite effect. The mechanism of EGFR-TKI resistance involving PD-L1 is pertinent to PD-1/PD-L1 axis-mediated immune escape [6]. In addition, Tung *et al.* [25] reported that PD-L1 can mediate Yes1 associated transcriptional regulator (YAP1) expression to confer EGFR-TKI resistance on NSCLC cells. Lin *et al.* [26] demonstrated that PD-L1 enhances tumor invasiveness and EGFR-TKI resistance by activating extracellular signal-regulated kinase (ERK) signaling. Therefore, blocking PD-L1 can inhibit the malignant phenotype of PC-9R cells, which has been demonstrated not only in human NSCLC cell line PC-9 in this study but also in other drug-resistant cell lines [27]. Moreover, in some clinical trials targeting NSCLC patients, PD-L1 inhibitors combined with EGFR-TKI have shown good therapeutic effects [28]. These findings suggested that blocking PD-L1 is crucial for improving the efficacy of EGFR-TKI.

The current study verified that the activated Wnt/β-catenin pathway is able to boost PD-L1 level [29]. Therefore, we studied how Wnt/β-catenin pathway affects the malignant phenotype of PC-9R cells. In previous preclinical studies, the activated Wnt/β-catenin pathway promotes the resistance of NSCLC cells to afatinib [30]. Coinciding

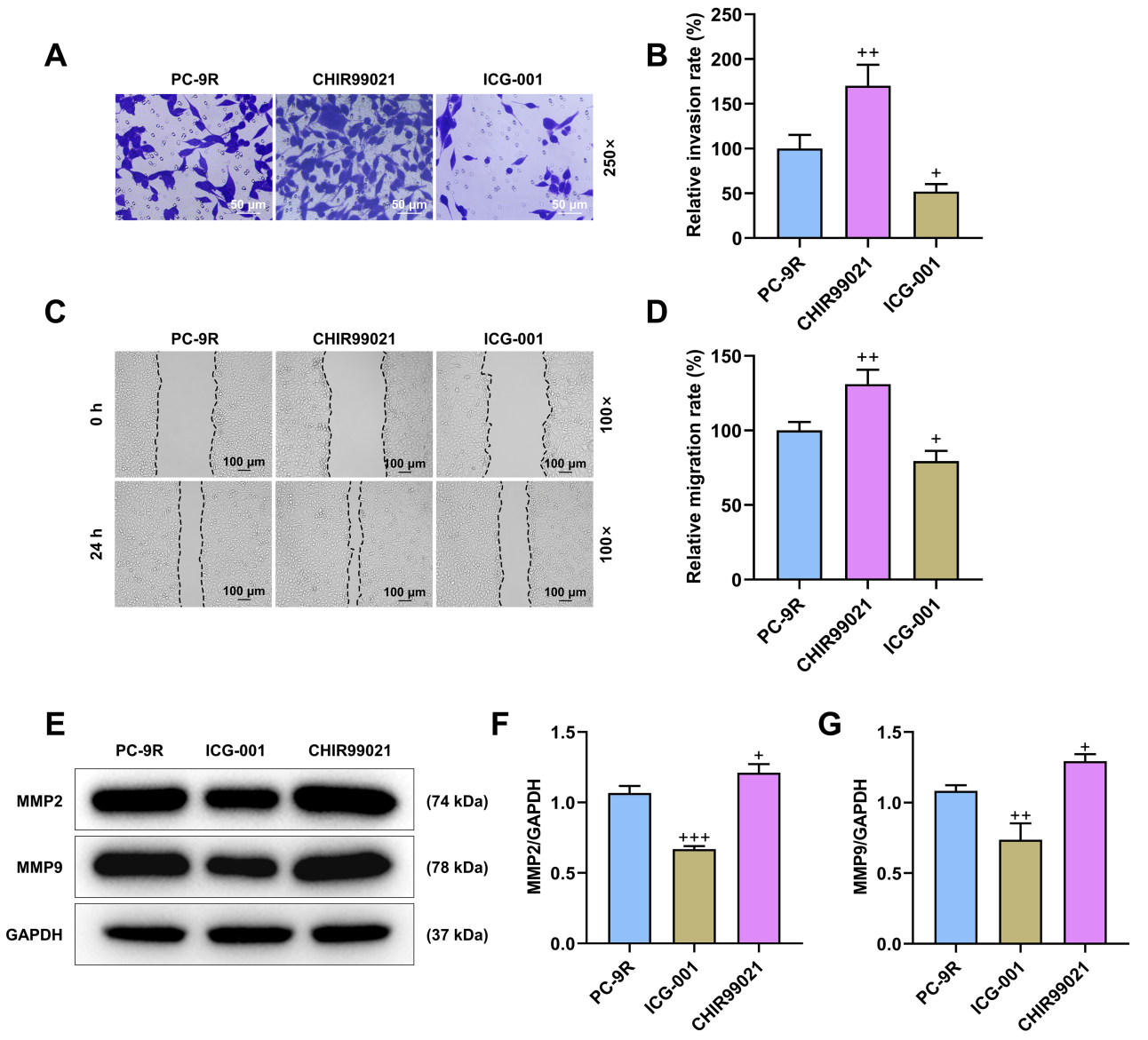

Fig. 5. Influences of CHIR99021 or ICG-001 on the migration, invasion, and MMP-2 and MMP-9 expression in PC-9R cells. PC-9R cells were exposed to Wnt pathway agonist CHIR99021 (6 μM) or β-catenin antagonist ICG-001 (20 μM). (A,B) Cell invasion was assessed by Transwell assay (magnification: 250×; scale bar = 50 μm). (C,D) Cell migration was assessed by wound healing assay (magnification: 100×; scale bar = 100 μm). (E–G) Protein levels of MMP-2 and MMP-9 were detected by Western blotting, with GAPDH being the internal control. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. PC-9R. n = 3.

with above studies, it has been found that activating Wnt/β-catenin pathway in NSCLC patients can promote gefitinib resistance [31]. Wnt/β-catenin pathway inhibitor can attenuate the emergence of osimertinib-resistant colonies from gefitinib-resistant NSCLC cells [32]. Consistently, our study reported that the IC_{50} of icotinib in PC-9R cells was elevated by the activation of Wnt/β-catenin pathway but reduced by its inhibition.

Conclusion

In conclusion, our study uncovered the mechanism of icotinib resistance in PC-9 cells, which is engendered by Wnt/β-catenin pathway activation-mediated PD-L1 upregulation, causing successful immune escape of tumor cells. This study presents a crucial research insight into exploring an innovative approach to treating NSCLC patients with EGFR-TKI resistance.

Availability of Data and Materials

The analyzed data sets generated during the study are available from the corresponding author upon reasonable request.

Author Contributions

ZDH designed the research study; JJW, ZHX and QL performed the research; ZYR and YFD collected and analyzed the data. All authors have been involved in drafting the manuscript and all authors have been involved in revising it critically for important intellectual content. All authors give final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202436190.211.

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