FGFR1 Promotes Tumor Immune Evasion via YAP-Mediated PD-L1 Expression Upregulation in Lung Squamous Cell Carcinoma

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**FGFR1 promotes tumor immune evasion via YAP-mediated PD-L1 expression
upregulation in lung squamous cell carcinoma**

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Abstract

Background: Variations in fibroblast growth factor receptor 1 (FGFR1), which occur frequently, are common driver mutations of lung squamous cell carcinoma. Immune checkpoint inhibitors targeting programmed death-1 (PD-1) and programmed death ligand-1 (PD-L1) are powerful anticancer weapons. Activation of FGFR1 leads to tumorigenesis through multiple downstream molecules, including Yes-associated protein (YAP), but whether and how FGFR1 regulates tumor immune evasion remain largely unclear.

Methods: H520 and HCC95 cells were treated with siRNA and plasmids to increase or decrease the expression of FGFR1, YAP and PD-L1, as assessed by molecular assays of protein and mRNA expression. The interaction between YAP and PD-L1 was verified by chromatin immunoprecipitation. After FGFR1 knockdown by shRNA, cancer cells were cocultured with Jurkat T cells, and then cell proliferation and activity were assessed. In C57BL/6 mice, the tumor immune microenvironment was analyzed by flow cytometry, immunofluorescence and immunohistochemistry after FGFR1 knockdown. The effect of the combination of FGFR1 knockdown and PD-1 blockade was explored both in vitro and in vivo.

Results: In H520 and HCC95 cells, FGFR1 upregulated PD-L1 expression via YAP, and YAP initiated the transcription of PD-L1 after binding to its promoter region. Both in vitro and in vivo, FGFR1 knockdown decreased tumor growth and reduced immune escape and reactivation of T cells. The combination of FGFR1 knockdown and PD-1 blockade synergistically exerted antitumor effects. In human LSQCC, the expression of fibroblast growth factor 2 (FGF2), the activator of FGFR1, was positively correlated with that of PD-L1 at the mRNA level.

Conclusions: The FGFR1/YAP/PD-L1 regulatory axis mediates tumor-associated immune suppression in lung squamous cell carcinoma, and FGFR1 knockdown reactivates T cells in the tumor microenvironment. Synergistic inhibition of both FGFR1 and PD-1/PD-L1 may be a possible treatment for lung cancer patients.

Keywords: FGFR1; PD-L1; driver mutation; tumor immune evasion; combination therapy.

Background

Fibroblast growth factor receptor 1 (FGFR1) is a receptor tyrosine kinase and one of the four members of the FGFR family (1). Variations in FGFR1 exist in nearly 20% of lung squamous cell carcinoma (LSQCC) patients (2–4), and they mediate proliferation, metastasis, epithelial-mesenchymal transition and stemness (5–7). However, the response rate to selective FGFR tyrosine kinase inhibitors (TKIs) and nonselective multitargeted FGFR TKIs is only 8%-12% in certain lung cancer patients (8–11). More evidence is needed to determine the unknown phenotypes of FGFR1 variations.

In the past decade, immune checkpoint inhibitors (ICIs), especially those targeting programmed death-1 (PD-1, or CD279) and programmed death ligand-1 (PD-L1, or CD274), have introduced the concept of immune therapy to lung cancer treatment (12). PD-L1 is abundantly expressed on cancer cells, macrophages, and dendritic cells (DCs). The interaction between PD-1 and PD-L1 causes T lymphocyte dysfunction, enabling cancer cells to escape immune surveillance (13–15). In several cancer types, pan-FGFR inhibitors positively remodel the immune profile within the tumor microenvironment (TME) (16–20), suggesting that FGFR1 may have an immunosuppressive effect.

Yes-associated protein (YAP) is the main downstream effector of the Hippo/LATS1 (also known as the Salvador-Warts-Hippo) transduction pathway (21). FGFR1 mediates the stemness of LSQCC cells by interacting with LATS1/YAP (6). When Hippo/LATS1 is inactivated, YAP is transported into the nucleus and forms a complex with transcriptional enhanced associate domain (TEAD) (22,23). Activation of YAP/TAZ (another cotranscription factor like YAP) promotes PD-L1 transcription in lung adenocarcinoma, large cell lung cancer, breast cancer and melanoma cell lines, increasing the immune escape of cancer cells^{24–28}. We wondered whether YAP also regulates the expression of PD-L1 in LSQCC.

In this study, we investigated the role of FGFR1 in the mechanisms of immune escape by LSQCC cells and, more specifically, on immune cells of the TME. Considering the limited response rate to targeted or immune therapy alone, the effect of the combination of FGFR1 inhibition and PD-1/PD-L1 blockade was explored both in vitro and in vivo. Our work uncovered a regulatory axis between FGFR1 and PD-L1 that may provide new insights into tumor immune evasion and clues for novel therapeutic interventions for LSQCC patients.

Materials and methods

Cell culture and reagents

H520, HCC95 and Jurkat T cells were purchased from ATCC and cultured using RPMI 1640 (HyClone, USA) with 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, USA). LLC cells were purchased from ATCC and cultured using DMEM (HyClone, USA) with 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, USA). FGF2 and IFN-γ were purchased from PeproTech. AZD4547 and nivolumab were purchased from Selleck. AZD4547 was dissolved in DMSO, aliquoted, and stored as a 10 mM stock solution at –20 °C. Nivolumab was stored at 4 °C. Verteporfin (Sigma-

Aldrich, USA) was stored at a concentration of 10 mM in DMSO at -20 °C. All cells were maintained in a humidified incubator at 37°C with 5% carbon dioxide.

RNA interference and plasmid transfection

siRNAs (short interfering RNAs) and plasmids were transfected into cells with Lipofectamine 3000 (Invitrogen, USA) with or without P3000 (Invitrogen, USA) following the manufacturer's instructions. FGFR1 siRNA and an FGFR1 overexpression plasmid were constructed by our team previously. YAP siRNA, a YAP overexpression plasmid and two PD-L1 overexpression plasmids were purchased from Shanghai Huzbio Biotechnology (China). A PD-L1 promoter luciferase plasmid expressing firefly and Renilla was overexpressed. The sequences were as follows:

siYAP-1:

sense 5'- GGUGAUACUAUCAACCAAAdTdT -3',

antisense 5'- UUUGGUUGAUAGUAUCACCCdTdT -3';

siYAP-2:

sense 5'- AGAUACUUCUUAUAAUCACAdTdT -3',

antisense 5'- UGUGAUUUAAGAAGUAUCUdTdT -3';

siRNA-NC:

sense 5'- GCCACCAAGCUAGAUAAAGAAAdTdT -3',

antisense 5'- UUCUUUAUCUAGCUUGGUGGCdTdT -3';

YAP-OE:

forward: 5'- CGCAAATGGGCGGTAGGCGTG -3',

reverse: 5'-AGTCCCGTCCTAAAATGTC -3';

PD-L1-OE:

forward: 5'- CGCAAATGGGCGGTAGGCGTG -3',

reverse: 5'-AGTCCCGTCCTAAAATGTC -3';

PD-L1-Luc:

forward: 5'- CGCCAGTCAAGTAACAAC -3',

reverse: 5'-TTCCACACCCTAACTGACAC -3'.

Lentivirus transduction and generation of stable cell lines

Human FGFR1 lentivirus and a negative control carrying GFP and a puromycin tag were provided by Shanghai Huzbio Biotechnology (China). Recombinant lentiviral vectors containing mouse FGFR1 shRNA and irrelevant sequences were purchased from Shanghai DANGEN Biotechnology (China). In addition to the lentivirus expression vectors, a vector expressing a green fluorescent protein (GFP) and a hygromycin B reporter gene driven by the CMV promoter was used. Human and mouse transduction and stable cell line selection were performed according to the manufacturers' instructions. The sequences were as follows:

Human shFGFR1-1:

CCGGGAGATGGAGGTGCTTCACTTACTCGAGTAAGTGAAGCACCTCCATCTCTTTTG,

Human shFGFR1-2:

CCGGTCTTGAAGACTGCTGGAGTTACTCGAGTAACTCCAGCAGTCTTCAAG

1 ATTTTGTG.

2 Mouse shFGFR1-1:

3 GATGTTGAAGTCCGACGCACTCGAGTGCCTCGGACTTCAACATCTTTT,

4 Mouse shFGFR1-2:

5 CCGGCCTGGAGCATCATAATGGATTCTCGAGAATCCATTATGATGCTCCAGG
6 TTTT,

7 Mouse shFGFR1-3:

8 CCAGATCCTGAAGACTGCTGGAGTTCTCGAGAACTCCAGCAGTCTTCAGG
9 ATCTGGTTTTT.

11 **Western blot analysis, RNA extraction, cDNA synthesis, and qPCR**

12 Cells were lysed for protein or RNA extraction, and the samples were subjected to
13 western blot analysis or used for cDNA synthesis and qPCR as previously described
14 (5). The primers used for qPCR were as follows:

15 FGFR1:

16 forward 5'- TAATGGACTCTGTGGTGGCCTC -3',

17 reverse 5'- ATGTGTGGTTGATGCTGCCG -3';

18 YAP:

19 forward 5'- CATGTTTCGAGCTCATGCCTCTCCAGCTTCTCTG -3',

20 reverse 5'- CAGAGAAGCTGGAGAGGCATGAGCTCGAACATG -3';

21 TAZ:

22 forward 5'- GTATCCCAGCCAAATCTCGTGATG -3',

23 reverse 5'- CAGCGCATTGGGCATACTCATG -3';

24 PD-L1:

25 forward 5'- GGTGCCGACTACAAGCGAAT-3',

26 reverse 5'- AGCCCTCAGCCTGACATGTC -3';

27 GAPDH:

28 forward 5'- ACAACTTTGGTATCGTGGAAGG',

29 reverse 5'- GCCATCACGCCACAGTTTC -3'.

31 **Immunoblotting**

32 Cell lysates were eluted with SDS-loading buffer (50 mM Tris-HCl, pH 6.8, 10%
33 glycerol, 1% SDS, 1% beta-mercaptoethanol). The eluates were separated by SDS-
34 PAGE and transferred to PVDF membranes (EMD Millipore, Germany). the
35 antibodies included FGF Receptor 1 (D8E4, CST, USA), phospho-FGF Receptor 1
36 (Tyr653/654) (D4X3D, CST, USA), LATS1 (C66B5, CST, USA), phospho-LATS1/2
37 (Ser909/Ser872, Affinity, China), phospho-LATS1 (Thr1079, Affinity, China), YAP
38 (D8H1X, CST, USA), phospho-YAP (Ser127) (D9W2I, CST, USA), YAP/TAZ
39 (D24E4, CST, USA), PD-L1 (E1L3N, CST, USA), and GAPDH (14C10, CST, USA).
40 Western blotting images were captured with a ChemiScope5600 instrument (Clinx,
41 Shanghai, China) using ECL substrate.

43 **Dual luciferase reporter assay**

44 H520 cells were seeded in a 24-well plate until the cells reached approximately 50-60%

confluence. For the luciferase assay, the cells were transiently transfected with the indicated plasmids, and Renilla and firefly luciferase were used as internal controls. The cells were lysed with passive lysis buffer (Promega, USA) 48 hours after transfection and the indicated treatment. Luciferase assays were performed using a dual luciferase assay kit (Promega, USA), and luciferase activity was normalized to that of the internal control Renilla.

Chromatin immunoprecipitation (ChIP)

Briefly, chromatin samples were incubated with specific antibodies in ChIP lysis buffer (20 mM Tris-HCl (pH 8.1), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 0.05% SDS) overnight at 4°C. The experiment was performed as previously reported (29). Protein-DNA complexes were immobilized on prewashed protein A/G beads (20 µL per reaction). The bound fractions were washed 3 times with lysis buffer, twice with low-salt wash buffer (10 mM Tris-HCl, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% Na-deoxycholate), and once with 10 mM Tris-HCl (pH 8.0). Elution and reverse crosslinking were carried out in elution buffer (50 mM Tris-HCl (pH 8.0), and 1% SDS) at 65°C for 5 hours. After treatment with RNase A (1 unit/µL) at 37°C for 1 hour and digestion with proteinase K (1 unit/µL) at 55°C, the DNA samples were purified using a PCR extraction kit (QIAGEN #28006, USA). The precipitated DNA samples were either analyzed by qPCR or prepared for DNA deep sequencing according to the manufacturer's guidelines (Vazyme #ND607, USA).

Coculture of cancer cells and T cells

Jurkat T cells were activated with 100 UI/ml IL-2 3 days before the experiment. Cancer cells were inoculated in 24-well plates or 96-well plates 2 days in advance. Complete culture medium containing 500 IU/ml IFN-γ was added to H520 and HCC95 cancer cells for 24 hours to induce PD-L1 expression. Suspended/adherent cells were added to the coculture system at a ratio of 2:1 to 8:1 and cultivated for 48-72 h. A total of 1.0×10^4 cells were seeded in 500 µL medium in each well (N = 3) of a 24-well plate. A total of 2.5×10^3 cells were seeded in 100 µL medium in each well (N = 5) of a 96-well plate. Then, Jurkat T cells were isolated from the culture supernatant for flow cytometry analysis, and adherent cancer cells were washed with PBS twice for crystal violet staining or the CCK-8 assay.

Viability of cancer cells

Surviving H520 or HCC95 cells in 24-well plates were fixed and stained with crystal violet solution, and pictures of the dried plates were taken. The viability of residual tumor cells was also tested with a Cell Counting Kit-8 (Dojindo Co., Japan) according to the manufacturer's protocol. The viability of the vehicle group was set as 100%. The cell survival rate (%) was calculated as (OD value of treatment group/OD value of vehicle group) × 100%. The data are presented as the mean ± SD and were graphed with Prism software (7.0, GraphPad Software).

Flow cytometry (FCM)

Cells from the coculture system or those obtained by tumor digestion were resuspended in PBS/EDTA/BSA containing Fc Block (BD Biosciences, USA), centrifuged, incubated for 30 min with surface marker antibodies, washed with PBS/EDTA/BSA and then resuspended in 300 μ L PBS/EDTA/BSA. For cytokine amplification and detection, cells were cultured with Cell Activation Cocktail (with Brefeldin A) for 6 hours, processed with the Fc γ 3/Transcription Factor Staining Buffer Set (eBioscience, USA) according to the manufacturer's instructions and then intracellularly stained. Human and mouse antibodies were purchased from Biolegend (USA) and BD Pharmingen (USA). All the stained cells were analyzed using a BD Fortessa flow cytometer. The data were analyzed using FlowJo V10.0.7 software.

In vivo subcutaneous lung cancer model

The mouse NSCLC cell line LLC was obtained from our laboratory. Hygromycin B (1 μ g/ml) was added to shFGFR1 and the control shRNA. Healthy male C57BL/6 mice aged 6-8 w were inoculated with FGFR1 knockdown or wild-type LLC cells. The cells (50×10^4) were digested, washed, and resuspended in 100 μ L PBS before being injected into the left armpit of each mouse. Beginning on day 6, the size of each tumor was measured with calipers every three days. The subcutaneous tumor volume was calculated as $(L^2 \times W)/2$, where V is the tumor volume, L is the longest diameter, and W is the shortest diameter. InVivoMab anti-mouse PD-1 (Bio X Cell, USA) or rat IgG2 α isotype (Bio X Cell, USA) was injected intraperitoneally at a dose of 100 μ g per mouse on day 6, 8, 10 and 12. The mice were sacrificed on day 15. The tumors were removed and then imaged and analyzed. The tissues were digested with collagenase A (0.33 U/ml), dispase (0.85 U/ml) and DNase I (144 U/ml) with rapid shaking at 37 $^{\circ}$ C. Large pieces of debris were removed by successive filtration through 70- μ m and 40- μ m strainers.

Immunofluorescence (IF) and immunohistochemistry (IHC)

Tumors were fixed and embedded in paraffin after being removed. Sections were cut at a thickness of 10 μ m and subjected to Opal multiple fluorescence staining as previously described (30). The sections were incubated with DAPI (4',6-diamidino-2-phenylindole, Dilactate, Biolegend), an FGF Receptor 1 antibody (D8E4, CST, USA), a YAP antibody (D8H1X, CST, USA) and a PD-L1 antibody (E1L3N, CST, USA). Other sections were deparaffinized, subjected to antigen retrieval and immunostained with a ki-67 antibody (Servicebio, GB111141, 1:500, China) and CD8 antibody (Abcam, ab237723, England) as previously described (31). The stained cells were observed and imaged under an immunofluorescence microscope (Leica DFC420C, Germany).

Bioinformatics analysis of public datasets

Relative FGF2 and PD-L1 copy number and mRNA level data from provisional LSQCC cohorts in public datasets from The Cancer Genome Atlas (TCGA) and Kaplan–Meier Plotter database were downloaded from cBioPortal. The data obtained from our institution was from 37 LSQCC patients and were reported previously (32).

Linear regression and Spearman correlation analysis of the mRNA levels of FGF2 and PD-L1 was conducted as previously reported (5). The log-rank test was used for statistical analysis. Single-cell RNA-seq gene expression data for tumor tissues from NSCLC patients were downloaded from Gene Expression Omnibus (GEO). The accession number of the dataset was GSE140819 (33). Seurat 4.0.1 was used to standardize, integrate, cluster, visualize, and annotate the original data using an R tool kit for single-cell genomics (33,34).

Statistical analysis

Statistical analyses were performed in Prism software (version 7.0a, GraphPad Software Inc.) and R software (version 4.0.3). Two-tailed, unpaired Student's t-test and Welch's correction were used. For multiple comparisons, one-way ANOVA followed by Bonferroni's multiple comparisons test was performed. P-values < 0.05 were considered significant (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Except where otherwise indicated, the experiments were repeated three times. Quantitative data are presented as the mean ± SEM. All images shown are representative.

Results

FGFR1 regulated PD-L1 expression through YAP in LSQCC

In H520 and HCC95 cells, protein and mRNA expression was measured after treatment with small interfering RNAs (siFGFR1-1, siFGFR1-2 or siCTRL; constructed and verified as previously reported (5)), an FGFR1/2/3 inhibitor (AZD4547) and an FGFR1 activator (bFGF2). siFGFR1 and AZD4547 inhibited FGFR1, pFGFR1 and PD-L1 expression (Figure 1A and B). There was no difference in LATS1 expression, but the phosphorylation of LATS1 at two amino acid residues (S909 and T1079) was decreased after FGFR1 inhibition (Figure 1A and B). As demonstrated previously, a decrease in phospho-LATS1 expression facilitated the phosphorylation of YAP at the S127 residue. When trapped in the cytoplasm, YAP was inactivated through phosphorylation (Figure 1A and B). The total mRNA levels and protein levels of FGFR1, YAP, TAZ and PD-L1 were similar (Figure 1C and D). During FGFR1 inhibition, less YAP was able to enter the nucleus, thus reducing the transcription levels of downstream molecules. On the other hand, PD-L1 was activated in a time-dependent manner by FGF2, consistent with YAP activation (Figure 1E).

Next, we investigated the underlying mechanism. A small interfering RNA targeting YAP was constructed, and its efficacy was verified by western blotting. siYAP inhibited the expression of YAP and PD-L1 (Figure 2A). A plasmid containing PD-L1 promoter region with a fluorescent label was first transfected into cells. Then, a YAP-overexpressing (YAP-OE) plasmid was added to the cells for YAP and PD-L1 amplification. The fluorescence intensity of the cancer cells was determined 48 hours later, and it was found to be increased in the YAP-OE group (p < 0.001) (Figure 2B). This indicated that YAP acted on the promoter region of PD-L1. To confirm this, H520 cells were cultured with IFN-γ to stimulate the expression of PD-L1 and then lysed, and DNA was extracted. Subsequently, chromatin immunoprecipitation was performed to

1 analyze the cDNA library. The chromatin region precipitated by the YAP antibody was
2 located between 5,440,000 and 5,460,000 bases in the PD-L1 region of chromosome 9.
3 Two known TEAD binding sites were differentially enriched in this region (Figure 2C).

4 To confirm the existence of the FGFR1/YAP/PD-L1 regulatory axis, siFGFR1-1
5 and YAP-OE plasmids were used. Overexpression of YAP reversed the expression of
6 PD-L1 after FGFR1 inhibition (Figure 2D). Conversely, an FGFR1 overexpression
7 plasmid (FGFR1-OE) and siYAP-2 were used, and it was found that PD-L1 expression
8 was also reversed by siYAP-2 after FGFR1-OE (Figure 2E).

9 In summary, FGFR1 was shown to positively regulate PD-L1 expression through
10 YAP in H520 and HCC95 cells, and YAP promoted PD-L1 transcription by binding to
11 the promoter region directly. Considering the results shown in Figures 1 to 3 together,
12 the FGFR1/YAP/PD-L1 axis promotes the immune escape of LSQCC in vitro.

13 14 **FGFR1 promoted tumor immune escape in the coculture system**

15 To construct stable FGFR1 knockdown strains, H520 and HCC95 cells were
16 transfected with shFGFR1-1, shFGFR1-2 or shCTRL, and then the effects of the
17 shRNAs were verified by western blotting and qPCR (Figure 3A and B). shFGFR1
18 decreased protein and mRNA levels in H520 and HCC95 cells, and sequence 1 was
19 chosen for subsequent study.

20 To mimic the interaction between cancer cells and immune cells, a coculture
21 system was established. Suspended Jurkat T cells were added to adherent shFGFR1 or
22 shCTRL cancer cells treated with a human PD-1 antibody (nivolumab) or control
23 antibody (isotype IgG). After coculture for 72 hours, the suspended cells were removed,
24 and cell proliferation was assessed by crystal violet staining and the Cell Counting Kit-
25 8 (CCK-8) assay. Cell viability was reduced when FGFR1 was knocked down and was
26 mostly decreased when FGFR1 knockdown was combined with nivolumab treatment
27 (Figure 3C and D). FGFR1 knockdown inhibited cell proliferation in the absence of
28 Jurkat T cells, as was previously reported (5). The toxicity of nivolumab to H520 and
29 HCC95 cells outside of a coculture system was limited, as previously reported (35).

30 Jurkat T cells were removed and stained for immune evasion markers and immune-
31 stimulating cytokines after coculture. In the experimental group (coculture with FGFR1
32 knockdown cancer cells), the expression of PD-1 and lymphocyte activation gene-3
33 (LAG-3) was decreased significantly ($p < 0.0001$), while the expression of interleukin-
34 2 (IL-2) was increased ($p < 0.05$). There was no significant difference in the expression
35 of interferon-gamma (IFN- γ) ($p = 0.7267$) or tumor necrosis factor-alpha (TNF- α) ($p =$
36 0.2386) (Figure 3E). These results indicated that FGFR1 inhibition partially reversed
37 tumor immune escape in vitro. In other words, if FGFR1 is activated, FGFR1 might
38 promote immune evasion in addition to causing the known phenotypes observed
39 previously. These results were further confirmed in a mouse model.

40 41 **FGFR1 knockdown remodeled the tumor microenvironment and exerted** 42 **antitumor effects in combination with PD-1 antibodies**

43 The interaction between cancer cells and surrounding immune cells is conducive
44 to the growth and development of cancer (36,37)(38). To explore the effect of FGFR1

on the tumor microenvironment (TME), stable FGFR1 knockdown (shFGFR1) or FGFR1 wild-type (shCTRL) Lewis lung carcinoma (LLC) strains were constructed by shRNA transfection and analyzed by western blotting. Lentivirus sequence 1 showed the strongest inhibitory effect on FGFR1 and PD-L1 expression and thus was amplified later for tumor inoculation (Figure 4A). C57BL/6 mice were injected with shFGFR1- or shCTRL-transfected LLC cells and injected with a mouse PD-1 monoclonal antibody (PD-1 mAb) or a control antibody (IgG), as shown in the flowchart (Figure 4B).

Photographs and growth curves of the tumors showed that the mouse PD-1 antibody alone only slightly inhibited tumor growth ($p = 0.0149$), while FGFR1 inhibition reduced the tumor volume more ($p < 0.0001$). In the two groups inoculated with the shFGFR1-transfected cell strains, the PD-1 antibody showed a synergistic inhibitory effect in combination with FGFR1 knockdown, as indicated by Bonferroni's multiple comparisons test ($p = 0.0156$). Therefore, FGFR1 knockdown combined with PD-1 antibody treatment mostly inhibited tumor growth ($p < 0.0001$) (Figure 4C and D).

Tumor tissues were embedded in paraffin and sectioned. Immunofluorescence staining showed that the expression levels of FGFR1, YAP and PD-L1 in the TME decreased after FGFR1 knockdown (Figure 4E). The trend in expression was consistent with what was observed in vitro (Figure 1A-D). Then, Ki-67 and CD8 were detected by immunohistochemistry. After FGFR1 knockdown, cancer cell proliferation decreased, as indicated by ki-67 staining (Figure 4F), while the infiltration of CD8⁺ T cells increased (Figure 4G).

After the mice were sacrificed, another part of the tumor tissue was processed for flow cytometry. Single-cell suspensions were stained with antibodies against different cell surface markers and cytokines as previously described (31). The analysis showed that FGFR1 knockdown increased the number of CD8⁺ T cells in the TME ($p = 0.005$) (Figure 5A). Further analysis of the CD8⁺ subset showed that the expression of the immune escape marker PD-1 was decreased ($p = 0.0057$) (Figure 5B) but that there was no significant difference in LAG-3 expression ($p = 0.5992$) (Figure 5C); however, the expression of the immune activating cytokines IL-2 ($p = 0.0041$) (Figure 5D), IFN- γ ($p = 0.0034$) (Figure 5E) and TNF- α ($p = 0.0042$) (Figure 5F) was increased. These results suggested that FGFR1 knockdown increased the number of, partially reduced the immune escape of, and reactivated the tumor killing ability of CD8⁺ T cells in the TME.

To comprehensively investigate the effect of FGFR1 on the immune profile of the TME, we detected immune cells other than CD8⁺ T cells. It is worth noting that the number of M1-like tumor-associated macrophages (M1-TAMs) (CD206⁺/CD45.2⁺MHCII⁺F480⁺) was significantly decreased ($p < 0.0001$) (Figure 5I) in the FGFR1 knockdown group compared with the control group. Classically activated macrophages promote the secretion of a variety of inflammatory cytokines and mediate the killing of microorganisms and tumor cells; thus, they have the opposite effects of M2-TAMs (39). After FGFR1 knockdown, the number of CD8⁺ T cells was increased and the number of M1-TAMs was decreased simultaneously, and the changes in the number of these cells played an opposite role in the antitumor effect. This might be one of the reasons why inhibition of FGFR1 alone was not found to be efficacious

1 in current clinical trials, as the human body is much more complicated than the mouse.
2 Although FGFR1 inhibition alone greatly reduced tumor growth in a mouse model, the
3 complexity of human tumors needs to be understood more deeply.

4 There was no significant difference in the number of total macrophages
5 (F480+/CD45.2+MHCII+) ($p = 0.8291$) (Figure 5G), M2-TAMs
6 (CD80+/CD45.2+MHCII+F480+) ($p = 0.8589$) (Figure 5H) or CD4+ T cells ($p =$
7 0.0973) (Figure 5J) between the two groups. In addition, there was only a slight
8 decrease in the number of monocytes, including CD11b+/CD45.2+MHCII+ cells ($p =$
9 0.0047) (Figure 5K) and CD11c+/CD45.2+MHCII+ cells ($p = 0.0287$) (Figure 5L), in
10 the shFGFR1 group.

11 In this part of the mouse experiment, we found that FGFR1 inhibition increased
12 the number of CD8+ T cells in the TME, inhibited immune escape, and stimulated the
13 production of immunoreactive cytokines. Moreover, the decrease in the number of
14 M1TAMs suggested that the effect of FGFR1 on the TME might be contradictory and
15 complicated. Tumor growth was more robustly inhibited by FGFR1 knockdown in
16 combination with PD-1 antibodies than by shFGFR1 alone or PD-1 mAbs alone. This
17 combination is a promising therapeutic strategy for LSQCC patients harboring sensitive
18 FGFR1 variations, although more research on the predictive biomarker is needed.

19 20 **FGFR1 expression was correlated with PD-L1 and FGFR1 was coexpressed with** 21 **PD-L1 in patients with LSQCC**

22 To evaluate the correlation between FGFR1 and PD-L1 expression in LSQCC
23 patients, we first analyzed the copy number and expression profiles of FGFR1 and PD-
24 L1 in our LSQCC cohort (32) and the provisional LSQCC cohort from TCGA. The
25 results showed no statistical correlation between FGFR1 and PD-L1 expression (Figure
26 6A and C), which was similar to the results of a previous study (40). To our surprise,
27 FGF2 expression and PD-L1 expression were positively correlated, and coamplification
28 of PD-L1 at the mRNA level was common in LSQCC patients with FGF2 amplification
29 (Figure 6B and D). Considering that FGF2 can activate FGFR1, these results suggest
30 an inequality between FGFR1 amplification and FGFR1 downstream pathway
31 activation, which might explain why FGFR1 amplification is not a sensitive predictive
32 biomarker for FGFR1 inhibitors (2,8).

33 We further investigated the distribution of these molecules in the TME due to
34 intratumoral heterogeneity. Selected single-cell sequencing data from the TCGA
35 database were analyzed and visualized with the Seurat package in R version 4.0.2. After
36 data standardization and reduction, the 1500 genes with the largest variation among
37 nearly 20,000 genes were group and annotated (41). Cells were groups according to
38 molecular profiles by t-SNE reduction (42) (Supplementary Figure 1).

39 As shown in the bubble graph, FGF2, FGFR1, YAP and PD-L1 coexpressed
40 mainly in endothelial cells and fibroblasts in the TME (Supplementary Figure 2A). This
41 suggested that FGF2/FGFR1/YAP/PD-L1 might act as a regulatory axis, mainly in
42 cancer cells in heterogeneous tumor tissues, according to the pathological
43 characteristics of LSQCC. Markers were visualized by t-SNE in scattering mode
44 (Supplementary Figure 2B). The FGFR1/YAP/PD-L1 regulatory axis existed in certain

subsets and all cancer cells within the TME, indicating the heterogeneity of the different cell groups in just one tumor.

Discussion

FGFR1 variations are among the most common mutations in LSQCC patients (43,44). However, ongoing clinical trials show that the response rate to FGFR1-targeted therapy is limited (Dai *et al.*, 2015; Javle *et al.*, 2018; Lim *et al.*, 2016). There are many possible reasons for this. One possible explanation is that there are unknown phenotypes of FGFR1 variations, which contribute to carcinogenesis and allow a limited response to currently used drugs. In this study, we identified that the FGFR1/YAP/PD-L1 signaling pathway is involved in the tumorigenesis of LSQCC through immune evasion (Figure 7). In C57BL/6 mice, the paradoxical regulatory effects of FGFR1 on CD8⁺ T cells and M1-TAMs in the TME might hinder the antitumor effect of FGFR1-targeted therapies in the sophisticated immune environment. To address this problem, we tried a combination strategy and found that FGFR1 knockdown combined with anti-PD-1 therapy mostly inhibited tumor growth.

FGFR1 variations, such as FGFR1 amplification, have been used as predictive biomarkers in previous clinical trials (47,48). Unfortunately, these biomarkers are not as sensitive as epidermal growth factor receptor (EGFR) mutations for lung adenocarcinoma (49). Interestingly, the expression of FGF2 mRNA, but not FGFR1 mRNA, correlated with the expression of PD-L1 mRNA (Figure 6). The heterogeneity between FGFR1 variations and FGFR1 pathway activation might indicate that FGFR1 amplification, fusion or point mutations are not effective predictive biomarkers because they do not indicate activation of downstream pathways of FGFR1 at the functional level (50). Higher FGF2 expression is more likely to indicate activation of the FGFR1 pathway, but this needs to be confirmed in clinical trials (1).

Several studies have shown the effect of pan-FGFR inhibitors on the TME in vivo. In a genetically engineered mouse model of primary KRAS-G12C mutant lung adenocarcinoma, the diversity of interacting T cell receptor (TCR) clones was shown to decrease after treatment with erdafitinib, while the number of TCR clones was shown to increase (51). In the Renca mouse renal carcinoma model, ODM-203 decreases PD-1 expression in CD8⁺ T cells and the number of NK cells but increases the ratio of total T cells to Tregs, indicating that most of the T cells that are increased in number are effector T cells (52). In a mouse model of 4T1 breast cancer, PD173074 reduces the number of myeloid-derived suppressor cells (MDSCs) in the blood, spleen and tumor and increases the infiltration of CD4⁺ and CD8⁺ T cells into the spleen and tumor (20). In a mouse model of MC38 colorectal adenocarcinoma, 3D185 increases the number of activated CD8⁺ T cells (IFN- γ +CD8⁺ T cells) while decreasing the number of TAMs and Tregs (17). Although the abovementioned studies investigated the immune-remodeling effects of FGFR inhibitors, none of them showed the exact influences of FGFR1 on the TME or the regulatory mechanism.

There are limitations to our work. For example, we revealed the ternary regulatory effect of FGFR1/YAP/PD-L1 and the specific site in the promoter region of PD-L1 to which YAP bound. However, it was not clear whether there are other molecules

involved in the interaction between FGFR1 and PD-L1. Due to limitations related to time and cost, we did not construct a mouse model of primary LSQCC or a transplantation tumor model of LSQCC patient tissues. If possible, immune cells modulation should be combined with FGFR1 regulation in vivo to identify additional mechanisms.

Conclusions

In summary, we verified the positive regulatory effect of FGFR1 on PD-L1 expression in vitro and in vivo. Furthermore, YAP was confirmed to bind to the promoter region of PD-L1 to initiate the transcription of the latter. In the coculture system and subcutaneous tumor model, we found that FGFR1 knockout increased the antitumor activity of CD8⁺ T cells and decreased the expression level of immune escape-related molecules. FGFR1 knockdown in combination with PD-1 antibody treatment significantly suppressed tumor growth in vitro and in vivo. These results provide new insights into the function of FGFR1 related to tumor immune escape and preclinical evidence for potential targeted therapies and immunotherapy for LSQCC patients.

Declarations

Ethics approval and consent to participate

All animal experiments were carried out in full accordance with protocols approved by the Institutional Ethics Committee of Shanghai Jiao Tong University.

Consent for publication

Consent to publish has been obtained from all authors.

Availability of supporting data

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

Competing interests

The authors declare no potential conflicts of interest.

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Authors' Contributions

Conception and design: Shun Lu, Weiliang Xia, Min Lu and Kaixuan Wang.

Acquisition of data: Min Lu and Kaixuan Wang.
Analysis and interpretation of data: Min Lu and Kaixuan Wang.
Writing, review, and/or revision of the manuscript: Min Lu, Kaixuan Wang, and Wenxiang Ji.
Administrative, technical, or material support: Yongfeng Yu, Ziming Li, Lan Shen, Weiliang Xia and Shun Lu.
Study supervision: Weiliang Xia and Shun Lu.
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Conflict of interest

The authors declare no conflict of interest.

Abbreviations

FGFR1	fibroblast growth factor receptor 1
LSQCC	lung squamous cell carcinoma
PD-1	programmed death-1
PD-L1	programmed death ligand-1
YAP	Yes-associated protein
FGF2	fibroblast growth factor 2
TKIs	tyrosine kinase inhibitors
ICIs	immune checkpoint inhibitors
DCs	dendritic cells
TME	tumor microenvironment
TEAD	transcriptional enhanced associate domain
siRNAs	short interfering RNAs
GFP	green fluorescent protein

1	ChIP	chromatin immunoprecipitation
2	CCK-8	cell Counting Kit-8
3	FCM	flow cytometry
4	IF	immunofluorescence
5	IHC	immunohistochemistry
6	TCGA	The Cancer Genome Atlas
7	GEO	Gene Expression Omnibus
8	LAG-3	lymphocyte activation gene-3
9	IL-2	interleukin-2
10	IFN- γ	interferon-gamma
11	TNF- α	tumor necrosis factor-alpha
12	TME	tumor microenvironment
13	M1-TAMs	M1 like tumor associated macrophages
14	M2-TAMs	M2 like tumor associated macrophages
15	EGFR	epidermal growth factor receptor
16	TCR	T cell receptor
17	MDSC	myeloid-derived suppressor cells

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Fig. 1

Figure 1. FGFR1 positively regulated YAP and PD-L1 expression at both the mRNA and protein levels. (A-D) shFGFR1-1 or AZD4547 was added to the H520 and HCC95 cell lines. (A, B) The levels of proteins related to the FGFR1/YAP/PD-L1 pathway were measured. (C, D) The levels of mRNAs related to the FGFR1/YAP/PD-L1 pathway were measured. $n = 3$, mean $\pm$ SEM, two-tailed Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (E, F) After activation of FGFR1 by 20 ng/ml bFGF2, the protein expression of YAP and PD-L1 was measured.

Fig. 2

Figure 2. FGFR1 promoted PD-L1 expression through YAP, which directly bound to the promoter region of PD-L1. (A) In the H520 and HCC95 cell lines, siYAP sequence 1 and sequence 2 (siCTRL was used as a control) were added to inhibit YAP, and Western blotting was used to measure the protein expression of YAP and PD-L1. Sequence 2 was used subsequently. (B) After transduction of the YAP-CTRL or YAP-OE (YAP overexpression) plasmid followed by the fluorescence-tagged PD-L1 promoter plasmid, relative fluorescence activity was determined (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (C) Chromatin immunoprecipitation (ChIP) revealed the interaction of YAP with a potential enhancer located upstream of the transcription starting site of PD-L1 on chromosome 9. Lysates were prepared from H520 cells and immunoprecipitated with IgG or 3-Flagged antibody. (D) siCTRL, siFGFR1-1, YAP-OE and siFGFR1 plus YAP-OE siRNA or plasmid were added. (E) siCTRL, FGFR1-OE (FGFR1 overexpression), siYAP-2 and FGFR1-OE plus siYAP-2 siRNA or plasmid were added.

Fig. 3

Figure 3. Knockdown of FGFR1 reversed immune evasion in vitro. (A) Western blot analysis of knockdown mediated by an shRNA-expressing lentivirus targeting FGFR1 (shFGFR1) in the H520 and HCC95 cell lines compared with the effect of the negative control lentivirus (shCTRL). (B) Confirmation of the knockdown mediated by shFGFR1 compared to the effect of shCTRL in H520 and HCC95 cells by qPCR. $n = 3$, mean $\pm$ SEM, two-tailed Student's t-test. (C, D) H520 and HCC95 cells transfected with shCTRL or shFGFR1 were cocultured with Jurkat T cells and then treated with isotype IgG or nivolumab (a human anti-PD-1 mAb). Suspended T cells were removed, and adherent cancer cells were stained with crystal violet (C) or evaluated with the CCK-8 assay (D). The experiment was repeated twice. (E) Jurkat T cells were collected and tested by flow cytometry. $n = 4$, mean $\pm$ SEM, two-tailed Student's t-test. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Fig. 4

Figure 4. FGFR1 knockdown inhibited the FGFR1/YAP/PD-L1 axis and reduced tumor growth, especially in the presence of PD-1 antibodies. (A) Stable LLC mouse lung cancer cell lines were constructed by transduction of shFGFR1 sequences 1, 2 and 3 (shCTRL as a control). Western blotting was used to measure the protein expression levels of FGFR1 and PD-L1. shFGFR1 sequence 1 was subsequently amplified. (B) Schematic diagram of tumor inoculation and drug delivery. (C, D) Tumor photographs and growth curves of the subcutaneous tumor model in the shCTRL+IgG, shFGFR1+IgG, shCTRL+anti-PD-1, and shFGFR1+anti-PD-1 groups. $n = 5$, mean $\pm$ SEM, one-way ANOVA followed by Bonferroni's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (E) Multiple immunofluorescence (MIF) staining for DAPI (blue), FGFR1 (pink), YAP (green) and PD-L1 (red) in tumor tissues from the shCTRL+IgG and shFGFR1+IgG groups. (F, G) Analysis of tumor tissues in the shCTRL+IgG and shFGFR1+IgG groups by immunohistochemistry (IHC). (F) IHC for ki-67. (G) IHC for CD8.

Fig. 5

2 **Figure 5. FGFR1 regulated CD8⁺ T cells and additional immune cells in the tumor**
3 **microenvironment.** (A-L) Subcutaneous tumors were processed as single-cell
4 suspensions and analyzed by flow cytometry. n = 5, mean ± SEM, one-way ANOVA
5 followed by Tukey's test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). (A-F)
6 Characteristics of CD8⁺ T cells are shown. (A) The percentage of CD8⁺ cells among
7 the CD3⁺ cell population. (B) The percentage of PD-1⁺ cells among the CD8⁺ cell
8 population. (C) The percentage of LAG3⁺ cells among the CD8⁺ cell population. (D)
9 The percentage of IL-2⁺ cells among the CD8⁺ cell population. (E) The percentage of
10 IFN-γ⁺ cells among the CD8⁺ cell population. (F) The percentage of TNF-α⁺ cells
11 among the CD8⁺ cell population. (G-I) Characteristics of macrophages. (G) The
12 percentage of F480⁺ cells among the CD45.2⁺MHCII⁺ cell population. (H) The
13 percentage of CD80⁺ cells among the CD45.2⁺MHCII⁺F480⁺ cell population. (I) The
14 percentage of CD206⁺ cells among the CD45.2⁺MHCII⁺F480⁺ cell population. (J)
15 The percentage of CD4⁺ cells among the CD3⁺ cell population. (K, L) Characteristics
16 of monocytes. (K) The percentage of CD11b⁺ cells among the CD45.2⁺MHCII⁺ cell
17 population. (L) The percentage of CD11c⁺ cells among the CD45.2⁺MHCII⁺ cell
18 population.

Fig. 6

2 **Figure 6. FGFR1 expression was correlated with PD-L1 expression and FGFR1**
3 **was coexpressed with PD-L1 in LSQCC patients from our institution. (A-D)**
4 Linear regression analysis and Pearson correlation of the mRNA levels of FGFR1 and
5 PD-L1. (A, C) No statistical correlation between the expression of FGFR1 and PD-L1
6 was observed in either our cohort or the selected cohort of TCGA LSQCC patients.
7 (B, D) A positive correlation between the expression of FGF2 and PD-L1 was
8 observed in both our cohort and the selected cohort from TCGA LSQCC patients.

Fig. 7

2 **Figure 7. Schematic illustration of FGFR1's role in tumor immune evasion.**

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Supplementary Figure 1

2 **Supplementary Figure S1. Cell type composition in the tumor**
3 **microenvironment.** Proportion of cells assigned to each cell type signature (color)
4 was visualized by tSNE. Endothelial cells, CD8+ T cells and fibroblasts were most
5 enriched as shown. Macrophages, epithelial cells, B cells, hematopoietic stem cells
6 (HSCs), natural killer cells (NK cells), and monocytes were also profiled.

7

8

Supplementary Figure 2

2 **Supplementary Figure S2. FGF2, FGFR1, YAP and PD-L1 were coexpressed in**
3 **the TME.** (A, B) Analysis of public single-cell sequencing data of LSQCC showed
4 that FGF2, FGFR1, YAP and PD-L1 were co-expressed mainly in epithelial cells and
5 fibroblasts in TME. (A) Bubble chart marked “FGF2”, “FGFR1”, “YAP1” (YAP) and
6 “CD274” (PD-L1) in different PCs. The bubble size was proportional to the
7 percentage of cells in the cluster that expresses the gene. The color intensity was
8 proportional to the average scaling of the gene in the cluster. (B) Dimensionality
9 reduction visualization map, highlighting “FGF2”, “FGFR1”, “YAP1”, and “CD274”
10 expression in “mediumvioletred”, “darkgreen”, “navyblue” and “firebrick” dots,
11 respectively.