Glucose metabolism inhibitor PFK-015 combined with immune checkpoint inhibitor is an effective treatment regimen in cancer

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ABSTRACT

Metabolic inhibition via PFKFB3 inhibition has demonstrated considerable tumor inhibitory effects in various studies; however, PFKFB3 inhibition did not show satisfactory tumor inhibition when used in clinical trials. PFKFB3 is a crucial metabolic enzyme that is highly upregulated in cancer cells and directly affects tumor glycolysis. Here, we showed that PFKFB3 inhibition suppresses tumors in vitro and in vivo in immune-deficient xenografts. However, this inhibition induces the upregulation of PD-L1 levels, which inactivated cocultured T-cells in vitro, compromises anti-tumor immunity in vivo, and reduced anti-tumor efficacy in an immune-competent mouse model. Functionally, PD-1 mAb treatment enhances the efficacy of PFKFB3 inhibition in immunocompetent and hu-PBMC NOG mouse models. Mechanistically, PFKFB3 inhibition increases phosphorylation of PFKFB3 at residue Ser461, which increases interaction with HIF-1α, and their colocalization into the nucleus, where HIF-1α transcriptionally upregulate PD-L1 expression and causes subsequent tumor immune evasion. Higher phos-PFKFB3 correlated with higher PD-L1 expression, lower CD8 and GRZMB levels, and shorter survival time in ESCC patients.

Introduction

Glycolysis is an essential enzymatic process in human cell metabolism. It participates in the production of substrates required in multiple biochemical pathways, such as the tricarboxylic acid cycle, pentose phosphate pathway, and fatty acids and cholesterol synthesis. In normal human cells, anaerobic reactions predominate in the metabolism under reduced oxygen conditions; however, in 1972, Otto Warburg reported an essential role of glycolysis in cancer cells regardless of oxygen concentration in the tumor microenvironment. This reprogramming of cancer cell metabolism is not only responsible for the aggressiveness of cancer growth but may also decrease reactive oxygen species production and accumulation, and increase the abundance of key metabolites required for rapid cell growth and proliferation.

Glycolysis intensity is regulated by the activity of three physiologically reversible enzymes: hexokinase, phosphofructokinase-1 (PFK-1), and pyruvate kinase. PFK-1 is the main rate-limiting enzyme of glycolysis and the activity of PFK-1 is regulated by metabolic products such as adenine triphosphate (ATP), adenosine diphosphate (ADP), and fructose 2–6 biphosphate. Of these compounds, F-2,6-BP is a reaction product catalyzed by 6-phosphofructokinase 2 (PFKFB3, also known as PFK-FB) and 6-phosphofructokinase 2,6-biphosphatase (PFK-2/FBPase-2/PFKFB3), which is also the most potent positive allosteric effector of PFK-1.

PFKFB is a bifunctional enzyme responsible for the catalyzation of both the synthesis and degradation of F-2,6-BP mediated through its N-terminal domain (2-kase) and C-terminal domain (2-pase) respectively. Of note, the active site of the 2-kase domain has 2 distinct key areas (the F-6-P binding loop and the ATP-binding loop) which are essential for PFKFB to function.

In humans, PFK-2/FBPase-2 is encoded by four different genes: PFKFB1, PFKFB2, PFKFB3 and PFKFB4. Thus, there are four different PFK-2/FBPase-2 isozymes that have been identified and characterized by tissue and functional specificity. PFKFB1 can be found predominantly in liver and skeletal muscles, PFKFB2 predominates in the cardiac muscles, PFKFB3 is ubiquitously expressed while PFKFB3 appears mainly in the testes. The overexpression of two out of four isozymes (PFKFB3 and PFKFB4) have been demonstrated in various solid tumors and hematological cancer cells, and have been attributed to be responsible for the tendency for glycolysis over aerobic respiration despite high oxygen availability. Furthermore, due to slight differences in amino acid sequences at key sites for enzymatic activity, all of the isozymes have a different affinity for the synthesis or degradation of F-2,6-BP. Their activity is expressed as the kinase/phosphatase ratio and this ratio is about 4.6/1 for PFKFB4 and 730/1 for PFKFB3, while it does not exceed 2.5/1 for PFKFB1 and
PFKFB2. Owing to the high activity ratio of PFKFB3 and its high expression in cancer cells, it has become the main target of research on potential selective inhibitors to be used as future molecular targeted therapy for the treatment of cancer.

PFKFB3 is upregulated in many cancer types and has been linked to various functions other than being a metabolic enzyme. Interestingly, PFKFB3, unlike many other metabolic enzymes, possesses a nuclear locating sequence that allows PFKFB3 to navigate into the nucleus, where many studies have revealed a plethora of unexpected roles of PFKFB3: promotion of cell proliferation by regulating the expression of cyclin-dependent kinases, increased angiogenic activity by upregulation human umbilical vein endothelial cell, and regulation of autophagy via ROS and adenosine-5’-monophosphate-activated protein kinase.

Reports on the importance of PFKFB3 in cancer development and the specificity and high activity ratio of the isozyme PFKFB3 in cancer strongly suggest that the isozyme PFKFB3 may represent a promising target as a potent molecular targeted therapy in cancer treatment. These characteristics of PFKFB3 prompted research groups and pharmaceutical companies to invest, develop and investigate the efficacy of selective inhibitors of PFKFB3. The first small molecule PFKFB3 inhibitor was 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one, also known as 3PO. 3PO belongs to the chalone group and showed marked inhibition of tumor cells in vitro and in vivo experiments; however, due to poor solubility and later poor inhibitive selectivity, the use of 3PO was limited to experimental research. Afterward, PFK-015, a derivative of 3PO, was developed with increased selectivity and inhibitory effectiveness (~100 fold). Unlike 3PO, PFK-015 did not inhibit other glycolysis-related enzymes such as PFK1 or hexokinases. In 2014, clinical trials of PFKFB3 inhibitors (NCT02044861) were initiated with no serious adverse effect after a year, still it was later stopped because “in human trials (PFKFB3 inhibitor) did not produce enough of an effect to retain interest from pharmaceutical companies”, with only six out of 19 patients’ tumor responding to the drug. Since the most prominent difference between in vivo experiments and clinical trials is the human immune landscape, we sought to find the underlying mechanism behind the failure of glucose metabolism inhibitor PFK-015 and whether it could be attributed to tumor immunity because recent studies have hinted that cell metabolism and tumor immunity might be highly intertwined.

Esophageal squamous cell carcinoma (ESCC) makes up more than 80% of all esophageal cancer worldwide and is mainly prevalent in southern China. According to the recent 2021 NCCN guidelines on esophageal cancer, there are still no molecular targeted therapies that are recommended in the treatment of esophageal cancer; however, immune checkpoint inhibitor has been listed as first-line therapy by the 2021 NCCN guidelines, median OS was only increased by 1–3 months (PD-L1 CPS ≥ 10). Therefore, we also aimed to investigate the effectiveness of selective PFKFB3 inhibitors in ESCC cells, possible effects on tumor immunity and combination therapies with immune checkpoint inhibitors.

Results

**PFK-015 impeded ESCC tumor growth in vitro and in immunodeficient in vivo models**

Previous studies have shown that inhibiting PFKFB3 using selective inhibitors such as PFK-015 can significantly impede tumor development in vitro and in vivo in multiple cancer types such as adenocarcinoma of the lung, stomach or colon, as well as hematological. Here, we confirmed the effectiveness of treatment with PFK-015 on esophageal cancer cell line in vitro, where treatment with PFK-015 could inhibit tumor growth in a dose-dependent manner in MTT assays and colony formation assays (Figure S1A-C). The IC of PFK-015 in esophageal cancer cell lines ranged from 4.01 to 5.08 μM, which is consistent with what has been reported in other studies. DMSO (0.05%) was used as normal control and exhibited minimal to no adverse effects on cell growth. Treatment using PFK-015 in immunodeficient (nude) mice in our in vivo study after sub-dermal flank inoculation with esophageal cancer cells confirmed with our in vitro experiments: showing a marked reduction in tumor volume and final tumor mass when compared with the vehicle group (Figure 1(a-c), S1D). Together, these results confirm that PFK-015 can indeed inhibit tumor growth both in vitro and in immunodeficient in vivo models.

**PFK-015 failed to inhibit tumor growth in immunocompetent models**

We hypothesized that the failure to complete phase II clinical trial of PFKFB3 selective inhibitor might have been attributed to the tumor microenvironment and anti-tumor immunity. Therefore, to test that hypothesis, we inoculated mouse-derived colon cancer cell line MC38 tumor cells into immunocompetent mice and repeated our previous in vivo experiment. One would expect similar results to our in vivo experiment in immunodeficient mice. However, there was no significant difference between the PFK-015 treated group and the vehicle group in immunocompetent mouse in terms of tumor volume and tumor mass (Figure 1(d-f), S1E). We showed that PFKFB3 inhibition using PFK-015 fails to inhibit tumor growth in immunocompetent mice. These results suggest that an intact immune system compromises the anti-tumor efficacy of selective inhibitor PFK-015.

**PFK-015 induced tumor PD-L1 expression and inhibited cytotoxic CD8 + T cells**

To find out the underlying reason why PFKFB3 selective inhibitors did not seem to be effective in an immunocompetent environment, we carried out an immune-related mRNA chip, which revealed an upregulation in several immune-related checkpoints, the most remarkable one being PD-L1 (Figure 1(g), S1F). To confirm if this increase was also reflected by its protein level, we used western blot and confirmed that this protein level expression of PD-L1 was also increased, which was most prominent after 48 hours of treatment with PFK-015 (Figure 1(h),S1G). The isolated tumors from both the immunocompetent mouse and the
immunodeficient mouse models were analyzed using flow-cytometry and immunofluorescence analysis and revealed an induction in the expression of PD-L1 (Figure 1(i-k)). Together, these findings suggest that treatment of PFK-015 on cancer cells induces PD-L1 expression.

Since treatment with PFK-015 was shown to increase PD-L1 expression and that PD-1 blockade on T cells is the main mechanism by which immune escape mediated by PD-L1 is carried out by tumor cells, we wanted to test whether the immune escape caused by PFK-015 was dependent on cytotoxic CD8+ T cells. We co-cultured human peripheral blood mononuclear cells (PBMC) together with cancer cells in the presence and absence of PFKFB3 inhibitor. Treatment with PFK-015 rendered cancer cells more resistant to cytotoxic CD8+ T cells in T cell-mediated tumor cell killing assays, as shown by our T-cells co-culture assays (Figure 2(a-c)). Furthermore, using flow-cytometry, we found that the activity of the co-cultured cytotoxic CD8+ T cells (IFN-γ+ CD8+ T cells) was significantly decreased in the PFK-015 treated group (Figure 1(d-e)), S1H). Consistently, ELISA assay using the culture medium aspirated from our co-culture wells showed decreased IFN-γ in the culture medium (Figure 2(f)). All those results indicate that inhibiting PFKFB3 using PFK-015 can cause a downregulation of immune activity against tumor cells mediated by CD8+ T cells. A previous study has shown that reverse PDL1 signaling in tumors could promote tumor survival,30 therefore to exclude that possibility, we cultured tumor cells with both PFK-015 and recombinant PD-1 and found no marked difference in phosphorylation of mTOR targets (p4EB-P1, pS6K, and pS6) in KYSE-70 tumor cells and tumor cell survival as demonstrate by MTT assays (Figure 2(g-h)).

**Synergistic effect of PFK-015 and PD-1 mAb therapy in vivo**

To test whether the immune escape caused by the inhibition of PFKFB3 using selective inhibitor PFK-015 could be attenuated using PD-1 mAb therapy, we utilized PD-1 mAb to treat

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**Figure 1.** PFK-015 impeded ESCC tumor growth in vitro and in immunodeficient in vivo models but failed to inhibit tumor growth in immunocompetent models due to increased PD-L1 expression in tumor cells.
immunocompetent mice in combination with PFK-015. All four groups (Vehicle+IgG, PFK-015+ IgG, Vehicle+PD1 mAb, and PFK-015 + PD-1 mAb) had no significant difference in mouse weight at the end of their respective treatments. We observed that the PFK-015 alone and the control group had no significant difference in terms of tumor volume and mass. Similarly, in B16 tumor-bearing mice, there was no significant difference in tumor volume and mass when treated with PD-1 mAb alone, while MC38 tumor-bearing mice showed decreased tumor volume and mass, both phenomena being consistent with previous studies. However, in the PFK-015 and PD-1 mAb combined treatment group, there was a significant decrease in tumor volume and tumor mass when compared with all other groups, even in the B16.F10 tumor-bearing mice (Figure 3(a-f)). At the end of the treatment, the tumor samples were harvested for further analysis. Flow cytometry of tumor-infiltrating lymphocytes showed that treatment with PD-1 mAb could rescue the inhibition in T-cell tumor-killing activity caused by PFK-015 (Figure 3(g-h), S2A-C). Immunohistochemistry staining of MC38 and B16 tumors harvested from the mice with antibodies specific to PD-L1, CD8 and Granzyme B showed an increase in PD-L1 expression and decreased CD8 and GRZMB, when cells were treated with PFK-015. When PFK-015 was combined with anti-PD1 mAb, CD8 and GRZMB levels increased significantly (Figure 3(i-j)). These findings support the concept that PD-1 mAb treatment can block the upregulated PD-L1 expression on cancer cells, caused by PFK-015 treatment, from blocking the PD-L1 to PD-1 interactions from inactivating T cells, thus restoring tumor immunity in the tumor microenvironment.

**PFK-015 increased HIF-1α mediated PD-L1 transcriptional activity**

A bioinformatic analysis of possible proteins that could both interact with PFKFB3 and act as a transcription factor that could interact with PD-L1 promoter regions was carried out. A list of positively correlated and negatively correlated transcription factors was obtained, where the most plausible candidates with positive correlation were FOX3 and HIF-1α (Figure 4(a)). Since FOX3 is known to be mainly expressed in immune cells, we decided to explore further the relations between PFKFB3 and HIF-1α, since HIF-1α is known to be able to bind directly to Hypoxia Responsive Elements (HRE) found on the promoter regions of PD-L1 gene. To determine whether HIF-1α interaction with PD-L1 promoter region increased under treatment
with PFK-015, we carried out CHIP assays, which confirmed the physical interaction of HIF-1α with the HRE regions on the PD-L1 promoter (Figure 4(b)). An interaction which when cells were treated with PFK-015. Furthermore, to determine whether PD-L1 was transcriptionally active, a luciferase assay was carried out in both 293 T and KYSE-520 cancer cell lines. Cells treated with PFK-015 had significantly higher PD-L1 transcriptional activation than the control group (Figure 4(c)). Also, silencing of HIF-1α using a transduced shRNA against HIF-1α showed that under PFK-015 treatment tumors cell were unable to upregulate PD-L1 expression (Figure S3A), suggesting that HIF-1α mediated PFK-015 in increasing PD-L1 expression. All of which highlights the evidence that treatment with selective PFKFB3 inhibitor PFK-015 can effectively increase the transcriptional activation of PD-L1 expression which was mediated by HIF-1α.

To validate the second part of our bioinformatics analysis, where the transcription factor could also interact with PFKFB3: co-immunoprecipitation experiments were carried out with KYSE-70 cells under treatment with PFKFB3 inhibitor PFK-015. In the CO-IP experiments, treatment with PFK-015 increased the interaction between PFKFB3 and HIF-1α and vice-versa (Figure 4(d)). For a transcription factor to be effective, the transcription factor HIF-1α. Finally, for HIF-1α to increase the expression of PD-L1, accumulation of HIF-1α in the nucleus is necessary. We carried out compartmental western blot assays to analyze the presence of HIF-1α in each compartment, and our results showed that under PFK-015 treatment, nuclear HIF-1α was markedly increased (Figure 4(e)). Compared with the amount of cytoplasmic HIF-1α, nuclear HIF-1α accounts for a smaller portion of HIF-1α, which may account for the insignificant reduction of
cytoplasmic HIF-1α following PFK-015 treatment. Those results confirm that PFK-015 treatment increases PD-L1 transcriptional activity and expression with HIF-1α as an intermediary.

**PKF3 phosphorylates and translocates into the nucleus under PFK-015 treatment**

There are several post-transcriptional modifications that PKF3 can undergo under different kinds of stress and stimuli. A previous study on PKF3 has shown that PKF3 underwent phosphorylation at serine 461 residue when treated with PFK-015. To determine if PKF3 phosphorylates under PFK-015 treatment: a specific antibody against phosphorylated PKF3 at Serine-461 residue was used. Tumor cells were exposed to PFK-015 and the phosphorylation of PKF3 protein was determined by western blots (Figure 4(e)). Furthermore, compartmental protein blot analysis showed a marked increase in phosphorylated nuclear PKF3, which pointed to the translocation of phosphorylated PKF3 into the nucleus (Figure 4(e)). PKF3 has been shown to conservatively maintain a nuclear localization signal (NLS) as “KKPR” (amino acid 472-475), which can be affected by post-transcriptional modifications such as phosphorylation or acetylation. Therefore, we focused on phosphorylated PKF3 and mutated serine 461 to either null mutation S461A or phosphorylation mimic S461D. The replacement of serine with aspartic acid can mimic the effect of phosphorylation. Flag-tagged WT-PKF3, S461A and S461D mutants were stably expressed in esophageal cancer cells and their distribution in the cell cytoplasm or the nucleus was examined using immunofluorescence and compartment protein blotting (Figure 4(f-g)). In contrast with wild-type PKF3 and null mutant S461A, phosphorylation mimic S461 showed higher nucleus localization, indicating that the phosphorylation of S461 may cause nuclear translocation. It is known that PKF3 translocates into the nucleus via interaction with the nucleus membrane protein importin-a5. We validated in our experiment that both treatment with PFK-015 and mutation to phosphorylation mimic S461D both increase interaction between PKF3 and importin-a5 (Figure 4(h-i)). Together, these results indicate that PFK-015 caused the phosphorylation of serine 461 on the protein PKF3, which increased the nuclear localization via importin-a5. Together, those results showed that when tumor cells are treated with PKF3 inhibitor PFK-015, PKF3 phosphorolates, interaction with HIP-1α increases, and together they translocate into the nucleus via...
importin α5. There, HIF-1α binds to the HRE regions found in the promoter sequence of PD-L1 gene, thereby increasing PD-L1 expression.

**Phosphorylated PFKFB3 expression levels correlated with the efficacy of PD-1 mAb therapy in ESCC patients**

To further validate our findings in human patient samples, we assessed protein expression of phos-PFKFB3, CD8, GRZMB and PD-L1 by IHC in a cohort of ESCC tissues (SYSUCC, \( n = 73 \)) (Figure 5(a)). The phos-PFKFB3 group exhibited higher PD-L1 expression and lower CD8 and GRZMB activity, and inversely the absent phos-PFKFB3 showed the opposite pattern with low PD-L1 expression and high CD8 and GRZMB activity (Figure 5(b)). Taken together, we speculated that phos-PFKFB3 could promote tumor immune evasion and ultimately affect the overall survival of ESCC patients. Therefore, we further investigated the effect of phos-PFKFB3 expression had on the survival of the patient, where patient with phos-PFKFB3 had worse prognosis than patient with absent phos-PFKFB3 (log-rank \( P < .01 \), Figure 5(c)). Phos-PFKFB3 expression was an independent prognostic indicator (HR (95% CI): 1.626 (1.594–7.177), \( P = .004 \)) to patient survival as shown by our univariate and multivariable analysis (Supplementary table 4–5). These data further confirm that phos-PFKFB3 could be used as a prognostic indicator and that the tumor immune evasion can occur via the phos-PFKFB3/HIF-1α/PD-L1 axis (Figure 5(d)).

**CDX transplantation into hu-PBMC NOG mice can mimic immunotherapy response in humans**

To further test our hypothesis in an experimental model that could approach the human immune system, human peripheral mononuclear cells (hu-PBMC) pre-primed for humanized mice model were injected intra-peritoneally into NOG mice.
to simulate the human immune system inside a mouse. The spleens from the sacrificed mouse were ground into single-cell suspensions, and lymphocytes cells were collected to be run through a flow-cytometer to compare the proportion of human against mouse CD45+ cells, and there were more human CD45+ cells than mouse cells; implying that the Hu-NOG mouse model was successful (Figure S4A). KYSE-520 and KYSE-70 esophageal cancer cell lines were inoculated into the flanks of our hu-PBMC NOG mice. When the tumors reached an appropriate size, the mice were randomized and separated into four groups: normal control (Vehicle + IgG), PFK-015 alone (PFK-015 and IgG), PD-1 mAb alone (Vehicle+PD-1 mAb) and finally, the combination treatment group (PFK-015 and PD-1 mAb). Consistent with our previous experiments in an immune-competent mouse model C57BL/6 with murine tumors, the combination group showed the most significant inhibition of tumor proliferation (Figure 6(a-c), S4B-D). FACS of TILs showed decreased IFN+ CD8+ T cells in tumors treated with PFK-015, and the highest level of IFN+ CD8+ T cells when PFK-015 was combined with PD-1 mAb (Figure 6(d-e), S4E-G). IHC staining of tumor slides showed a marked upregulation of PD-L1 and downregulation of CD8 and GRZMB in tumors treated with PFK-015, which could be rescued using anti-PD-1 mAb (Figure 6(g-h)). Therefore, these data confirm that the use of PFKFB3 specific inhibitor increases PD-L1 expression on tumor cells, which inhibits the host immune system from clearing tumor cells, a phenomenon which can be remedied by using PD-1 mAb, thus underlining the synergistic effect of combination therapy using PFKFB3 specific inhibitors and PD-1 mAb.

**Discussion**

Our study revealed that selectively inhibiting PFKFB3 in cancer cells using PFK-015 induces the expression of tumor PD-L1 via the phos-PFKFB3/HIF-1α axis. Furthermore, combining PD-1 mAb with PFKFB3 selective inhibitor PFK-015 enhances the

**Figure 6.** PFK-015 combined PD-1 mAb therapy significantly inhibits tumor growth in human PBMC-engrafted NOG mice.
Treatment efficacy of ESCC by enhancing CD8 + T-cell activity in immunocompetent mouse models such as C57BL/6 and hu-PBMC-NOG. Therefore, this study shows that inhibiting glycolysis using PFK-015 can upregulate PD-L1 levels leading to tumor evasion and uncovered a combination therapeutic strategy for the treatment of ESCC.

Glucose metabolism is relied heavily upon by tumor cells to fulfill their high metabolic demands during continuous proliferation.12 The bi-functional enzyme PFKFB3 plays a crucial role in the regulation of glycolysis in cancer cells. Moreover, PFKFB3 is the only member of the PFKFB family with the highest kinase/phosphatase ratio (740:1) and is a key tumor-associated isoform.14 Thus, selective inhibition of PFKFB3 has gained substantial interest as an attractive strategy for cancer therapy in academic research but also in the pharmaceutical industry due to its important role in regulating glucose metabolism in cancer. As expected, promising in-vitro results showed that inhibiting PFKFB3 in cancer cells can cause tumor cell apoptosis, necroptosis, cell cycle arrest.38 Giving rise to clinical trials involving selective PFKFB3 inhibitors.17,39

Translational medicine usually refers to the "bench-to-bedside" enterprise of harnessing new knowledge, mechanisms and techniques generated by advances in basic research into new approaches for the prevention, diagnosis and treatment of disease where the end-point is the production of a promising new treatment that can be implemented clinically.40 However, most translational research (54%) of translational research fails, where the most common cause was "lack of efficiency".41-43 Clinical trial (NCT02044861) of PFKFB3 selective inhibitor seems to have stopped with just 6/19 evaluable patients responding to the drug. The main reason for clinical trial failure usually is human subjects not responding to the drug the way laboratory animal models do, which is usually true for fields like immuno-oncology, where truly translatable animal models are often lacking.44 This seems to be underlined by the fact that more and more studies are making the connection between cancer cell metabolism and their immune evasion ability.45-48

Glycolysis plays a pivotal role in cancer proliferation, metabolism and other life activities. When selective inhibitors are applied to key metabolic enzyme PFKFB3, glycolysis levels are attenuated, resulting in low glucose processing ability and ultimately in low production of ATP: the main building block of cells in the body. Theoretically, targeted therapy aiming to disrupt the glycolysis pathway should be a very effective anti-cancer therapy; however, from our study, we can observe two things: firstly, once a selective PFKFB3 inhibitor was applied, tumor cells produce an early response where it leads to PFKFB3 phosphorylation, an allosteric activation process which greatly increases the potential enzymatic function of protein PFKFB3, showing the sensitivity of cells to low glucose availability and an attempt to restore energy availability to resume cellular proliferation and other life activities, which is consistent with previous reports.35 Here, it is noteworthy to point out that tumor cells generally have higher phosphorylated PFKFB3 expressions when compared with normal cells and that some cellular stress and stimuli such as glucose starvation and chemotherapy can also induce an increase in phosphorylation levels of PFKFB3.10,49,50 Secondly, after inhibiting PFKFB3; although the level of glycolysis decreased, which inhibited cell proliferation, it activated the immune escape pathway of tumors by shuttling HIF-1α into the nucleus via its interaction with phos-PFKFB3, thereby upregulating PD-L1 and activating the immune escape pathway of tumors. With recent advances in the field of tumor immunotherapy, the importance of the immune clearance of tumor cells by the immune system has been clearly underlined, oncologic therapy can only decrease tumor burden to a certain point, to reach complete response, the body’s immune system needs to clear out the remaining weakened cancer cells.29,51

There are many studies reporting the effect of HIF-1α on PFKFB3 and vice-versa. HIF-1α acts as a transcriptional factor of PFKFB3 and PFKFB3 has a reverse feedback loop acting on HIF-1α.52,53 According to our bioinformatic analysis, there was a possible protein interaction between HIF-1α and PFKFB3 which was confirmed using Co-IP experiments. In our study, we also found that PFK-015 induced the phosphorylation of PFKFB3 which increased its nuclear localization. PFKFB3 contains a highly conserved NLS as “KKPR” which mediates its nuclear import via the importin family.36 This nuclear import can be affected by post-transcriptional modification, for example, acetylation of the residue causes it cytoplasmic retention.37 In our study, we found that phosphorylation increased its binding with importin-α5 and subsequent nuclear localization, which is in line with a study by Dana C. et al. where phosphorylation of ERKs upon stimulation increased binding to importin 7 and caused subsequent nuclear localization.54 Finally, we showed that upon PFK-015 treatment, HIF-1α and phosphorylated PFKFB3 localizes into the nucleus. Those results do not rule out other factors increasing the import of HIF-1α via other methods such as its innate nuclear import system via the importin family, and would require further detailed studies to clarify.55

Cancer cells with elevated glycolysis not only consume large amount of glucose from the tumor microenvironment but also releases elevated levels of metabolic by-products, especially lactic acid into the tumor microenvironment.36 An increasing number of studies have demonstrated that lactate is not only an end product of glycolysis, but also an important regulator that participates in multiple signaling pathways in normal tissues and cells.56 For example, tumor-infiltrating Treg cells can take up lactate to maintain their suppressive functions by increasing the expression of FOXP3 and MCT1.57 High expression of FOXP3 reprograms Treg cell into a more suppressive form and more adaptable to a high lactic acid microenvironment.57 Another study showed that glycolysis plays also plays a key role in T-cell differentiation, thus when cancer cells out-compete T-cell for glucose, T-cell differentiation into effector T-cells is inhibited. Therefore, tumor excessive glycolysis promotes tumor cell growth by starving TILs, with the resulting metabolites indirectly regulating the tumor microenvironment and ensuring tumor immune evasion. Considering that PFK-015 severely inhibits glycolysis in tumor cells, tumor cells lose their ability to competitively starve TILs in the tumor microenvironment and suppresses the amount of metabolites such as lactic acid released by the tumor cells into the tumor microenvironment.
Hence, the TILs in the TME could regain more tumor suppressive functions which could explain the greater combined effect of PFK-015 with PD-1 mAb. However, a limitation of this study is that we have not yet studied the effect inhibiting tumor glycolysis on tumor infiltrating lymphocytes in the tumor microenvironment from our experiments in details, which we aim to do in future studies focusing on the immunological side of glucose inhibition in tumor cells.

In conclusion, this “beside-to-bench” study was inspired from an inconclusive clinical trial of glycolysis enzyme PFKFB3 selective inhibitor, where a thorough investigation of possible cause and mechanism was carried out, finding out that upon inhibition of glucose metabolism in-vitro results are astounding: significant inhibition of cell proliferation and decrease in tumor volume and mass in immune-deficient mouse models. However, when carried out in more human translatable models such as Hu-NOG mouse, upregulation of PD-L1 immune escape occurs, shrinking away the cytotoxic effect of PFKFB3 selective inhibitor. An effect which can be remedied by combining immune checkpoint inhibitor (anti-PD-1 mAb) treatment. These results could provide theoretical guidance for future drug trials and combinational therapy.

Materials and methods

Cell lines

293 T, MC38 and BL16-F10 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). KYSE-520 and KYSE-70 cells were obtained from German Cell Culture Collection (DSMZ, Braunschweig, Germany). TE-11 cells were obtained from the Cell Bank of Shanghai Institute of Cell Biology (Chinese Academy of Medical Sciences, Shanghai, China). The cells were cultured in RPMI-1640 or DMEM (for 293 T, MC38 and B16.F10) (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (HyClone) at 37°C with 5% CO₂. Based on short tandem repeat (STR) profiling by vendors, no cells used in this study are found in the database of commonly misidentified cell lines. All cells were further authenticated via STR-PCR DNA profiling by Guangzhou Cellcook Biotech Co.,Ltd. (Guangzhou, China) and determined to be free of mycoplasma contamination.

Reagents, plasmid construction, and site-directed mutagenesis

The PFKFB3 selective inhibitor PFK-015 was purchased from Selleckchem (Houston, TX, United States). The lentiviruses were synthesized by OBIO Technology Co., Ltd. (Shanghai, China). Target cells were infected for 48 hr with lentivirus particles in the presence of 5 mg/ml polybrene and screened with puromycin to establish stable cells expression FLAG-tagged PFKFB3. Site-directed mutants (S461A and S461D) were provided by OBIO Technology (Shanghai, China). Transfection was performed using Lipofectamine 3000 Transfection Reagent according to the recommended protocol.

Cell proliferation and colony formation assay

Cells were seeded at 1,500 cells per well in 96-well culture plates, and MTS assays (Qiagen, Hilden, Germany) were performed according to the manufacturer’s guidelines. The absorbance was measured at a wavelength of 490 nm on a Synergy™ Multi-Mode Microplate Reader (Biotek, Vermont, USA). The colony formation assay was carried out as approximately 500 cells were seeded per well in six-well-plates. After 14 days, the cells were fixed in methanol and stained with 0.2% crystal violet. The number of colonies was determined using Quantity One software (Bio-Rad, Hercules, CA, USA).

The co-culture system of lymphocytes and tumor cells

PBMC were obtained from Shanghai AoNeng Biotechnology Co., Ltd. Then, cancer cells were cultured in different intervention groups in 6-well plates: namely normal control or with cells pre-treated with PFK-015. Living cancer cells were then quantified by crystal violet staining and counting using quantity one software (Bio-Rad, Hercules, CA, USA).

Lactase Dehydrogenase assay and Elisa

Lactate dehydrogenase (LDH) activity was used as an index of cytotoxicity. The samples were placed in 24-well plates with three wells in each group. After treatment, the culture medium was collected and centrifuged for the determination of LDH activity using an LDH activity detection kit (ab102526, Abcam, U.K.). LDH activity was determined spectrophotometrically (at 440 nm) according to the manufacturer’s instructions. Supernatant collected from wells with treated cells were kept at −80°C. Human IFN-β ELISA kit (ELabscience, Wuhan, China) were used according to manufacturer’s instructions.

Western blot and qPCR analysis

Western blot analysis was performed according to the manufacturer’s instructions. Cells or tissues were lysed in RIPA buffer. The protein concentrations were normalized with a BCA assay kit (Thermo Fisher Scientific, Carlsbad, CA, USA). Anti-β-actin (1:1000, Cell Signaling Technology, Beverly, USA, 4967), anti-vinculin (1:1000, Cell Signaling Technology, 13901), anti-lamin B, anti-PFKFB3 (1:1000, Abcam, Cambridge, MA, USA, ab181861), anti-PFKFB3 (phosphor-S461) (1:1000, ab232498), anti-HIF1α (1:1000, Abcam, Cambridge, MA, USA, ab228649), anti-FLAG tag (1:1000, Cell Signaling Technology, 8146) were used in this study. RNA levels were measured by qPCR analysis according to the manufacturer’s instructions. The specific primer sequences are listed in Supplementary Table 1.

Co-immunoprecipitation (CO-IP)

Co-IP was performed as following, briefly, cells were collected and lysed in lysis buffer with PMSF freshly added to a final concentration of 1 mM, and 1x protease inhibitor cocktail. After quantification using a BCA protein assay kit (Thermo Fisher, #23225), 2 mg of total protein were used for Co-IP and
incubated for overnight with 5 mg of anti-FLAG antibodies, and normal IgG as a negative IP control, respectively. The mixtures were incubated for another 2–4 hr with protein A/G agarose beads, and then beads were washed at least 5 times, and treated with 1x SDS sample buffer (Bio-Rad, #161-0737) and boiled at 100°C for 10 min. Cell lysates were also treated with equal volume of 5x SDS sample buffer and resolved by SDS-PAGE under denaturing conditions and transferred onto PVDF membranes (Bio-Rad, #162-0177). The membranes were blocked with 5% nonfat milk (LabScientific, #M0841) in 1x PBST at RT for 2 hr and incubated with primary antibody overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 hr at RT. To visualize proteins in the membrane, enhanced chemiluminescence (SuperSignal ECL, ThermoFisher Scientific, Carlsbad, USA) was used.

**Bioinformatics analysis**

Transcription factors that could bind and activate promoter regions of PD-L1/CD274 was listed from the registry AnimalTFDB (v3.0) (http://bioinfo.life.hust.edu.cn/AnimalTFDB/), and they were scored according to their possible interaction with protein PFKFB3. Then, they were cross-examined against their correlation with PD-L1 expression levels using TCGA database (https://www.tcgag.org/), obtaining a list of possible transcription factors that correlated with PD-L1 expression and PFKFB3 interaction. This list of transcription factors that support the findings of this study are available from the corresponding author upon reasonable request.

**ChIP and luciferase reporter assays**

ChIP assays were performed with a ChIP kit from Merck Millipore (Billerica, MA, USA) according to the manufacturer’s instructions. Briefly, 1 x 10^7 cells were cross-linked with a final concentration of 1.42% formaldehyde in growth medium for 15 min at RT, and cross-linking was quenched by the addition of glycine to a final concentration of 125 mM and incubation for 5 min at RT. Cells were harvested and lysed in IP buffer supplemented with 1 mM PMSF and 1 x protease inhibitor cocktail and sonicated to shear the chromatin to yield DNA fragment sizes of 0.5 to 1 kb. Samples were preincubated for 1 hr with 40 µl of protein A/G agarose beads. A 10% portion of the precleared samples was used as input DNA. Then, approximately 5 mg of HIF-1α antibody or normal immunoglobulin (IgG) was added to the remainder of the samples and incubated for 1 hr at 4°C, 40 µl of protein A/G agarose beads were added, and the mixture was incubated for 4 hr at 4°C. Beads were washed six times with cold IP buffer, the total input DNA was also isolated. Quantification was performed using real-time PCR. Control IgG and input DNA signal values were used to normalize the values from the HIF-1α ChIP to target genes. The primers sequences for target genes were HRE-1-PDL1: TCTCTTATGGAACCAAGGGAC; HRE-2-3-PDL1: TGGAGAAGTCACCAATCC; HRE-4-PDL1: GTTTCACAGACACCGGAGGT. qPCR analysis was performed to detect the DNA fragments immunoprecipitated with HIF-1α. An anti-HIF-1α antibody (1:20, Abcam, ab228649) was used in the ChIP assays. Luciferase reporter assays were performed according to the manufacturer’s instructions (Promega, WI, USA). Firefly and Renilla luciferase activities were examined by the Dual-Luciferase Reporter Assay System, and firefly activity was normalized to Renilla activity.

**Cell-based xenograft mouse models**

Cancer cell lines (2 x 10^6) or murine cancer cell lines (5 x 10^5) were injected subcutaneously into the dorsal flanks of 4-week-old female BALB/c nu/nu mice (five mice per group) or C57BL/6 mice respectively. Tumor growth was monitored every 3 days after transplantation using calipers. When the study finished, the mice were anesthetized, and the tumor volume and weight were measured. All tissues from the cell-based xenografts underwent further analysis.

**Humanization of NOG mice**

Human peripheral blood mononuclear cells (hu-PBMC), primed for humanized mouse model, were obtained from Shanghai AoNeng Biotechnology Co., Ltd. Prior to engraftment, the PBMCs were resuspended into RPMI-1640 supplemented with 10% fetal calf serum at a density of 5 x 10^7 cells/ml. Then, the PBMC solution was transplanted into 4–5 weeks old female NOG mice by IP injection. Successfulness of huPBMC-NOG mouse models was determined by flow cytometry as previously reported.

**Immunohistochemistry and immunofluorescence**

For the IHC assays, staining and analysis were performed according to the manufacturer’s instructions. Respective antibodies used in this study are listed in supplementary table 2. For quantification analysis, we evaluated the extent and intensity of all markers. Immunofluorescence staining was performed according to the manufacturer’s instructions, and an anti-PD-L1 antibody (1:200, Abcam, ab238697) and anti-PFKFB3 antibody (1:150, Abcam, ab181861) was used in this study.

**Flow cytometry analysis**

In this study, all flow cytometry antibodies and agents described above were purchased from BioLegend, San Diego, CA, USA. For mouse samples, single cell suspension of MC38/B16 /KYSE-520/KYSE-70-xenograft tumors were obtained by rapid and gentle stripping, physical grinding and filter filtration. After getting rid of dead cells with Zombie Aqua Fixable Viability Kit (77143), Zombie Red Fixable Viability Kit (77475), cells were stained with respective antibodies for 20 min as shown in supplementary table 3. After fixation and permeabilization by BD Cytofix/Cytoperm™ Fixation and Permeabilization Solution as per manufacturer’s instruction. Stained cells were analyzed by CytoFLEX flow cytometer (Beckman Coulter). Data were further analyzed by FlowJo 10.0 software.
Statistics and reproducibility
All experiments were carried out at least three times, for immunofluorescence staining, IHC, and western blot assays, representative images are shown. The results are presented as the mean ± S.D of at least three independent experiments after analysis by Student's t test or one-way ANOVA using GraphPad Prism 8.0.1 (GraphPad, La Jolla, CA, USA). Relative gene expression was analyzed using the 2^−ΔΔCt or 2^−ΔΔCt method. Correlations between phos-PFKFB3 levels and PD-L1, CD8 and Granzyme B expression were analyzed with Pearson’s correlation analysis. Survival analyses were performed using the Kaplan–Meier method and assessed using the log-rank test. All the statistical tests were two-sided, P < .05 was considered statistically significant.

Study ethics and approval
The study was approved by the Medical Ethics Committee of SYSUCC. Written informed consent was obtained from the patients who provided samples. All research on human samples and data have been performed in accordance with the principles stated in the Declaration of Helsinki. The animal studies were approved by and conducted according to the Institutional Animal Care and Use Committee of Sun Yat-Sen University.

Disclosure statement
The authors declare no potential conflicts of interest.

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Data availability statement
List of transcription factors obtained from our bioinformatic analysis can be found in the supplementary data. The data that support the bioinformatic findings of this study were derived from the following resources available in the public domain: the registry AnimalTFDB (v3.0) (http://bioinfo.life.hust.edu.cn/AnimalTFDB/) and the TCGA database (https://www.tcgasample.org/). All data and materials that support the results and analyses presented in the manuscript can be made freely available upon reasonable request to the corresponding author.

References
1. Warburg O. On the origin of cancer cells. Science. 1956 Feb 24;123 (3191):309–314. doi:10.1126/science.123.3191.309.
2. Koppenol WH, Bounds PL, Dang CV, Otto Warburg’s contributions to current concepts of cancer metabolism. Nat Rev Cancer. 2011 May 01;11(5):325–337. doi:10.1038/nrc3038.
3. Wang Y, Qu C, Liu T, Wang C. PFKFB3 inhibitors as potential anticaner agents: mechanisms of action, current developments, and structure-activity relationships. Eur J Med Chem. 2020 Oct 1;203:112612. doi:10.1016/j.ejmech.2020.112612.
4. Pilkis SJ, Claus TH, Kurland JJ, Lange AF. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: a metabolic signaling enzyme. Annu Rev Biochem. 1995;64(1):799–835. doi:10.1146/annurev.bi.64.070195.004055.
5. Yalcin A, Telang S, Clem B, Chesney J. Regulation of glucose metabolism by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in cancer. Exp Mol Pathol. 2009 Jun;86(3):174–179. doi:10.1016/j.exmp.2009.01.003.
6. Van Schaftingen E, Jett MF, Hue L, Hers HG. Control of liver 6-phosphofructokinase by fructose 2,6-bisphosphate and other effectors. Proceedings of the National Academy of Sciences. 1981;78(6):3483–3486. doi:10.1073/pnas.78.6.3483.
7. RIDER MH, BERTRAND L, VERTOMMEN D,Michels PA, Rousseau GG, HUE L. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis. Biochemical Journal. 2004;381(3):561–579. doi:10.1042/bj20040752.
8. Darville ME, Crepin KM, Vandekerckhove J, Van Damme J, Octave JN, Rider MH, et al. Complete nucleotide sequence coding for rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase derived from a cDNA clone. FEBS Lett. 1987;224(2):317–321. doi:10.1016/0014-5793(87)80476-3.
9. Okar DA, Lange AJ, Manzano Á, Navarro-Sabaté A, Riera L, Bartrons R. PFK-2/FBPase-2: maker and breaker of the essential bifactor fructose-2,6-bisphosphate. Trends Biochem Sci. 2001 January 01;26(1):30–35. doi:10.1016/S0968-0004(00)01699-6.
10. Bando H, Atsumi T, Nishio T, Niwa H, Mishima S, Shimizu C, et al. Phosphorylation of the 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase/PFKFB3 family of glycolytic regulators in human cancer. Clin Cancer Res. 2005 Aug 15;11(16):5784–5792. doi:10.1158/1078-0432.Ccr-05-0149.
11. Atsumi T, Chesney J, Metz C, Leng L, Donnelly S, Makita Z, et al. High expression of inducible 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (IPFK-2; PFKFB3) in human cancers. Cancer Res. 2002 Oct 15;62(20):5881–5887.
12. Reddy MM, Fernandes MS, Deshpande A, Weisberg E, Inogilizian HV, Abdel-Wahab O, Kung AL, Levine RL, Griffen JD, Sattler M, et al. The JAK2V617F oncogene requires expression of inducible phosphofructokinase/fructose-bisphosphatase 3 for cell growth and increased metabolic activity. Leukemia. 2012;26(3):481–489. doi:10.1038/leu.2011.225.
13. Kotowski K, Rosik J, Machaj F, Suplitt S, Wiczew D, Jabłońska K, Wiechee E, Ghavami S, Dziegiel P. Role of PFKFB3 and PFKFB4 in cancer: genetic basis, impact on disease development/progression, and potential as therapeutic targets. Cancers. 2021;13(4):909. https://www.mdpi.com/2072-6699/13/4/909.
14. Yi M, Ban Y, Tan Y, Xiong W, Li G, Xiang B. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 and 4: a pair of valves for fine-tuning of glucose metabolism in human cancer. Molecular Metabolism. 2019 February 01;20:1–13. doi:10.1016/j.molmet.2018.11.013.
15. Bartrons R, Simon-Molás H, Rodriguez-García A, Castaño E, Navarro-Sabaté À, Manzano A, Martinez-Outschoorn UE. Fructose 2,6-Bisphosphate in cancer cell metabolism. Front Oncol. 2018;September-04 2018;8(331): doi:10.3389/fonc.2018.00331.
16. Fortunato S, Bononi G, Granchi C, Minutolo F. An update on patents covering agents that interfere with the cancer glycolytic cascade. ChemMedChem. 2018 Nov 6;13(21):2251–2265. doi:10.1002/cmdc.201800447.
17. Clem B, Telang S, Clem A, Yalcin A, Meier J, Simmons A, Rasku MA, Arumugam S, Dean WL, Eaton J, et al. Small-molecule inhibitors of 6-phosphofructo-2-kinase activity suppresses glycolytic flux and tumor growth. Mol Cancer Ther. 2008;7(1):110–120. doi:10.1158/1535-7163.Mct-07-0482.
50. Desideri E, Vegliante R, Cardaci S, Nepravishita R, Paci M, Ciriolo MR. MAPK14/p38α-dependent modulation of glucose metabolism affects ROS levels and autophagy during starvation. Autophagy. 2014 September 19;10(9):1652–1665. doi:10.4161/auto.29456.

51. Hargadon KM, Johnson CE, Williams CJ. Immune checkpoint blockade therapy for cancer: an overview of FDA-approved immune checkpoint inhibitors. Int Immunopharmacol. 2018 Sep;62:29–39. doi:10.1016/j.intimp.2018.06.001.

52. Long Q, Zou X, Song Y, Duan Z, Liu L. PFKFB3/HIF-1α feedback loop modulates sorafenib resistance in hepatocellular carcinoma cells. Biochem Biophys Res Commun. 2019;513(3):642–650. doi:10.1016/j.bbrc.2019.03.109.

53. Tawakol A, Singh P, Mojena M, Pimentel-Santillana M, Emami H, MacNabb M, et al. HIF-1α and PFKFB3 mediate a tight relationship between proinflammatory activation and anaerobic metabolism in atherosclerotic macrophages. Arterioscler Thromb Vasc Biol. 2015 Jun;35 (6):1463–1471. doi:10.1161/ATVBAHA.115.305551.

54. Chuderland D, Konson A, Seger R. Identification and characterization of a general nuclear translocation signal in signaling proteins. Mol Cell. 2008 September 26;31(6):850–861. doi:10.1016/j.molcel.2008.08.007.

55. Chachami G, Paraskeva E, Mingoz G, Braliou GG, Görlich D, Simos G. Transport of hypoxia-inducible factor HIF-1α into the nucleus involves importins 4 and 7. Biochem Biophys Res Commun. 2009 Dec 11;390(2):235–240. doi:10.1016/j.bbrc.2009.09.093.

56. Angélin A, Gil-de-Gómez L, Dahiya S, Jiao J, Guo L, Levine MH, et al. Foxp3 reprograms T Cell metabolism to function in low-glucose, high-lactate environments. Cell Metab. 2017 Jun 6;25(6):1282–1293.e1287. doi:10.1016/j.cmet.2016.12.018.

57. Kumagai S, Koyama S, Itahashi K, Tanegashima T, Lin Y-T, Togashi Y, Kamada T, Irie T, Okumura G, Kono H. Lactic acid promotes PD-1 expression in regulatory T cells in highly glycolytic tumor microenvironments. Cancer Cell. 2022;40(2):201–218.e209. doi:10.1016/j.ccell.2022.01.001.

58. Brehm MA, Cuthbert A, Yang C, et al. Parameters for establishing humanized mouse models to study human immunity: analysis of human hematopoietic stem cell engraftment in three immunodeficient strains of mice bearing the IL2rynull mutation. Clinical Immunology. 2010;135(1):84–98. doi:10.1016/j.clim.2009.12.008.