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The mechanism of *ABL1* upregulating the expression of *PD-L1* and the therapeutic effect of PD-L1 and STAT3 inhibitors in lung adenocarcinoma

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The upregulation of programmed cell death-ligand 1 (PD-L1) and continuous mutation of EGFR could induce chemoresistance in somatic cancers, however, the molecular mechanism of oncogene ABL1 in regulating the expression of PD-L1 in lung adenocarcinoma (LAD) remains unclear. In addition, the therapeutic effect of STAT3 and PD-L1 inhibitors in LAD is not fully understood. The ABL1 lentiviruses were used to transfect LAD cell lines (H1975, PC-9) with different EGFR mutation subtypes. Next, the expression of the JAK/STAT3 and PD-L1 pathway was detected followed by the treatment with STAT3 and PD-L1 inhibitors. Lastly, we observed the apoptosis and expression of STAT3 and PD-L1 before and after treatments in transfected and knocked down cell lines. The expression of ABL1 was upregulated by more than 3.71-fold and the expression of PD-L1 increased by 4.85-fold in lung cancer tissues compared with para-cancer tissues (both $p<0.01$), the ABL1 could induce upregulation of PD-L1 in LAD cell lines. Furthermore, the STAT3 inhibitor might induce more apoptosis than the PD-L1 inhibitor in both H1975 and PC-9 cell lines (both $p<0.01$) The STAT3 inhibitor combined with PD-L1 inhibitor had a synergistic effect on the PC-9 cell line, and the antagonistic effect was observed on the H1975 cell line. Furthermore, the expression of PD-L1 decreased almost equally after the PD-L1 inhibitor combined with a STAT3 inhibitor, or the STAT3 inhibitor alone ($p>0.05$). In addition, the STAT3 and PD-L1 decreased significantly after the STAT3 inhibitor compared with other treatments on the H1975 cell line (both $p<0.01$). To conclude, the EGFR mutation subtypes might influence the therapeutic efficacy in the treatment with PD-L1 inhibitor combined with STAT3 inhibitor on LAD cell lines.

Key words: lung adenocarcinoma, ABL1, JAK/STAT3, PD-L1

The major attribution to poor prognosis of non-small cell lung cancer (NSCLC) patients are cancer metastasis and chemoresistance [1], of which the possible reasons are the re-mutation of epidermal growth factor receptor (EGFR) which is insensitive to epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) or the upregulation of programmed cell death-ligand 1 (PD-L1). Therefore, the subsequent therapeutic choices after chemoresistance are rare [2, 3], thus it is necessary to further explore the mechanism of chemoresistance and further treatment strategy.

It is well known that the upregulation of PD-L1 would be accompanied by chemoresistance in the NSCLC [4]. Studies have demonstrated that the PD-L1 could contribute to primary resistance to EGFR-TKI in EGFR-mutant NSCLC cells, which may be mediated through the induction of epithelial-mesenchymal transition (EMT) via activating of the TGF-β/Smad canonical signaling pathway [5], and PD-L1 expression serves as a prognostic role in advanced NSCLC [6]. We have revealed that the miR-3127-5p/p-STAT3 axis could upregulate the expression of PD-L1 which may induce the chemoresistance [7], however, the complexity of PD-L1 made it difficult to identify the specific cells that contribute to the evasion from immune surveillance, therefore, further exploring the mechanism which induce the immune evasion may be helpful to identify the patients who are sensitive to immunotherapy [8]. Up to date, Atezolizumab (PD-L1 inhibitor) has been used in the treatment of platinum-resistant metastatic NSCLC and urothelial cancer based on phase II and the preliminary phase III studies which have shown significant improvement in objective response rate and median overall survival (OS) [9, 10], which shows great potency in cancer therapeutics.

Abelson murine leukemia viral oncogene homolog 1 (*ABL1*) was identified as an oncogene in leukemia at first [11]. Cytoplasmic c-Abl is associated with growth factor receptor signaling that influences cell mobility and adhesion [12]. Studies have shown that the c-ABL protein could regulate non-receptor tyrosine kinase involving in cell proliferation, survival, migration, and apoptosis [13]. We have reported that reduced miR-3127-5p could promote the NSCLC invasion via the c-Abl/Ras/ERK pathway in the previous study [14]. Furthermore, multiple JAK-independent mechanisms, which induce STAT3 activation, have been identified [15, 16], and STAT3 inhibition displays an attractive cancer strategy [17]. One study has revealed the apoptosis could be induced in NSCLC cells with acquired erlotinib resistance through direct inhibiting STAT3 [16]. Another study also illustrated that the STAT3 inhibitor could induce apoptosis and pro-death autophagy in A549 cells (NSCLC cell line). It has been reported that a single-agent of AZD9150 (STAT3 inhibitor) is effective in patients with highly treatment-refractory lymphoma and NSCLC in phase 1 dose-escalation study [18].

Based on the above analysis, in the present study we intended to study on: 1/ How the oncogene *ABL1* to regulate the expression of PD-L1 via JAK/STAT3 pathway; 2/ The therapeutic effects of PD-L1 and STAT3 inhibitors on different EGFR mutation subtypes of the LAD cell lines; 3/ The molecular mechanisms of different therapeutic effect of PD-L1 and STAT3 inhibitors on LAD cell lines.

Patients and methods

Human samples. Human cancer and corresponding non-cancer tissues were collected at the time of surgery from 73 patients with NSCLC from May 2017 to April 2018 at the Department of Thoracic Surgery of the Huadong Hospital Affiliated to Fudan University. Human tissues were immediately frozen in liquid nitrogen and stored at –80 °C. Signed informed consent was obtained from all patients and the study was approved by the Clinical Research Ethics Committee of Fudan University.

Cell culture and reagents. Human NSCLC cell lines (PC-9: Del19 mutation, H1975: T790M/L858R mutation) were purchased from the Cell Resource Center, Shanghai Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences. PC-9 cell line with. Cells were maintained at 37 °C in a humidified air atmosphere containing 5% carbon dioxide in RPMI1640 supplemented with 10% FBS. Both PD-L1 inhibitor (BMS202, S7912) and STAT3 inhibitor (Stattic, S7024) were bought from Selleck Company.

Cell proliferation assay. Cells were seeded into 96-well plates (6.0×10^3 cells/well). On day 0, 3, 5, and 7, cell viability was assessed by the cell-counting kit-8 assay (Beyotime Institute of Biotechnology, Shanghai, China). The absorbance of each well was read on a spectrophotometer (Thermo, Shanghai) at 450 nm (A450). Three independent experiments were performed in quintuplicate.

Virus packaging and infection. The fluorescent lentiviral vectors pLenti-III-blank, pLenti-III-*ABL1*, and pLenti-III-*ABL1*-off were purchased from Shanghai Genechem Company. To produce lentivirus, all vectors were transfected into HEK-293T cells with virus packaging plasmids using LentiFectin™ reagent. To generate stably transduced cell lines, the PC-9 and H1975 cell lines were infected with each lentivirus and subjected to puromycin selection (PC-9: 0.8 mg/ml; H1975: 1.0 mg/ml) for two weeks.

RNA extraction and quantitative real-time PCR. Total RNA was extracted from cultured cells using the Isolation Kit (Ambion; Life Technologies), formalin-fixed and paraffin-embedded normal and human lung cancer specimens using the Recover All Total Nucleic Acid Isolation Kit (Ambion; Life Technologies). cDNA was synthesized from total RNA with specific stem-loop primers and the TaqMan Reverse Transcription Kit (Applied Biosystems; Life Technologies). The sequences of the primers were as shown in Table 1.

Immunofluorescence (IF). Cells were fixed with 4% formaldehyde, permeabilized with 0.2% Triton, and blocked with 2% BSA for the indicated times. Next, the prepared cells were stained with antibodies purchased from Cell Signaling Technology anti-ABL1 (1:100, #2862), anti-STAT3 (1:100, #9139), anti-PD-L1 (1:100, #13684) at 4 °C for 12 h. The next day, the cells were incubated with FITC-conjugated secondary antibody for 1 h and then observed under a fluorescence microscope. To label the nuclei, cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen), and visualized using a confocal microscope (Olympus FV-1000).

Microscopy. Cells were plated at low confluence in 24-well plates (5×10^4 cells/well). Cells were washed with PBS and treated with 4% paraformaldehyde/PBS for 20 minutes at room temperature, washed, and then permeabilized with 0.1% Triton X-100 for 5 min. Cells were then blocked with 5% normal goat serum (Cell Signaling Technology) containing 0.3% Triton X-100 in PBS for 60 minutes. The diluted primary antibody was applied in blocking buffer overnight at 4 °C. Alexa Fluor-594/488 secondary antibodies diluted in 1% normal goat serum in PBS were added for 1 h at room temperature. Cells were fixed using Vectashield hard-set mounting medium containing DAPI dye (Vector Laboratories). Images were acquired using confocal microscopy (Olympus FV-1000) and overlaid using ImageJ.

Table 1. Primers used for quantitative real-time PCR.

ABL1	forward: 5'-AAGCCGCTCGTTGGAACTC-3'
	reverse: 5'-AGACCCGGAGCTTTTCACCT-3'
STAT3	forward: 5'-CAGCAGCTTGACACACGGTA-3'
	reverse: 5'-AAACACCAAAGTGGCATGTGA-3'
PD-L1	forward: 5'-TGGCATTTGCTGAACGCATTT-3'
	reverse: 5'-TGCAGCCAGGTCTAATTGTTTT-3'
GAPDH (internal control)	forward: 5'-GGAGCGAGATCCCTCCAAAAT-3'
	reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'

Flow cytometric analysis. Cells were collected by trypsinization and washed with ice-cold PBS. The cells were then stained with PE-labeled anti-PD-L1 mAb (29E.2A3; BioLegend, San Diego, CA), APC-labeled anti-PD-L1 mAb (EH12.2H7; BioLegend), or isotype control. Labeled cells were analyzed by a FACSCalibur flow cytometer (BD, San Jose, CA) and FlowJo (Tree Star Inc., Oregon, USA). Each assay was done in triplicate.

For cells were plated in 75 cm^2 dishes and treated with either starvation; STAT3 inhibitor or PD-L1 inhibitor, STAT3 inhibitor combined with PD-L1 inhibitor treatments and analyzed for relative DNA content on a dual laser flow cytometer (Beckman Coulter Epics Elite; Beckman). Each assay was done in triplicate.

Flow cytometry after annexin-V/propidium iodide (PI) staining. The collected cells were centrifuged at 650×g for 5 min and then washed twice with pre-cooled PBS. The washed cells were centrifuged and collected, and the treated cells (1×10^6) were transferred into an Eppendorf (EP) tube and 500 μl of bidding buffer was added into each EP tube, and the mixture was mixed evenly. PI (5 μl) and Annexin V (5 μl) (KeyGEN BioTECH, Nanjing, China) were added into each EP tube, followed by 10–15 min incubation in the dark The incubated cell suspension was filtered through 400 mesh cell screens to filter off the cell clumps. After filtration, the cells were placed on ice and analyzed within 30 min using a flow cytometer. AnnexinV+PI– indicated early apoptosis and AnnexinV+PI+ indicated late apoptotic. Apoptotic rate = early apoptosis rate + late apoptosis rate.

Statistical analysis. Data was shown as mean ± SD, Student-t test was used for statistical analysis, and all statistical analyses were performed with the SAS 9.4 software. A p-value <0.05 was considered significant.

Results

***ABL1* upregulated the expression of PD-L1.** The basic expression of PD-L1 in cell lines was confirmed by flow cytometry (Figure 1A). Next, *ABL1*-lentiviruses were transfected into the H1975 and PC-9 cell lines. As a result, we found that the expression of PD-L1 was upregulated by exogenous *ABL1* in the H1975 and PC-9 cell lines; in contrast, the expression of PD-L1 was suppressed when *ABL1* was knocked down compared with the control (Figure 1B). Furthermore, the *ABL1* transfected cells began to show growth advantages after 4 days compared with the knocked down or control groups (Figure 1C). We also employed immunofluorescence to show the location of ABL1 and PD-L1. As a result, a higher expression of PD-L1, which was induced by *ABL1* presented on the membrane of cell lines (Figure 2). These results indicated that *ABL1* might induce the expression of PD-L1.

Next, we detect the expression of *ABL1* in 73 pairs of primary lung cancer and corresponding non-tumorous tissues. The characteristics of patients are shown in Table 2; we found that there was no difference in terms of TNM stage, histology, age, gender, and smoking history. Figure 3A showed that the expression of *ABL1* was upregulated by more than 3.71-fold and PD-L1 increased 4.85-fold in cancer tissues compared with non-tumorous tissues ($p<0.001$). Moreover, Figure 3B showed that *ABL1* and *PD-L1* elevated significantly in advanced-stage patients (stage III) than early stages (stage I and II) ($p<0.01$).

ABL1 could promote the expression of PD-L1 through activating STAT3. We explored the molecular mechanism of *ABL1* in regulating the expression of PD-L1. At first, we detected the expression of STAT3 in *ABL1* transfected H1975 and PC-9 cell lines, as a result, the expression of STAT3 increased in the *ABL1* transfected cell lines compared with the knocked down and control groups. Moreover, the expression of STAT3 decreased significantly in the knocked down group compared with the control (Figures 3C, 3D). Therefore, we speculated that the *ABL1* could promote the expression of PD-L1 through activating STAT3.

The therapeutic efficacy of PD-L1 inhibitor combined with STAT3 inhibitor. We have demonstrated that the upregulation of PD-L1 could induce immune evasion resulting in cisplatin-resistance in lung cancer in the previous study. Given that: 1/ the expression of PD-L1 in H1975 and PC-9 cell lines were higher than that in the wild-type cell lines; 2/ the *ABL1* could promote STAT3 to induce the expression of PD-L1. Therefore, we intended to explore the therapeutic efficacy of PD-L1 inhibitor (BMS202) combined with

Table 2. The basic characteristics of NSCLC patient.

Variable	Patients (n)
Gender	
Male	36
Female	37
Age	
<64 years	39
≥64 years	34
Smoking	
Never	31
Ever	42
Histology	
Adenocarcinoma	44
Squamous cell carcinoma	29
TNM stage	
I	26
II	20
III	27
EGFR mutation	
Y	28
N	45
Chemoresistance	
Y	33
N	40

Abbreviations: NSCLC – non-small cell line cancer; EGFR – epidermal growth factor receptor

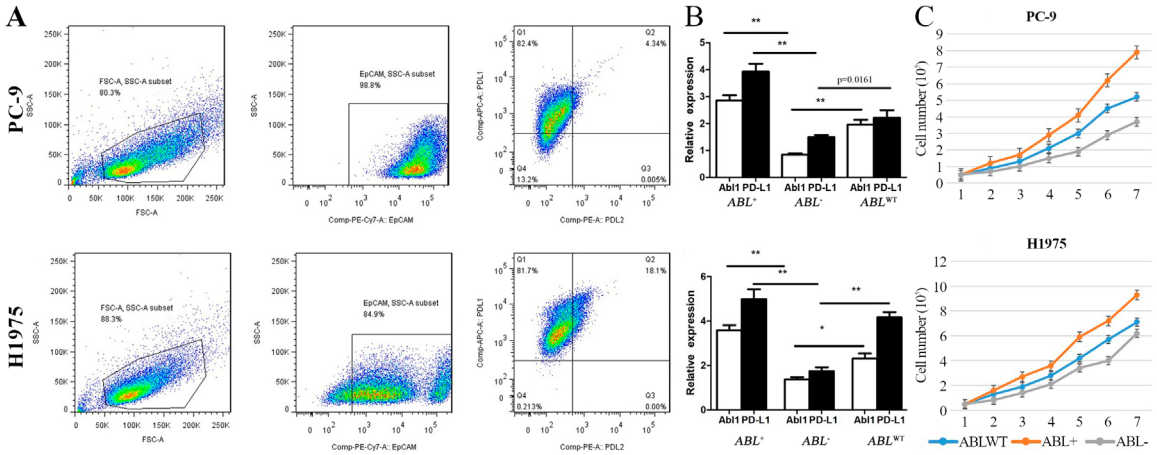

Figure 1. The basic expression of PD-L1 in cell lines. A) Flow cytometry showed the expression of PD-L1 in the PC-9 cell line and the expression of PD-L1 in the H1975 cell line; B) qRT-PCR showed the expression of *ABL1* and *PD-L1* when *ABL1* was transfected or knocked down in the PC-9 cell line and in the H1975 cell line; C) The *ABL1*-transfected PC-9 and H1975 cells show growth advantages after 4 days compared with the knocked down cells or control. *p<0.05; **p<0.01

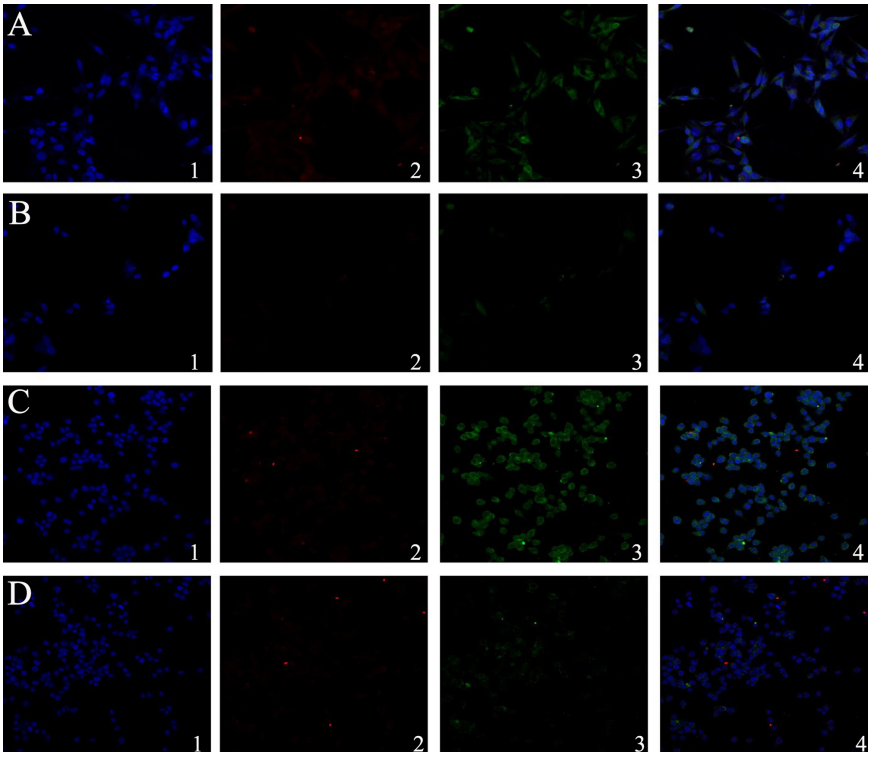

Figure 2. Immunofluorescence showed the expression of ABL1 and PD-L1 in the *ABL1*-transfected cell lines. A) The ABL1 and PD-L1 expression in the *ABL1*-transfected H1975 cell line; B) The ABL1 and PD-L1 expression when the *ABL1* was knocked down in the H1975 cell line; C) The ABL1 and PD-L1 expression when the *ABL1* was transfected in the PC-9 cell line; D) The ABL1 and PD-L1 expression when the *ABL1* was knocked down in the PC-9 cell line. Abbreviations: 1 – Nucleus; 2 – ABL1 expression; 3 – PD-L1 expression; 4 – merge

STAT3 inhibitor (Stattic) in the treatment of LAD cell lines with different EGFR mutation subtypes. At first, we detected the IC50 values of the PD-L1 inhibitor and STAT3 inhibitor on H1975 and PC-9 cell lines. As a result, the dose (24 h) of the PD-L1 inhibitor was 24.4 μM for H1975, 6.7 μM for PC-9. The dose (24 h) of the STAT3 inhibitor was 32.2 μM for H1975, 8.9 μM for the PC-9 cell line.

In order to detect therapeutic efficacy of the PD-L1 inhibitor combined with STAT3 inhibitor on the ABL1-transfected or knocked down H1975 and PC-9 cell lines, we performed

six assays for each cell line: 1/ Blank, 2/ STAT3 inhibitor (IC50), 3/ PD-L1 inhibitor (IC50), 4/ 1/2 STAT3 inhibitor (1/2 IC50)+PD-L1 inhibitor (IC50), 5/ STAT3 inhibitor (IC50)+1/2 PD-L1 inhibitor (1/2 IC50), 6/ STAT3 inhibitor (IC50)+PD-L1 inhibitors (IC50). Then we observed the apoptosis after 24 hours.

We found that: 1/ the STAT3 inhibitor could induce more apoptosis than PD-L1 inhibitor for both PC-9 cell line (Figures 4A-2/3 – 4C-2/3, $p<0.05$) and H1975 cell line (Figures 4D-2/3 – 4F-2/3, $p<0.05$). 2/ The STAT3 inhibitor combined with PD-L1 inhibitor has a synergistic effect on the PC-9 cell line, that is, the PD-L1 inhibitor combined with the STAT3 inhibitor induced more apoptosis than 1/2 STAT3 inhibitor+PD-L1 inhibitor or STAT3 inhibitor+1/2 PD-L1 inhibitor (Figures 4A-4 – 4C-4). 3/ 1/2 STAT3 inhibitor+PD-L1 inhibitor or STAT3 inhibitor+1/2 PD-L1 inhibitor could induce more apoptosis than STAT3 inhibitor or PD-L1 inhibitor alone on the PC-9 cell line. 4/ The antagonistic effect was observed on the H1975 cell line, that is, the PD-L1 inhibitor combined with the STAT3 inhibitor could induce less apoptosis than the PD-L1 inhibitor alone (Figures 4D-4 – 4F-4).

We also examined the therapeutic effect of STAT3 inhibitor (IC50) and PD-L1 inhibitor (IC50) on the ABL1-transfected cell lines. As a result, we found that: 1/ the STAT3 inhibitor, PD-L1 inhibitor induced more apoptosis in *ABL1*-transfected cell line (PC-9) (Figure 4A) than the control (Figure 4B) or knocked down cell line (Figure 4C). 2/ The *ABL1*-knocked down cell line (PC-9) has less apoptosis compared with the control group with the treatment of STAT3 inhibitor or PD-L1 inhibitor ($p<0.05$). 3/ The results in the H1975 cell line are shown in Figures 4D–4F.

The change of STAT3 and PD-L1 expression after PD-L1 inhibitor and STAT3 inhibitor treatment. We compared the different therapeutic effects of the STAT3 inhibitor (IC50), PD-L1 inhibitor (IC50), and STAT3 inhibitor (IC50) combined with PD-L1 inhibitor (IC50) on *ABL1*-transfected and knocked down PC-9 (Supplementary Figure S1A-S1C) and H1975 cell lines (Supplementary Figures S1D–S1F); as a result, we found that the STAT3 inhibitor combined with PD-L1 inhibitor has a significant synergistic therapeutic effect on the PC-9 cell line, antagonistic effects were observed on the H1975 cell line.

Next, we detected the expression of STAT3 and PD-L1 before and after treatments. At first, the expression of STAT3 in the PC-9 cell line was detected. We found that: 1/ The expression of STAT3 decreased nearly equally after PD-L1 inhibitor combined with STAT3 inhibitor or STAT3 inhibitor treatment alone, however, the expression of STAT3 decreased more in both treatments compared with PD-L1 inhibitor

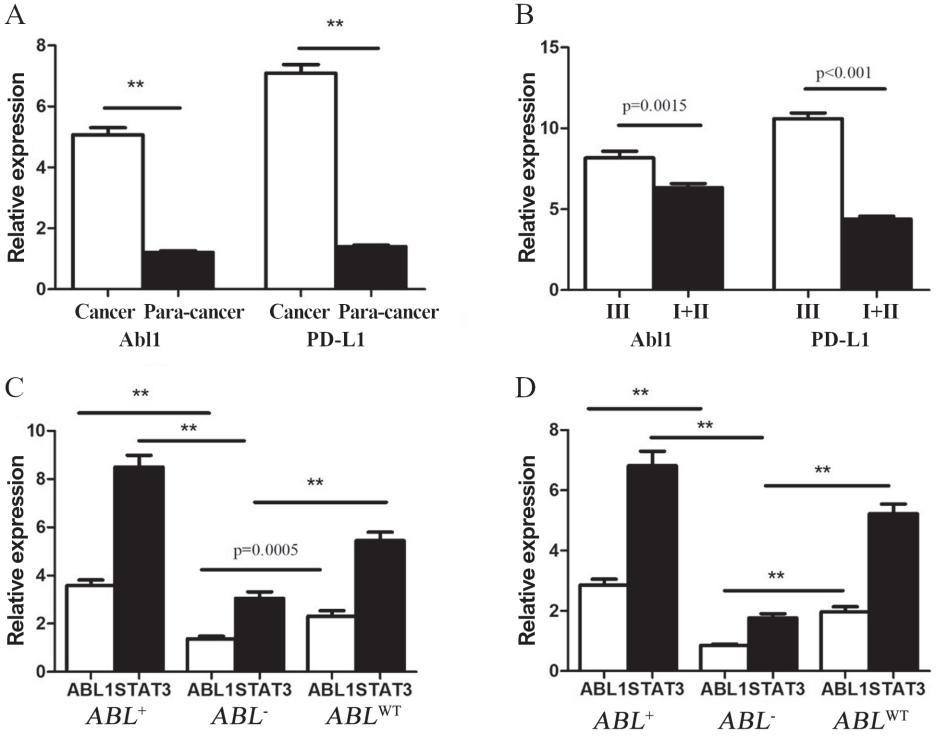

Figure 3. The expression of *ABL1*, *STAT3*, and *PD-L1* in the lung cancer tissues and transfected cell lines. A) The expression of *ABL1* was upregulated by more than 3.71-fold and PD-L1 increased 4.85-fold in lung cancer compared with non-tumorous tissues; B) The *ABL1* and PD-L1 increased in advanced-stage patients (stage III) than early-stages (stage I and II); C) The STAT3 increased in *ABL1*-transfected cell lines compared with knocked down and empty controls in the H1975 cell line; D) The STAT3 increased in *ABL1*-transfected cell lines compared with knocked down and empty controls in the PC-9 cell line. **p<0.01.

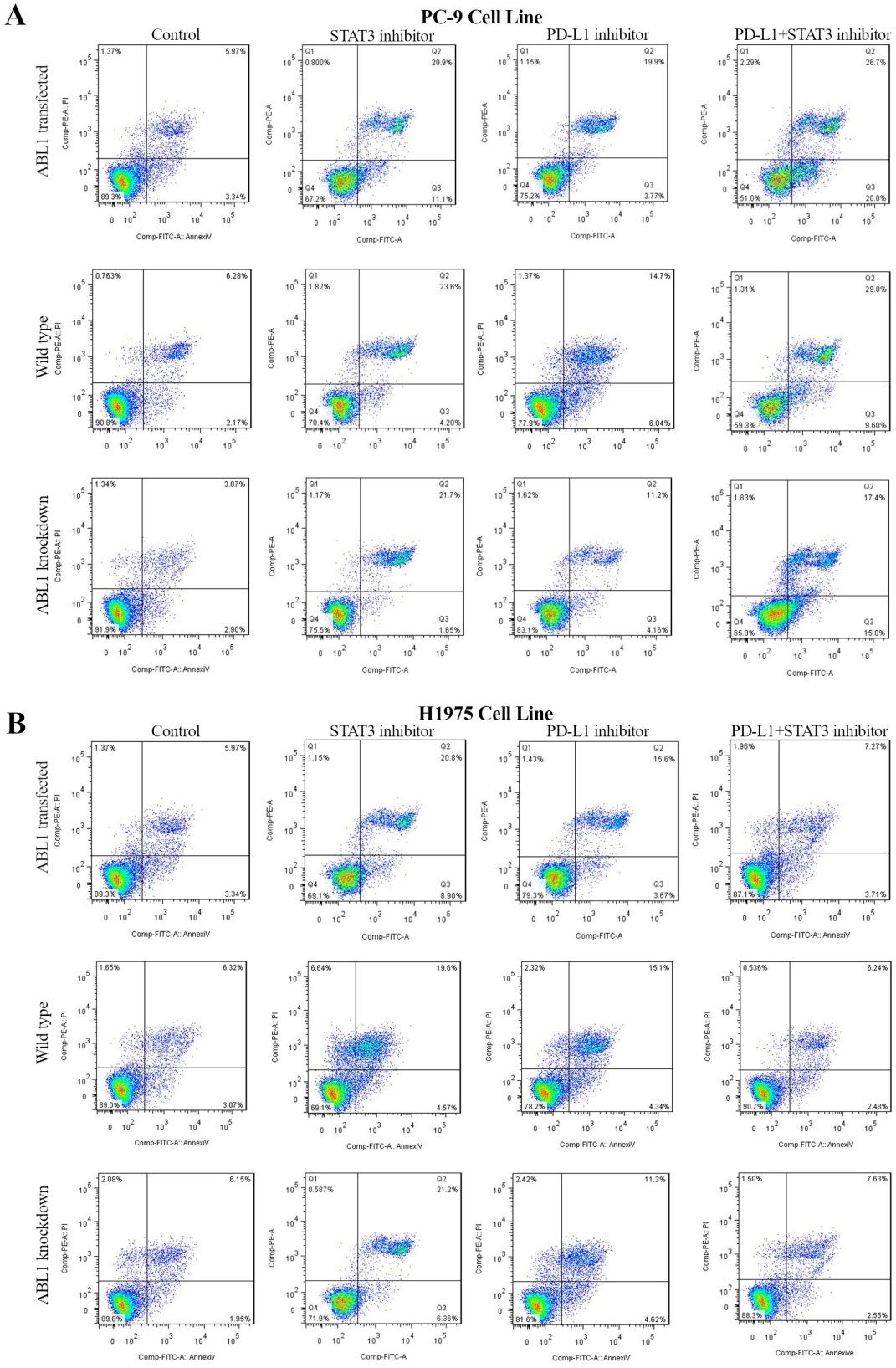

Figure 4. Treatment efficacy of the PD-L1 inhibitor and the STAT3 inhibitor on the apoptotic rate in *ABL1*-transfected PC-9 (A) and H1975 (B) cell lines.

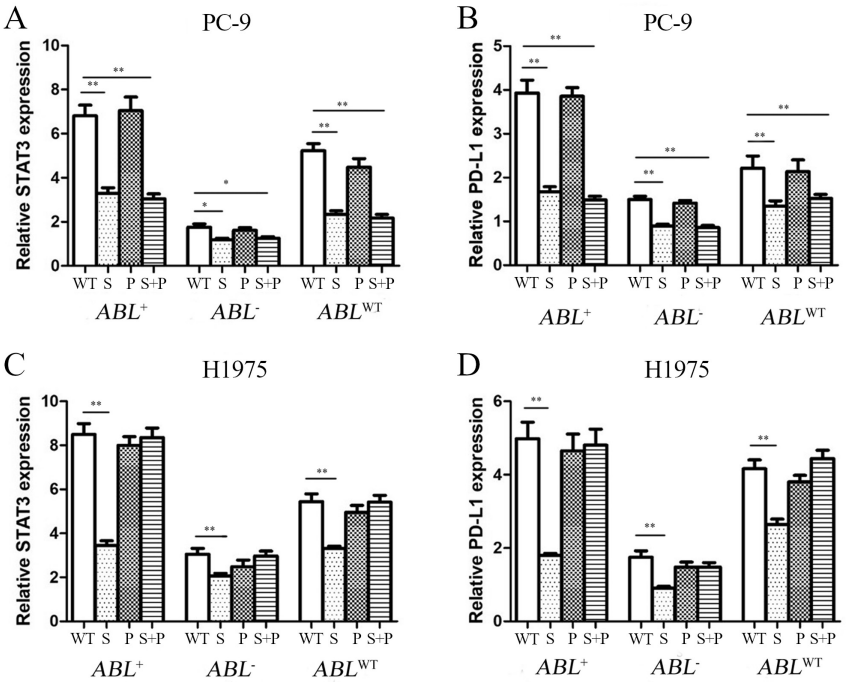

Figure 5. The change of PD-L1 and STAT3 in cell lines after PD-L1 inhibitor and STAT3 inhibitor treatment. A) The change of STAT3 on PC-9 cells was detected after PD-L1 inhibitor and STAT3 inhibitor treatment when ABL1 was transfected or knocked down. B) The change of PD-L1 on PC-9 cells was detected after PD-L1 inhibitor and STAT3 inhibitor treatment when ABL1 was transfected or knocked down. C) The change of STAT3 on H1975 cells was detected after PD-L1 inhibitor and STAT3 inhibitor treatment when ABL1 was transfected or knocked down. D) The change of STAT3 on H1975 cells was detected after PD-L1 inhibitor and STAT3 inhibitor treatment when ABL1 was transfected or knocked down. *p<0.05; **p<0.01.

alone; 2/ The influence of the STAT3 inhibitor on the expression of STAT3 was more than that of the PD-L1 inhibitor; 3/ The expression of STAT3 decreased more after 1/2 PD-L1 inhibitor+STAT3 inhibitor than PD-L1 inhibitor+1/2 STAT3 inhibitor (Figure 5A).

Then the expression of PD-L1 was detected in the PC-9 cell line and we found that: 1/ The expression of PD-L1 decreased nearly equally with the treatment of PD-L1 inhibitor combined with STAT3 inhibitor or STAT3 inhibitor alone, however, the PD-L1 decreased more in both compared with PD-L1 inhibitor treatment alone. 2/ The effect of STAT3 inhibitor on the expression of PD-L1 was greater than that of PD-L1 inhibitor. 3/ The expression of PD-L1 decreased more with the treatment of 1/2 PD-L1 inhibitor+STAT3 inhibitor than PD-L1 inhibitor+1/2 STAT3 inhibitor. Finally, we found that the change on the expression of STAT3 and PD-L1 was greater in the *ABL1*-transfected cell line than control, the change on knocked down cell line was the weakest (Figure 5B).

Next, we detected the expression of STAT3 in the H1975 cell line. As a result, we found that: 1/ The expression of STAT3 decreased significantly after the STAT3 inhibitor compared with PD-L1 inhibitor alone or PD-L1 inhibitor combined with STAT3 inhibitor treatment; 2/ The influence of the STAT3 inhibitor on the expression of STAT3 was greater than that of the PD-L1 inhibitor (Figure 5C).

At last, the expression of PD-L1 in the H1975 cell line was detected, we found that: 1/ the expression of PD-L1 decreased more after STAT3 inhibitor compared with PD-L1 inhibitor combined with STAT3 inhibitor or PD-L1 inhibitor alone; 2/ the influence of STAT3 inhibitor on the expression of PD-L1 was more than that of PD-L1 inhibitor. Finally, we found that the change on the expression of STAT3 and PD-L1 was more in the *ABL1*-transfected cell line than that in the control or knocked down cell lines (Figure 5D).

Mutation analyses. We investigated the mutation status of *ABL1* in lung tumor tissues through data collected from the public database of the cBioPortal for Cancer Genomics. As shown in Supplementary Figures S2A and S2B, *ABL1* had a low somatic mutation rate in lung adenocarcinoma (mutation rate = 1.67% [11/660]) in the Pan-Lung Cancer (TCGA, Nat Genet 2016); and lung squamous carcinoma (mutation rate = 1.45% [7/484]) in the Pan-Lung Cancer (TCGA, Nat Genet 2016). The putative copy-number alterations are mainly comprised of shallow deletion, diploid and gain, and *ABL1* is a common target for abnormal activation in exons encoding the tyrosine kinase (TK) domain (Supplementary Figures S2C, S2D). In addition, we investigated the protein-protein interaction (PPI) network between ABL1 and STAT3 to identify the hub genes. The PPI network was constructed by using the STRING online database (https://string-db.org/cgi/input.pl?sessionId=WG9mHyppXW0F&input_page_

show_search=on). Then the specific node genes were identified (Supplementary Figure S2E) and the top five highly scored genes were *JAK2, ABI2, EGFR, IL6,* and *ABI1*.

Discussion

In the present study, we found that the *ABL1* could promote upregulation of PD-L1 through activating JAK/STAT3 pathway; EGFR mutation subtypes might influence the therapeutic efficacy of PD-L1 and STAT3 inhibitors in NSCLC cell lines; furthermore, the STAT3 inhibitor could induce more apoptosis which may due to it inhibit the STAT3/PD-L1 axis expression, whereas the PD-L1 inhibitor has no such therapeutic effect.

It is well known that the reactivity of EGFR-TKI varied widely for patients with different EGFR mutation subtypes [19, 20]. Uchibori et al. have reported that patients with Del19/21 are sensitive to the first-generation EGFR-TKI, while patients with T790M are resistant [21]. It also has been reported that the EGFR re-mutation (i.e. L858R) would be resistant to the first-generation EGFR-TKI [22, 23]. Although the third-generation EGFR-TKI (i.e. osimertinib) could overcome the problem, while as, the new re-mutation (i.e. T797C) appears again [24–26]. How to settle with chemoresistance induced by the continuous gene re-mutation is urgent [27, 28]. In the present study, we found that the PD-L1 and STAT3 inhibitors have an obvious therapeutic effect on LAD cell lines; and the EGFR mutation subtypes might influence the therapeutic efficacy. One case report has found that the L858R mutation is an acquired resistance mutation to nivolumab (PD-L1 inhibitor) [29], and patients with high PD-L1 expression may exhibit a poor response to EGFR-TKI. In addition, it has reported that the JAK/STAT3 pathway plays an important role in cancer proliferation and invasion, which could promote cancer aggression through inflammation or immunosuppression [30, 31]. Kim et al. have reported that Afatinib might activate the interleukin-6 receptor (IL-6R)/JAK1/STAT3 signaling via autocrine IL-6 secretion in the NSCLC with T790M and inhibiting it could increase the sensitivity to Afatinib [32]. Therefore, we intended to explore the therapeutic effect of PD-L1 and STAT3 inhibitors in the EGFR mutant LAD cell lines with a higher expression of PD-L1. As a result, we found that the PC-9 cell line with Del19 was sensitive to both inhibitors and a synergistic effect was observed when they were combined in the treatment, whereas the effect was antagonized for the cell lines with T790M/L858R mutation. So, we speculated that the EGFR mutation subtypes might affect the therapeutic effect. Fujita et al. have shown that the immune escape induced by the upregulation of PD-L1 could promote cancer metastasis and chemoresistance [33]. The study has found that pembrolizumab (PD-L1 inhibitor) is safer than paclitaxel for advanced gastrointestinal cancer with positive PD-L1 expression, however, pembrolizumab did not improve the overall survival (OS) compared with paclitaxel as second-line therapy [34]. While another study has reported that atezolizumab (PD-L1 inhibitor) could improve the clinically OS in the NSCLC patients who had received chemotherapy previously [35]. The Checkmate-057 study has shown that the nivolumab (PD-L1 inhibitor) could improve the OS compared with conventional docetaxel chemotherapy for non-squamous NSCLC patients with increasing PD-L1 expression; however, the Checkmate-017 study revealed that superior OS was obtained regardless of PD-L1 expression [36, 37]. Although PD-L1 inhibitors show superior promise, no specific beneficiaries have been identified yet [38]. There are two possible reasons for the unsatisfied results: 1/ no specific population has been identified [39]; 2/ the target of PD-L1 inhibitors is not specific, so more researches should be focused on the immune status of patients such as the distribution of sub-lymphocyte [40–42].

Undated, there is no better solution for patients without EGFR mutation who are resistant to chemotherapy regime or KRAS-mutant LAD. Spigel et al. have reported that the Nivolumab and Crizotinib may have some effect, however, there are still serious toxic and side effects [43]. Based on the results in the present study, in addition, Guru et al. [44] have reported that in the *BCR/ABL1* negative myeloproliferative neoplasm patients, JAK2 (V617F) mutation percentage or burden is associated with increased mRNA expression of PD-L1 and the upregulation of PD-L1 is mediated via STAT3/STAT5 activation and STAT3 is mainly co-expressed with PD-L1 and STAT5 has a minor role in the PD-L1 induction. Therefore, we hypothesized that if the expression of STAT3 and PD-L1 has been detected before treatment and the patients are adopted appropriate treatments according to the expression, the STAT3 and PD-L1 inhibitors would bring better wish for such patients [38].

There are still some limitations to the present study. At first, it is unknown whether the PD-L1 inhibitor activates the immune system *in vivo*; next, we did not detect other pathways which may be activated by *ABL1*; at last, whether the autophagy or pyroptosis are associated with the treatment efficacy of PD-L1 inhibitors and STAT3 inhibitors should be detected in further work?

In conclusion, the study demonstrated that the STAT3 inhibitor combined with PD-L1 inhibitor had a synergistic effect on PC-9 cells, the antagonistic effect was observed on H1975 cells. The EGFR mutation subtypes could influence the efficacy of PD-L1 and STAT3 inhibitors in the treatment of LAD.

Supplementary information is available in the online version of the paper.

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The mechanism of *ABL1* upregulating the expression of *PD-L1* and the therapeutic effect of PD-L1 and STAT3 inhibitors in lung adenocarcinoma

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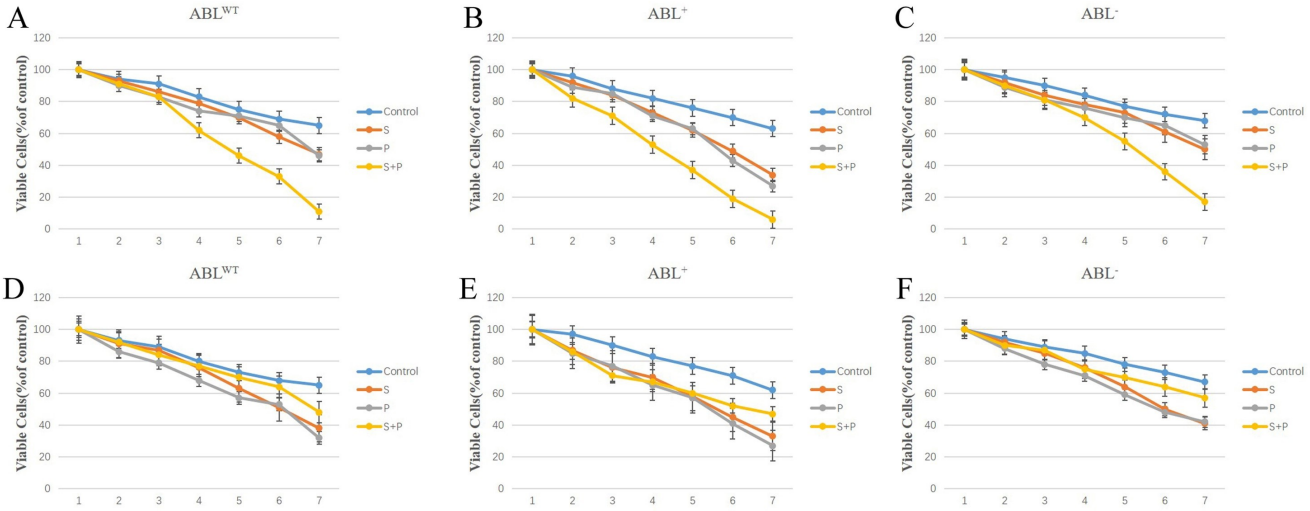
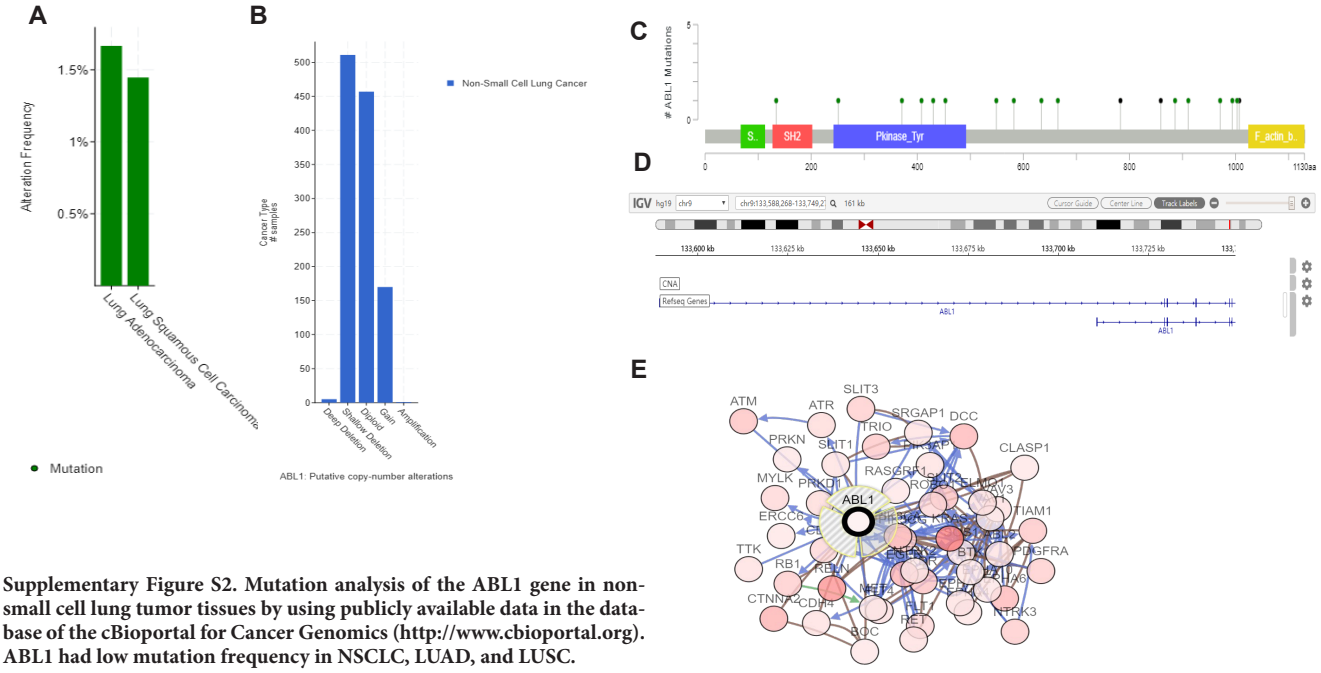
Supplementary Information



Supplementary Figure S1. The treatment effects of STAT3 inhibitor, PD-1/PD-L1 inhibitor, and STAT3 inhibitor combined with PD-1/PD-L1 inhibitor on *ABL1*-transfected and knocked down PC-9 and H1975 cells. A–C) PC-9 cells, D–F) H1975 cells.



Supplementary Figure S2. Mutation analysis of the ABL1 gene in non-small cell lung tumor tissues by using publicly available data in the database of the cBioportal for Cancer Genomics (http://www.cbioportal.org). ABL1 had low mutation frequency in NSCLC, LUAD, and LUSC.