Targeting PARP1 activies cGAS-STING signaling pathway to promote tumor cells apoptosis and reshape tumor immune microenvironment in non-small cell lung cancer

Yanqi Feng
Tongji Hospital

Xinyue Liu
Tongji Hospital

Shu Xia
Tongji Hospital

Yiming Li
Tongji Hospital

Piao Li
Tongji Hospital

Xiangtian Xiao
Tongji Hospital

Yuelin Han
University of Science and Technology

Shu Xia (✉ xiashutj@hust.edu.cn)
Tongji Hospital

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Abstract

Lung adenocarcinoma (LUAD) is the most common pathological subtype of non-small cell lung cancer. Although the application of immune checkpoint inhibitors has greatly improved the therapy of solid tumors, treatment of lots of patients with lung adenocarcinoma is still not satisfactory. For most diseases with low immunogenicity, it’s urgent to seek for new combination treatment strategies.

Platinum is a widely used DNA damage agent. PARP inhibitors are more effective for tumors with defects in DNA damage and repair. In the context of inhibition of PARP1, tumor cells are easier to form more immunogenic tumor antigen libraries and increase immunogenicity. Nonetheless, the efficacy of PARP inhibitors, combined with platinum and immune checkpoint inhibitors is still undefined. In this research, we have demonstrated that inhibiting PARP1 activated cGAS-STING pathway to up-regulate PD-L1 expression in lung adenocarcinoma cells by real-time quantitative PCR and immunoblotting. Then, we constructed a LLC tumor model to verify that PARP inhibitors, combined with platinum and immune checkpoint inhibitors could change the lymphocyte infiltration in tumor microenvironment, increase the proportion of CD8+T cells and activated DC cells, and enhance anti-tumor immunity.

To sum up, we confirmed that PARP inhibitors combined with platinum can enhance the immune checkpoint effect by activating cGAS-STING pathway, further increase the infiltration of CD8+ T and DC cells and reshape tumor immune microenvironment. Therefore, this research provides a novel strategy for the treatment of lung adenocarcinoma patients with poor prognosis.

1. Introduction

Lung cancer is the most common type of malignant cancer (about 12.6% of all cases) and the leading cause of cancer death[1]. Non-small cell lung cancer (NSCLC) accounts for about 80%-85% of lung cancer. Lung adenocarcinoma (LUAD) is the main pathological type of LC in China[2–5]. Programmed cell death protein 1 (PD-1) / programmed death ligand 1 (PD-L1) inhibitor monotherapy and PD-1/PD-L1 inhibitor combined chemotherapy have become the standard treatment for advanced NSCLC in China[4, 6, 7]. Along with the emergence of targeted therapy and immunotherapy, the treatment of NSCLC has been greatly improved, but the overall cure rate and survival rate of NSCLC are still very low[8, 9]. More and more evidence confirms that the immunophenotype of tumors greatly affects the efficacy of ICB. In tumors with low oncogenicity, the tumor mutation load is lower, and high mutation load is a potential factor to improve immune response[10, 11]. Therefore, the treatment strategy of effectively increasing the mutation load of tumor microenvironment of lung adenocarcinoma is very significant to improve the curative effect of most patients.

Platinum was a traditional chemotherapeutic drug for NSCLC before early immunotherapy was approved [12], and now it is mainly used in combination with immune checkpoint inhibitor (ICls) for standard treatment of advanced NSCLC [13]. Platinum mainly changes the structure of DNA by binding to DNA, damages tumor cell DNA and kills tumor cells[14–16]
PARP inhibitors increase tumor DNA damage by inhibiting single-stranded DNA damage repair, which is more effective for tumors with defects in DNA damage repair[17–22]. Inhibition of tumor DNA damage repair is closely related to increasing tumor mutation load, and to some extent enhance the efficiency of ICIIs[23–26]. It has been reported that PARP inhibitors combined with platinum can significantly improve the gastrointestinal neurotoxicity of platinum[27]. PARP inhibitors combined with platinum has become the cornerstone of maintenance therapy for patients with epithelial ovarian cancer[28–30]. However, the efficacy of PARP inhibitor combined with immune checkpoint inhibitors on NSCLC is still unknown.

In summary, we hypothesized that PARP inhibitor combined with ICIIs can generate a synergistic anti-tumor effect on the basis of the application of platinum in NSCLC. Through the research, we found that PARP inhibitor can activate cGAS-STING pathway and up-regulate the expression of PD-L1 in NSCLC. By establishing the tumor-bearing model of LLC in C57BL/6 mice, we confirmed that platinum combined with PARP inhibitor could cooperate with αPD-L1 to suppress tumor growth, increase the new tumor antigen in NSCLC and enhance the immune response of tumor to ICIIs. After treatment, the volume of subcutaneous tumor reduced, and we found the infiltration of D8 cells and DC cells in tumor microenvironment increased by flow cytometry. This could provide a new treatment strategy for clinical patients with NSCLC.

2. MATERIALS AND METHODS

2.1 Cell culture and Reagent

Human NSCLC cell lines H1975 and H1299 and murine LC cell lines LLC were obtained from the Tongji Hospital Oncology Laboratory (Wuhan, China). H1975 and H1299 cells were cultured in RPMI1640 medium (Cytiva, Hyclone Laboratories, Logan, USA) with 10% fetal bovine serum (FBS, Gibco, Life Technologies Limited, UK). LLC cells were cultured in DMEM-high glucose (Cytiva, Hyclone Laboratories, Logan, USA) containing 10% FBS with 5% CO2 at 37°C.

Anti-mouse PD-L1 (B7-H1) antibody Olaparib(AZD2281) and Cisplatin(NSC 119875) were purchased from Selleck Chemicals (Houston, TX, USA).

2.2 Mice

Female C57BL/6 mice (6–8 weeks) were acquired from Vital River Laboratories (Beijing, China) and raised under specific pathogen-free (SPF) conditions. The in vivo experiments were performed in SPF condition and accorded with the guidelines of the International Guiding Principles for Animal Research and approved by the Ethics Committee of Huazhong University of Science and Technology.

2.3 Immunoblotting

Tumor cells proteins were extracted using RIPA buffer (Beyotime, Shanghai, China) mixed with 1% phenylmethylsulfonyl fluoride and 1% phosphatase inhibitor cocktail (Servicebio, Wuhan, China), and the concentration of protein was quantified using a BCA assay kit (Beyotime, Shanghai, China). After mixing
with 5× sodium dodecyl sulfate (SDS) loading buffer, the protein sample (30 µg) was separated using SDS-PAGE and wet-transferred to a PVDF membrane (Millipore, Burlington, MA, USA). The membrane was blocking with 5% non-fat milk powder for 1 h, and then incubated with the following primary antibodies: GAPDH (1:20000, 60004-1-Ig, Proteintech, Wuhan, China), TUBLIN (1:20000, 11114-1-AP, Proteintech), PD-L1 (1:1000, E1L3N, Cell Signaling Technology, Boston, Massachusetts, USA), Phospho -STING (1:1000, E9A9K, Cell Signaling Technology), Phospho-TBK1 (1:1000, D52C2, Cell Signaling Technology), Phospho-IRF3 (1:1000, D601M, Cell Signaling Technology), STING (1:1000, 19851-1-AP, Proteintech), PARP1 (1:1000, 13371-1-AP, Proteintech) and Histone H2A.X (1:1000, D17A3, Cell Signaling Technology), at 4°C overnight. Next, the membrane was incubated with secondary antibodies (Promoter, Wuhan, China) for 1 h and visualized using an ECL HRP substrate kit (#K22020, Abbkine, Inc.). The signal from the blots was detected using the G:BOX Chemi X system (Syngene, Cambridge, UK) and analyzed using ImageJ software.

**2.4 Immunofluorescence (IF)**

Tumor cells were cultured on 24-well chamber slides and multiplied to 80%. Then cells were washed with phosphate buffer saline (PBS) for 3 times and 5 minutes each time and fixed with 4% paraformaldehyde for 20 minutes. Wash cells with PBS for 3 times again. The cells were incubated with 0.5% Triton (40ulTriton + 8000ulPBS) for 20 minutes at room temperature, and then blocked with 5% BSA for 30 minutes. Next, cells were incubated with Histone H2A.X (1:400, D17A3, Cell Signaling Technology) at 4°C overnight and washed with PBS for 3 times. After incubating with Alexa Fluor secondary antibodies at room temperature for 1 h, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) and visualized with SP8 confocal laser scanning microscope (Leica, Germany) or IX 73 DP80 fluorescent microscope (Olympus, Tokyo, Japan). Choose three fields under the microscope randomly, and use the ImageJ software to detect the relative fluorescence intensity (RFI).

**2.5 Real-time PCR (RT-PCR)**

Cells were planted on 6-well plates. The RNA of cells was isolated using Trizol reagent (NO. 9766, Takara Bio, Shiga, Japan) and then reversed transcribed to cDNA with the PrimeScript 1st Strand cDNA Synthesis Kit (NO.6110A, Takara Bio). Gene-specific primers used are listed: GAPDH, 5’-AGGTCGGTGTAACGGATTTG-3’(forward), 5’-TGTAGACCAGTTGAGGTCA-3’(reverse); PARP1, 5’-GCCCTAAGGCTCAGACGA-3’(forward), 5’-CTACTCGGTCCAAGATCGCC-3’(reverse); PD-L1, 5’-GGAGATTAGTCTGAGGAAAACCA-3’(forward), 5’-AACGGAAGATGACTGCTA-3’(reverse). Gene expression levels was detected by RT-PCR using SDS 2.1 software (Applied Biosystems). GAPDH expression levels were used to normalize the mRNA expression levels of the target genes, and then we analyzed the mRNA expression levels of them using the $2^{-\Delta\Delta Ct}$ method.

**2.6 Immunohistochemistry**

Fix tumor tissue with 4% paraformaldehyde (PFA) at 25°C for 48 h. After performed with antigen retrieval, the sections were incubated with Hydrogen Peroxide Block and blocked with Ultra V Block. Then, the sections were incubated with the following primary antibodies: anti-Ki67 (1:200, D3B5, Cell Signaling
Technology), γH2AX(1: 300, 20E3, Cell Signaling Technology), pSTING (1:100, E9A9K, Cell Signaling Technology), pTBK1(1:100, D52C2, Cell Signaling Technology), pIRF3(1:100, D6O1M, Cell Signaling Technology) overnight. After washed for three times, the sections were incubated with secondary antibody. We stained the sections with 3,3-diaminobenzidine and counterstained them with hematoxylin. The score of the sections accorded with the staining intensity.

2.7 Flow cytometry

Tumor tissue, isolated from the xenograft mice of each group, was cut, ground, and digested with the mixture of DMEM, type IV collagenase (Promoter), hyaluronidase (Biosharp), and DNase (Biosharp) at 37°C for 40 min. After filtered with 200-mesh gauze, the suspensions were treated with RBC lysis buffer and then neutralized with PBS. We separated live cells from the mixture with Zombie (Zombie Aqua™, Fixable Viability Kit, BioLegend, USA) for 30 min at room temperature. Next, we used the following fluorescent antibodies: APC-cy7-anti-mouse CD45 (103116, BioLegend), PC-cy5.5-anti-mouse CD8a (100734, BioLegend), APC-anti-mouse CD4 (100516, BioLegend), FITC-anti-mouse CD3 (100204, BioLegend), FITC-anti-mouse CD11c (117305, BioLegend), APC-anti-mouse CD86 (105114, BioLegend), PE-anti-mouse I-A/I-E (107607, BioLegend), for surface staining. After demonstrated with the flow cytometer (CytoFLEX-3 laser 13 colors, Beckman Coulter), the results were analyzed by FlowJo v10 software.

2.8 Statistical analysis

All statistical analysis were demonstrated with GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). All data were appeared as mean ± SEM or SD. Differences between 2 groups were decided through student’s t-test, and the results of multiple comparisons were performed by one-way ANOVA. For all analysis, p < 0.05 were considered statistically significant.

3. Result

3.1 PARP inhibitor up-regulated the expression of PD-L1 in NSCLC cells

Firstly, we used Olaparib(1µM) to dose NSCLC cell lines H1975 and H1299. Westernblot showed Olaparib up-regulated the expression of PD-L1 in a time-dependent manner, and the content of mRNA was detected by qPCR was consistent with this result (Fig. 1A-B). In addition, in order to further confirm that the up-regulated PD-L1 expression of the targeting effect of PARP inhibitor, siRNA was used to knock down the PARP1 gene in NSCLC cell lines. The expression of PD-L1 in the low expression group of PARP1 was much higher than that in the control group (Fig. 1C-D). Therefore, we confirmed that PARP inhibitor targeted up-regulated the expression of PD-L1 in NSCLC cells.

3.2 PARP inhibitor upregulated the expression of PD-L1 by activating cGAS-STING pathway.
cGAS-STING pathway is a dsDNA sensor, activated by perceptual self-DNA and involved in pathogen detection and anti-tumor effect. We examined H1975 and H1299 cell lines treated with Olaparib by immunofluorescence and WesternBlot. Interestingly, the expression of γH2AX was also up-regulated in a time-dependent manner, indicating that intracellular DNA damage increased after PARP inhibitor treatment (Fig.2A-B). At the same time, phosphorylated STING, phosphorylated TBK1 and phosphorylated IRF3 were also up-regulated in a time-dependent manner, which confirmed that cGAS-STING pathway was activated after PARP inhibitor treatment. And its trend was consistent with the tendency of PD-L1 expression. Early studies have confirmed that cGAS-STING pathway mediates the upregulation of PD-L1 through type I interferon response[31]. From this we can conclude that PARP inhibitor up-regulated the expression of PD-L1 in NSCLC cells by activating cGAS-STING pathway.

3.3 On the basis of platinum application, PARP inhibitor combined with αPD-L1 could significantly inhibit tumor growth.

The anti-tumor effect of combined drugs was observed on LLC tumor model in vivo. According to some early studies, PARP inhibitor was mainly used for the maintenance of DNA damage, and it was generally used in combination with platinum as a DNA damage agent[28, 32]. 7–9 days after subcutaneous injection of tumor cells (tumors volume were about 100mm^3), the mice were divided into five groups (n = 6) and treated. Except for the blank group, the other four treatment groups were given low dose cisplatin to sensitize mice on the first day of treatment (Fig. 3A). Cisplatin alone did not show inhibitory effect on LLC tumor model. Although PARP inhibitor and αPD-L1 groups partially inhibited tumor growth, the combined group had significant anti-tumor effect (Fig. 3B). In addition, tumor weight and tumor growth curve also reflected the inhibitory effect of combined therapy on tumor (Fig. 3C-D). The reason why the anti-tumor effect of cisplatin alone was not obvious might be that cisplatin was only used in a single low dose, which had little effect on tumor DNA damage and lasted for a short time. To further explore the effect of combination therapy on the tumor cells in vivo, we collected the tumors isolated from LLC tumor model of each treatment group, and stained with Ki67 and TUNNEL. The expression of Ki67 in the combination group was much lower than that in other treatment groups, while the rate of TUNNEL positive cells in the combination group was much higher than that in other groups (Fig. 3, E-G). In the combined treatment group, the proliferative activity of tumor cells decreased significantly and the level of necrosis and apoptosis increased. From the above results, we could draw a conclusion that PARP inhibitor, with the application of platinum, can significantly improve the effect of immunotherapy in non-small cell lung cancer by enhancing the apoptosis and necrosis of tumor cells.

3.4 PARP inhibitor increased CD8^+ T cells in tumor microenvironment by activating cGAS-STING in tumor cells in vivo.

To further investigate the mechanism of the tumor growth inhibition, we detected the activation of cGAS-STING pathway in subcutaneously transplanted tumors isolated from each treatment group. The results showed that the expression of γH2AX, p-STING p-TBK1 and p-IRF3, the markers of cGAS-STING pathway excitation, in subcutaneous tumors of the combination group was significantly higher than that in other
The results confirmed that, consistent with the conclusion of experiment in vitro, combined therapy could enhance the activation of cGAS-STING pathway. In the meantime, we found that the infiltration of CD3$^+$ and CD8$^+$ T cells in the subcutaneous tumor tissue in the combined treatment group was higher than that in the monotherapy group (Fig. 4B-D). Therefore, we could conclude that the combination of PARP inhibitor, platinum and αPD-L1 can enhance the inflammation of tumor microenvironment and enhance the immune activity of tumor by activating cGAS-STING pathway and increasing CD8$^+$ T cells infiltration.

### 3.5 PARP inhibitor plus platinum combined with αPD-L1 can regulate lymphocyte infiltration in tumor microenvironment.

In order to confirm the effect of PARP inhibitor combined therapy on lymphocyte infiltration in tumor microenvironment, we performed immunohistochemical detection of CD3$^+$ and CD8$^+$ T cells in LLC tumors. There was almost no CD3$^+$ and CD8$^+$ T cell infiltration in LLC tumor in blank group and cisplatin-treated group, and a small number of CD3$^+$ and CD8$^+$ T cells were found in PARP inhibitor-treated and αPD-L1-treated groups, while CD3$^+$ and CD8$^+$ T cell infiltration increased significantly in combined group (Fig. 4E-F). The results of flow cytometry further was consistent with the conclusion of IHC (Fig. 5A-B). In addition, the combination therapy also significantly increased the penetration of DC cells in the immune microenvironment (Fig. 5C-D) and further promoted their activation (Fig. 5E-F). These results showed that the combination of cisplatin, PARP inhibitor and αPD-L1 significantly increased the penetration and activity of DC cells and CD8$^+$ T cells in the immune microenvironment of NSCLC.

### 4. Discussion

It is reported that PARP inhibitor has not achieved a good curative effect in BRCA-enriched tumors[33]. The research and application of PARP inhibitor are mainly focused on ovarian cancer and breast cancer with BRCA1/2 mutation[34, 35]. At present, only a few studies have confirmed that PARP inhibitor is effective in NSCLC with DNA repair defect[19]. This research originally confirmed that PARP inhibitor combined with platinum could enhance the anti-tumor immune response of αPD-L1, increase the tumor mutation load and improve the immune activity of tumor microenvironment in NSCLC, which provided a new treatment strategy for patients with NSCLC.

Previous studies have confirmed that PARP inhibitor upregulated the expression of PD-L1 through ATM/GSK3β in breast cancer [36]. In this study, we confirmed that PARP inhibitor upregulated the expression of PD-L1 in NSCLC in a time-dependent manner and activated the intracellular cGAS-STING pathway in tumor cells with the same trend. The type I interferon response mediated by cGAS-STING pathway mediates the upregulation of PD-L1[31], which indicates that PARP inhibitor partly up-regulates the expression of PD-L1 by activating cGAS-STING pathway. At present, a variety of evidence shows that the efficacy of PARP inhibitor alone in BRCA-proficient tumors is not ideal[33], and platinum, as the most classical DNA damage agent, its auxiliary effect on PARP inhibitor has been confirmed[32], and cisplatin
may up-regulate the expression of PD-L1 through mTOR/PI3K/AKT and other signal pathways[37].
Therefore, based on the single application of low dose, the immunoreactive mice were randomly divided into 5 groups. Interestingly, the results of in vitro studies showed that the tumor in the combined treatment group grew slowly, the tumor size at the end point of treatment was very small, and the tumor inhibition was significant. The analysis of tumor microenvironment showed that the inhibition of tumor proliferation and activation apoptosis and necrosis in the combined treatment group were significantly higher than those in the single drug group, and the infiltration of activated DC cells and CD8 + T cells in the tumor was also significantly increased. The above results suggest that on the basis of platinum application, PARP inhibitor could cooperate with ICIS to generate more significant anti-tumor immune response, which may be related to the increase of tumor mutation load, which may transform cold tumors into hot tumors, and provide new therapeutic ideas for more patients with non-small cell lung cancer.

In fact, our study also had some limitations. First of all, we lacked some clinical samples to prove the feasibility of combined use of drugs. Secondly, we only constructed the subcutaneous tumor model of LLC. If we further construct the mouse model of carcinoma in situ, the conclusion will be more rigorous and reliable.

5. Conclusions

This research revealed that PARP inhibitors combined with platinum can activate cGAS-STING pathway and further increase the infiltration of CD8 + T cells and DC to enhance the immune checkpoint effect in NSCLC. In vitro experiment also demonstrated the significant tumor suppression of combination therapy. In consideration of this consequence, our study provides a useful strategy for the combination treatment of lung adenocarcinoma patients with low immunogenicity.

Declarations

Acknowledgements

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Competing Interests

All authors declare there is no conflicts of interests.

Ethics Approval

This work was approved by the Laboratory Animal Welfare & Ethics Committee of Tongji Hospital Huazhong University of Science and Technology.

Consent for publication
Authors' contributions

Shu Xia designed and directed this study. Yanqi Feng conducted most experiment and analyzed the results. Xinyue Liu, Shuxi Yao and Yuelin Han performed the animal experiment. Yanqi Feng, Yiming Li, Xiangtian Xiao and Piao Li wrote and revised the manuscript. All authors have approved the final manuscript.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

References


Figures
Figure 1

PARP inhibitor up-regulates the expression of PD-L1 in NSCLC cells. (A) Representative immunoblotting of PD-L1 in H1975 and H1299 cells 0h, 24h and 48h after Olaparib (1μM). (B) PD-L1 mRNA levels of H1975 and H1299 cells upregulated 0h, 24h and 48h after Olaparib. (C) PARP1 and correspondent PD-L1 mRNA levels in H1975 and H1299 cells after siPARP1. (D) Image of immunoblotting of PARP1 and PD-L1 in H1975 and H1299 cells after siPARP1. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001
Figure 2

PARP inhibitor activated cGAS-STING pathway. (A) Representative immunofluorescence of γH2AX in H1975 and H1299 cells 0h, 24h and 48h after Olaparib (1μM). (B) Immunoblotting and quantification of γH2AX in H1975 and H1299 cells 0h, 24h and 48h later. (C) Representative immunoblotting of p-STING, STING, p-TBK1 and p-IRF3 in H1975 and H1299 cells 0h, 24h and 48h after Olaparib (1μM). (D) Quantification of p-STING. The white scale bar is 50μM
Figure 3

On the basis of platinum application, PARP inhibitor combined with αPD-L1 could significantly inhibit tumor growth. (A) Schematic diagram of design and treatment of the xenograft tumor-bearing mice mode. (B) Image of extracted subcutaneous tumors. (C) Weight of tumors at the end of treatment. (D) Growth curves of subcutaneous tumors. (E) Images of tumor tissue stained with Ki67, and TUNEL. Quantitative
analysis of the (F) Ki67 positive area and (G) TUNEL positive area. The black and white scale bar are 100μM.

Figure 4
PARP inhibitor increased CD8+ T cells in tumor microenvironment by activating cGAS-STING in tumor cells in vivo. (A) Image of IHC of γH2AX pSTING,pTBK1,pIRF3 after treatment. (B) Representative IHC of
CD8\(^+\) and CD3\(^+\) T cells. The black scale bar is 50\(\mu\)M, and the red scale bar is 20\(\mu\)M.

Figure 5

The image of LLC tumor of flow cytometry. (A) Images and quantitative analysis (B) of CD8\(^+\) T cells (gated on CD45\(^+\) cells) in extracted tumors of each group by flow cytometry. (C) Images and quantitative
analysis(D) of MHC^ +CD11c^ cells (gated on CD45^ cells). (E) Images and quantitative(F) analysis of CD86^ CD11c^ cells.