

Differential Expression Factors in Radiation Induced Lung Injury and Immune Checkpoint Inhibitor associated Pneumonia in Lung Cancer

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Abstract. Radiotherapy and immune checkpoint inhibitors (ICIs) are both important treatments for lung cancer patients. However, radiation induced lung injury (RILI) and immune checkpoint inhibitor associated pneumonia (CIP) are severe dose-limited pulmonary inflammation. The prediction or early diagnosis of them can help improve the therapeutic effect and avoid lung injury, thus improve the quality of life (QOL) for patients with lung cancer. This study obtained datasets GSE41789 and GSE184000 from National Center for Biotechnology Information (NCBI), Gene Expression Omnibus (GEO). Using DESeq2 in R packages to analysis differential gene expression (DEGs). T-test and Fold Change were used to screen DEGs. By comparing the differential expressed genes between the two datasets, the contemporary DEGs were elected and analyzed. Four DEGs in both RILI high risk group and CIP high risk group were identified, including PER3, DBP, CXCL5 and SPON2, indicated that these gene may be potential biomarkers for RILI and CIP, during the radiation an ICIs treatment process. Molecular and clinical experiments will be conducted in the next stage to verify the results.

Keywords: Lung cancer, radiotherapy, ICIs, RILI, CIP.

1. Introduction

Lung cancer is a notorious kind of malignant diseases [1]. In 2020, there were more than two million newly diagnosed cases of this disease and 1.8 million deaths due to its cause [1]. Unfortunately, the prognosis for patients with lung cancer is not satisfactory, around half of the patients has been distant metastasis at the time of diagnosis [2]. This highlights the importance and exigency of improving the overall survival (OS) and the quality of life (QOL) of patients suffering from this disease.

Radiotherapy is one kind of the dominating remedy methods of lung cancer. Chemoradiotherapy as the standard treatment mode, could get better local control and reduce the metastasis rate for inoperable lung cancers [3]. Whereas, radiation induced lung injury (RILI) is a most sort of severe dose-limited toxicities, the incidence rate of RILI is about 5%-20% [4]. RILI is also an independent negative correlate of lung cancer prognosis, it could seriously affect patients' QOL and their survival [4]. RILI is an evolving process, including radiation pneumonia (RP) and radiation pulmonary fibrosis (RF), its underlying mechanisms may include gene changes, signal pathways regulation, cytokines involvement [5]. Previous studies have shown that oxidative stress and a variety of cytokines can participate in the process of occurrence and development of RILI, and amplify the acute response [5-7]. At present, there are only three main known signal pathways involved in RILI and there is still no clear biomarker [8].

The mainly clinical manifestations of RILI are cough, chest tightness, shortness of breath, low fever, pulmonary dysfunction and imaging changes [4]. However, the diagnosis of RILI is mainly based on the chest irradiation history, imaging changes and patient symptoms. Clinicians has to diagnose and judge the changes of the disease through repeated CT examinations. There is still no conveniently clear predictive factors or identification indicators for RILI.

Recently, immune checkpoint inhibitors (ICIs) have proceeded apace and has changed the traditional strategies to treat lung cancer. Studies have shown that programmed death receptor-1 (PD-1) is highly expressed in many kinds of cancers. The inhibitor has achieved certain efficacy in multitudinous of solid tumors, for example, esophageal cancer, melanoma (KEYNOTE-006) and some other kinds of cancer [9, 10]. Pembrolizumab as the first-line therapy with or without chemotherapy for metastatic and recurrent head and neck cancer showed that pembrolizumab significantly prolonged the mOS of patients in PD-L1 CPS $\geq$ 20 sub-group [9]. The results of KEYNOTE-181 study showed that Pembrolizumab combined chemotherapy prolonged the mOS of Asian esophageal squamous cell carcinoma (ESCC) patients compared with chemotherapy (HR 0.63; nominal $P < 0.0001$) [10].

Although the participation of ICIs brings innovation to the treatment regimen of lung cancer, the efficacy of anti-PD-1 inhibitors alone to treat most cancers is still very limited. A part of the patients may face the drug resistance problem during their treatment. Therefore, combined therapies need to be explored to get better efficacy and help delay drug resistance.

PACIFIC study first proved the synergistic effect of immunity and radiotherapy, based on this, the standard treatment mode for stage III unresectable NSCLC adds the combination of PD-L1 antibodies to radiotherapy/chemotherapy [11]. As PEMBRO-RT study reported their results, radiotherapy combined with immunotherapy has become one of the research hotspots [12]. The IMPOWER study and the CASPIAN study, inspired us that chemotherapy combined with atezolizumab or durvalumab can be a more optimal treatment strategy and benefit the survival of extensive SCLC patients [13, 14]. Theeleneven announced that radiotherapy in combination with pembrolizumab could promote the prognosis of NSCLC patients significantly compared with pembrolizumab alone [15].

Radiotherapy could directly damage tumor cells, when combined with ICIs, they could induce a series of immune-mediated anti-tumor effects, such as increasing the release of cancer specific antigens, promoting the activation of immunity, increasing the density of tumor infiltrating lymphocytes, promoting T cells to recognize tumor cells. By the above ways, radiotherapy could improve the ability of immune system to recognize and killing cancer cells [16]. In addition, radiotherapy can induce the reprogram of tumor microenvironment. The less infiltrated "cold" cancer cells could transform into lymphocyte infiltrated "hot" cancer cells, thus supply conditions for ICIs response [16].

With the development of radiotherapy techniques and methods, radiotherapy has becoming more and more accurate, and the adverse reactions have been gradually reduced, especially RILI. However, the involvement of ICIs may lead to potential immune-related adverse event (irAE), such as CIP. At present, no study shows whether ICIs could increase the risk of RILI during the remedy of lung cancer. And still no specific discriminant factor for RILI and CIP. What's more, how to screen the potential pneumonia risk groups are the key point that we need to further explore.

Radiation-induced cell injury and immunotherapy-induced T cells reactivation together lead the secretion multiple of cytokines. Through the activation of some granulocytes and lymphocytes, they can indirectly harm lung tissue in addition to directly damaging it through the TGF- β /Smad, TNF- α /NF- κ B, and other signal pathways [8]. The production of cytokines by various cells can be induced by both RILI and CIP, and among these cytokines, some members of the interleukin family have been linked to both RILI and anti-PD-1 antibodies [8]. Other studies have recommended that irradiation combined with anti-PD-1 antibodies may worsen RILI by preventing the release of IL-17A or by enhancing the production of TGF- β 1 in concert [17]. In addition, some signaling pathways related to lung injury, such as ROS/RNS, cGAS-STING signal pathways, and cell death pathway, may be also involved in the pathogenesis of RILI and CIP [8].

Therefore, the purpose of this project is to screen out differentially expressed genes in both RILI and in CIP by multiple variation method and t-test to provide a theoretical basis for clinical identification of patients who may benefit from radiotherapy combined with ICIs and find better combined treatment regimens to improve the therapeutic efficiency and reduce the adverse reactions.

2. Methodology

2.1. Data Resources and Selection

Two gene expression datasets were obtained from NCBI GEO (<http://www.ncbi.nlm.nih.gov/geo/>). The irritated and control groups of mRNA expression information in mice lung tissues were selected from dataset GSE41789 (Table 1). The irradiated groups were received 5Gy and 17.5Gy of whole thorax irradiation, as RILI high risk group. Samples in the two groups were obtained at 16 weeks after irradiation. The mRNA expression information of lung tissues from mice with anti-PD-1 inhibitor treated aged group (aged PD) and the aged control group (aged control) were selected from Dataset GSE184000 (Fig. 1). Since the aged PD group had a significantly higher risk of irAE, especially CIP. Therefore, these two groups were select as CIP high risk group and control group.

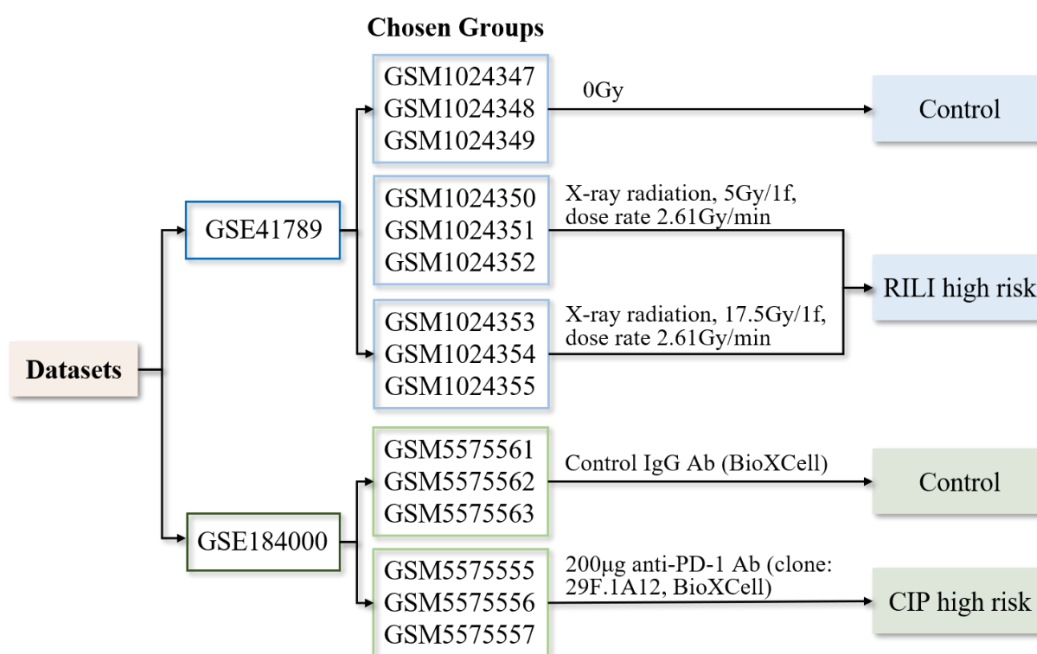


Figure 1. Selected samples of the datasets

2.2. Differential Gene Expression Analysis

In processing GSE41789 datasets using the GEO2R online analysis tool that comes with the GEO database, 0Gy irritated group at week 16 was selected as the control group and 5Gy and 17.5Gy irritated groups were chosen as the experimental groups. The expression profile data in GSE184000 dataset were normalized by the DESeq2 installation package of R software, and the aged mouse control group was used as the control group and the aged mice treated with anti-PD-1 antibody group was selected as the experimental group. T-test and Fold Change were used to screen differentially expressed genes, and genes meeting FDA (adjusted p value) < 0.05 and $|\log_2(\text{Fold Change})| \geq 1$ were set as differentially expressed genes, and the differential gene volcano plot was drawn.

3. Results and Discussion

3.1. Screening of DEGs in RILI High Risk Group and Control Group

By comparing the three groups of randomly selected data sets from GSE41789 database two by two, differentially expressed genes (DEGs) were identified and plotted by Volcano plots. $P < 0.05$ was used as the cut-off point for significant differences. DEGs analysis shows that a total of 46 DEGs were identified in the irradiated group and control group including 18 up-regulated genes and 28 down-regulated genes. Significant DEGs were conservatively defined as fold change ratios $\geq \pm 2$ and $P < 0.05$ after adjustment for false discovery. (Fig. 2).

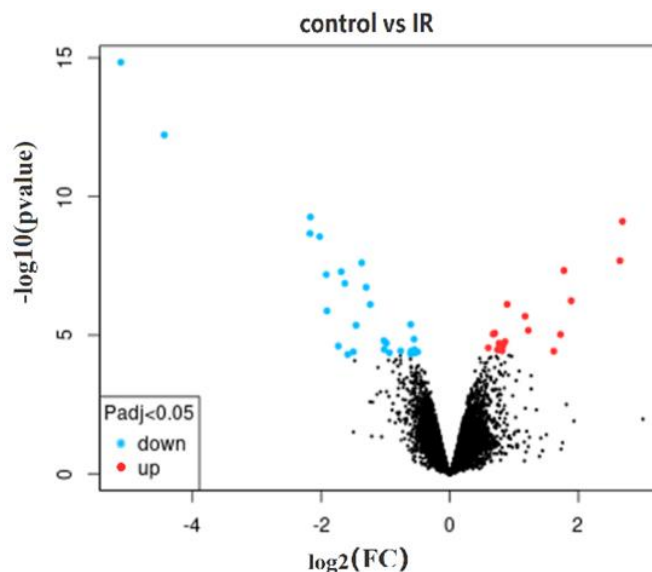


Figure 2. Differentially expressed genes in the groups.

Volcano map of control group (con) vs. irradiated group (IR). Red dots: Significantly upregulated genes. Blue dots: Significantly downregulated genes. Black dots: non-differentially expressed genes. Y coordinate was $|\log_2(\text{fold change})|$ and the X coordinate was $-\log_{10}(\text{p value})$.

3.2. Screening of DEGs in CIP High Risk Group and Control Group

Two groups of data sets were selected from GSE184000, which were Aged PD group and aged control group. Aged mice showed irAE in lung after PD-1 treatment, compared with young mice. Therefore, after PD-1 treatment, aged mice were considered to have a higher risk of CIP. Three sets of DEGs were identified and plotted by Volcano plots (Fig. 3). A total of 746 DEGs were identified in the anti-PD-1 antibody treated set and control set including 446 (59.76%) up-regulated genes and 300 (40.24%) down-regulated genes. Significantly DEGs were conservatively defined as fold change ratios $\geq \pm 2$ and $P < 0.05$ after adjustment for false discovery. (Fig. 3).

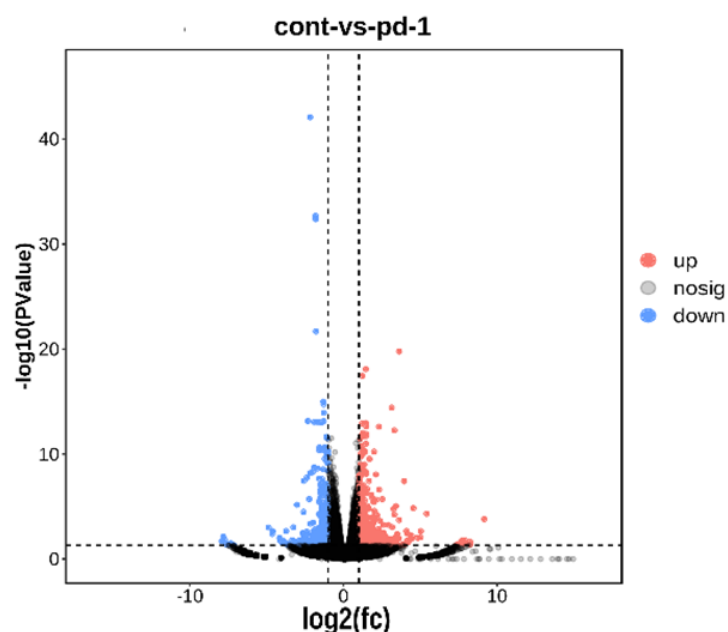


Figure 3. Volcano map of DEGs in anti-PD-1 Ab treated group and control group

Volcano map of control group (cont) vs. anti-PD-1 Ab treated (pd-1). Red dots: Significantly upregulated genes. Blue dots: Significantly downregulated genes. Black dots: non-differentially expressed genes. Y coordinate was $|\log_2(\text{fold change})|$ and the X coordinate was $-\log_{10}(\text{FDR})$.

3.3. Analysis of Genes Expressed in both RILI and CIP

Using Venn diagram to analysis the up-regulated and down-regulated genes in both RILI high risk group and CIP high risk group. Four DEGs in both RILI high risk group and CIP high risk group were identified, these gene symbols were including PER3, DBP, CXCL5 and SPON2 (Fig. 4). These four genes were all involved in protein coding. Suggesting that these factors may be participated in the modulation of both RILI and CIP. The genes' specific information has been listed in table 1.

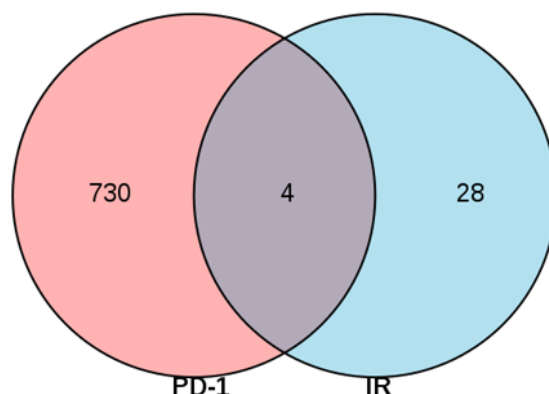


Figure 4. DEGs of both RILI and CIP in Venn diagram

Table 1. DEGs information in both RILI and CIP

Gene symbol	Chr	Start	End	Strand	Gene biotype
SPON2	chr5	33198184	33218455	-	Protein_coding
DBP	chr7	45705088	45710203	+	Protein_coding
CXCL5	chr5	90759360	90761624	+	Protein_coding
PER3	chr4	151003652	151044665	-	Protein_coding

Chr: chromosome number, Start: the starting position of the gene on the chromosome, End: the termination position of the gene on the chromosome, Strand: positive or negative chain.

3.4. Discussion

Radiotherapy and ICIs are important treatment schemes in clinical practice. Their grievous dose limited side effect was RILI and CIP, they could all cause pulmonary inflammation. To make better arrangement of these two great regimens and to predict or distinguish RILI and CIP in an early time can help improve treatment outcome and avoid lung injury for lung cancer patients. In this study, DEGs between RILI high risk group vs control group and CIP high risk group vs control group were evaluated respectively. And genes expressed in both RILI high group and CIP high group were identified, including Period Circadian Regulator 3 (PER3), D-Box Binding PAR BZIP Transcription Factor (DBP), C-X-C Motif Chemokine Ligand 5 (CXCL5) and Spondin 2 (SPON2). Interestingly, the situation of these four genes is not accordance in the two datasets (Table 2).

Table 2. Expression of common DEGs in the two datasets

Gene symbol	GSE184000	GSE41789
SPON2	down	up
CXCL5	down	up
DBP	up	down
PER3	up	down

PER3 and DBP are both mainly involved in regulating circadian rhythm. Previous study has shown that they were significantly rhythmic in both day and night shift schedule. Circadian dysregulation, especially the night shift, can generate endogenous DNA damage and irradiation can induce exogenous DNA damage. PER3 is one of the important DNA repair genes, the abnormally expression

of it could enhance the radiation induced DNA damage. Studies also reported that in NSCLC, PER3 is a therapeutic target and tumor suppressor, related to the prognosis. PER3 is also highly expressed in CD8 T and CD8 T is one of the key targets of ICIs. Research shows that, CD8 T cells can affect the antigens' presentation ability of CD8 T by regulating T cell proliferation and activation through PER3 and other circadian related genes. Report shows that, normal lung tissue PD-L1 expression is associated with circadian. Those critical genes involving in circadian can regulate the tumor microenvironment of lung cancer by affecting the biological clock state. This study found that PER3 was down-regulated in the radiation group and upregulated in the anti-PD-1 Ab treated group, similar with the above research results. In consequence, the over expression of PER3 in lung cancer patients possibly indicating positive therapeutic effect and prognosis of radiotherapy and anti-PD-1 Ab treatment, meanwhile, they may also have a lower risk of lung injury. We can also suggest lung cancer patients to start ICIs treatment before radiotherapy. Detecting the expression of PER3 and DBP during ICIs treatment, and radiotherapy can be processed after they were increased. This treatment pattern may reduce the risk of lung injury (Fig. 5).

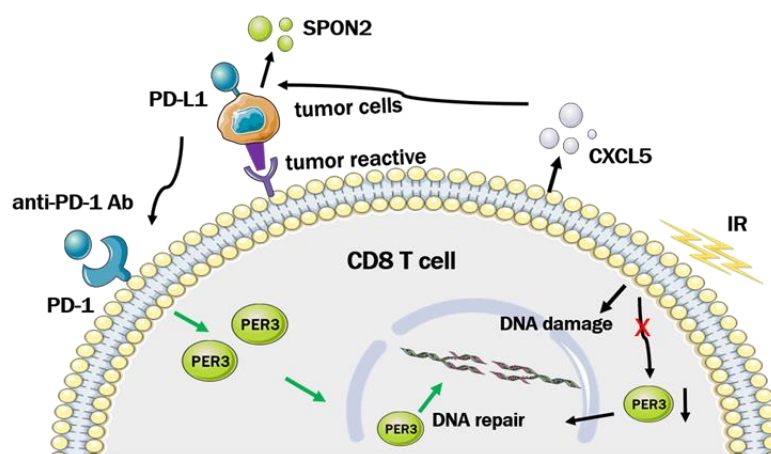


Figure 5. Schematic diagram of the mechanism (Using Power Point)

CXCL5 is related to chemokine activity. According to some reports, CXCL5 could promote PD-L1 expression in cancer, and it is related to the prognosis of ICIs therapy in NSCLC. SPON2 is related to antigen binding and lipopolysaccharide binding. It is a protein secreted by extracellular matrix that participates in and affects the tumor microenvironment. The expression of SPON2 is increased in lung cancer, gastric cancer, liver cancer and other cancers. The present study revealed that after irradiation the expression of CXCL5 and SPON2 could be upregulated, which indicated poor prognosis. CXCL5 promotes cancer progression by promoting PD-L1 expression in cancer cells so that it could not be recognized. According to the results of this study, patients with CXCL5 and SPON2 over-expression should be treated with ICIs at first. Detected the expression level of them after ICIs treatment, if the expression decreased, radiotherapy could then begin.

4. Conclusion

In summary, PER3, DBP, CXCL5 and SPON2 may be potential risk biomarkers for lung cancer patients receiving radiotherapy in combination with ICIs. According to the results of this study, patients with PER3 and DBP down regulated or with CXCL5 and SPON2 over-expression should be treated with ICIs before radiotherapy. Radiotherapy can be processed after PER3 and DBP were increased or CXCL5 and SPON2 decreased. This treatment pattern may improve the therapeutic effect and reduce the risk of lung injury. However, data of the present study were all public datasets, molecular and clinical experiments will be conducted in the next stage to verify the results.

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