The role of GLUT-1 in upregulation of PD-L1 expression after radiotherapy and PD-L1 is associated with a favourable overall survival in hypopharyngeal cancer

Li-Fang Shen (11418276@126.com)
Zhejiang University School of Medicine First Affiliated Hospital https://orcid.org/0000-0001-5884-1345
Shui-Hong Zhou
Zhejiang University School of Medicine First Affiliated Hospital
Yu Guo
Zhejiang University School of Medicine First Affiliated Hospital

Research

Keywords: glucose transporter 1, programmed cell death ligand 1, radiotherapy, prognosis, hypopharyngeal cancer

Posted Date: April 15th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-22536/v1

Abstract

Purpose: Although the alteration of tumor immunity after radiotherapy (RT) has been studied widely in recent years, how radiotherapy mediates tumor immunity and whether glycolysis is involved in the mediation in hypopharyngeal cancer are still unclear. This study aimes to determine whether radiotherapy regulates programmed cell death ligand 1(PD-L1) partly via glucose transporter 1(GLUT-1) expression and whether PD-L1 expression predicts overall survival (OS) in patients with hypopharyngeal cancer.

Methods: PD-L1, Glut-1 expression and C D4+, CD8+ T cell were detected by immunohistochemistry analysis on 47 pre-RT and 25 post-RT specimens of hypopharyngeal cancer. The changes of these indicators before and after radiotherapy were compared, and their association with overall survival of patients were analyzed. Moreover, we used siRNA-GLUT-1 to inhibit GLUT-1 expression and determined whether GLUT-1 was a key factor involved in mediation of PD-L1 expression by RT in vitro.

Results: In multivariate analysis, patients with higher PD-L1 expression (P=0.037), higher CD4+ T cell infiltration (P=0.016) and earlier clinical stage (P=0.019) had favourable OS. The PD-L1 expression and CD4+, CD8+ T cell increased significantly after RT. PD-L1 expression was correlated with Glut-1 in pre-RT (P=0.002), but not after-RT(P=0.051). The PD-L1 expression of FaDu cells was upregulated after RT, especially at 96h after RT in vitro. However, the PD-L1 expression of siRNA-GLUT-1 FaDu cells was significantly decreased at 96h after RT when compared with FaDu cells.

Conclusion: The patients with high PD-L1 expression and CD4+ T cell infiltration might have favourable OS in hypopharyngeal cancer. RT could increase PD-L1 expression and alter tumor immunity, the expression of PD-L1 was correlated with Glut-1, and inhibiting GLUT-1 expression might decrease the expression of PD-L1. GLUT-1 might participate in the alteration of tumor immunity after RT.

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents about 6% of all cancers, and there are approximately 644,000 new cases and 352,000 cancer deaths every year in the world[1]. Despite comprehensive treatments such as surgery, radiotherapy, and chemotherapy, the 5-year survival rate of HNSCC has not significantly improved. Since 1975, 5-year survival of laryngeal cancer has fallen from 67–61%, while oral cavity and pharynx cancer has increased merely from 53–63%[2]. Therefore, there is an urgent need to find more effective treatments. Recently, targeted therapy for programmed cell death 1 (PD-1) and PD-L1 has shown enormous prospects for tumor treatment. Even after traditional therapies have failed, immune checkpoints blockade treatments could increased the progression free survival of advanced stage patients more than two years[3]. Despite encouraging results, many cancer patients have low respond rates to clinical checkpoint blockade, the response to anti-PD-1/PD-L1 therapy is only approximately 18%-25% response rate in HNSCC[4, 5]. To overcome the unsatisfactory respond rates, combining other strategies, including radiotherapy, chemotherapy, other immunotherapy and targeted therapy, has become a commonly used strategy for HNSCC.

Preclinical studies have shown that RT could upregulate PD-L1 expression on tumor cells, RT and anti-PD-L1 therapy had synergistically antitumor effect[6]. In the clinical studies, patients who received RT before anti-PD-L1 therapy had a better prognosis than those who did not receive RT in Lung Cancer[7, 8], mammary cancer[9]. However, the knowledge about PD-L1 upregulation after RT in hypopharyngeal cancer is scarce[10], this has led us to interest in studying the expression of PD-L1 after RT in hypopharyngeal cancer. Moreover, little is known about how RT-mediated immune responses alter the tumor immunity. The alteration of PD-1/PD-L1 expression after RT is dependent on multiple factors such as signaling cascades, general somatic mutation prevalence, individual genetic background, tumor environment, therefore it cannot be generalized. Previous studies have found that tumor glycolysis and tumor immune evasion are interdependent. Glycolytic activity was a stronger predictor for tumor immunity in a number of cancers, glycolysis could increase PD-L1 expression in tumors[11]. Li D et al assessed Glut-1 and hexokinase 2(HK-2) expression in metabolic reprogramming after irradiation. GLUT-1 controls glucose uptake and HK-2 encodes the key kinase involved in glycolysis. They found that after radiation the two genes expression declined while the PD-1 expression elevated in both activated CD4 + and CD8 + populations relative to the unirradiated wild type C57BL/6 male mice[12]. So how irradiation mediate tumor immunity of hypopharyngeal cancer and whether glycolysis is involved in the mediation are need to be studied further.

Moreover, PDL1 expression level has been associated with the therapeutic response and prognosis in diverse cancers. Jiang C et al demonstrated that positive PDL1 expression was associated with a higher survival in patients of esophageal squamous cell carcinoma (ESCC) who underwent RT, they found that the patients with high PDL1 expression had an increased infiltration of Tumor Infiltrating Lymphocytes (TILs), these patients had highly immunogenic tumors prior to RT, it was a independently predictor of favourable prognosis for patients with ESCC[13]. However, the data on the prognostic value of PD-L1 expression in hypopharyngeal cancer remains limited, so it is important to demonstrate the clinical significance of PD-L1 expression in patients with hypopharyngeal cancer.

The present study aimed to focus on the immune related changes of tumor micro-environment after RT in hypopharyngeal cancer and whether RT regulates PD-L1 expression partly via GLUT-1 expression, and to determine the association of PD-L1 expression with OS in patients of hypopharyngeal cancer.

Materials And Methods

Ethics Statement

The study has been approved by the appropriate institutional committee and have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The institutional review board of The First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, Zhejiang, China) approved the present study. Written informed consent was obtained from all individual participants included in the study.

Clinical data

Formalin-fixed paraffin-embedded tissue of 61 patients with locally advanced hypopharyngeal cancer treated with (chemo)radiotherapy and surgery at the First Affiliated hospital, College of Medicine, Zhejiang University between 2008 and 2017 was collected, among them, 14 patients were subsequently excluded due to the unavailable tumor samples or missing clinical data. Excluded criteria: Patients with distant metastasis at diagnosis, patients who received preoperative targeted therapy, immune therapy or chemotherapy, patients with more than one malignancy.

All patient datas were reviewed for the following baseline characteristics: gender, age, primary tumor histologic subtype, tumor location, clinical stages. RT parameters were also recorded, including total dose, dose per fraction, time between RT and sample resection.

Follow-up

OS data for patients were obtained by outpatient service or telephone. OS was determined from the date of diagnosis to the death of patients. A follow-up examination was performed every month during the first year, every three months during the second year, and every six months after the third year. In addition to routine physical examinations, patients underwent laryngoscopy, cervical CT or magnetic resonance imaging (MRI), or whole body PET/CT.

Immunohistochemical analysis and evaluation

we analysed the specimens of pre-RT and the post-RT. The expression of PD-L1, Glut-1, and the numbers of CD4+ and CD8+ T cells in the tumor tissues were detected by immunohistochemistry in 47 cases of hypopharyngeal cancer, Formalin-fixed paraffin-embedded specimens were obtained from the predominant lesions in each subject. Immunohistochemical staining was performed to detect expression of PD-L1(1:100 dilution; catalog no: 66248-1-Ig, Proteintech), Glut-1(dilution, 1:100; catalog no: ab14683; Abcam, Cambridge, UK) on tumor cells, and CD4+ T cells(1:50 dilution; catalog no: DF6451, Affinity), CD8+ T cells(1:200 dilution; catalog no: 66868-1-1, Proteintech) in the tumor specimens. Serial sections (4μm) subjected to immunohistological staining were fixed with 3% H_2O_2, and treated with antigen retrieval solution for 15 min. Monoclonal antibody incubated the sections for 30 min at 36–38°C, followed by secondary antibody (K5007, Dako) incubation for 15 min at 20–25°C. The final reaction product was developed by exposure to 0.03% diaminobenzidine, and the nuclei were counterstained with hematoxylin.

We scored the percentage of tumor cells with PD-L1 positive stained, increasing by 5% increments, and used a semiquantitative scoring method to evaluate PD-L1 expression, high PD-L1 expression was defined at a minimum of 10% of stained cells.

The staining intensity of Glut-1 was classified as no staining, weak, moderate, and strong intensity for 0, 1, 2, or 3 scores, respectively. The percentage of stained cells was classified as follows: 0–25% stained cells for 1 score, 26–50% stained cells for 2 score, 51–75% stained cells for 3 score, and >75% stained cells for 4 score. The Glut-1 expression was assessed semi-quantitatively using the product of these

scores (intensity × percentage of stained cells): 0—5 points = negative expression and 6—12 points = positive expression.

To evaluate CD4+, CD8+ T cells infiltration in tumor, we counted the numbers of CD4+ and CD8+ T cells in a selected hotspot under 400×magnification and selected the median number of CD4+, CD8+ T cells as the cut-off point for CD4+ and CD8+ T cells density.

Two experienced pathologists calculated the staining intensity and the percentage of stained cells independently.

Cell culture and reagents

FaDu cell line was purchased from the Cell Research Institute of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM medium (Sigma, USA), supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin (Gibco, USA) and 10% heat-inactivated FCS, at 37°C in a 5% CO_2 atmosphere.

Sequences of GLUT-1 and PD-L1 entire coding regions were obtained from GenBank, and primers were designed using ClustalX and the Omega 2.0 software. The high-purity total RNA rapid extraction kit was purchased from Generay (Cat No: GK3016, Batch: 1703G01), the reverse transcription kit HiScript-II Q RT SuperMix for qPCR was purchased from Vazyme (Cat No: R222-01, Batch: 7E092G6), PrimeScript^{TM} RT reagent Kit was purchased from TaKaRa (Cat No: RR037A, Batch: AK5302-1), qPCR reagent ChamQ SYBR Color qPCR Master Mix was purchased from Vazyme (Cat No: Q411-02, Batch: 7E092H6). The quantitative PCR instrument was CFX connect Real-Time PCR System. PVDF membrane was purchased from Millipore (Cat No: IPVH00010, Batch: K5JA5013L).

Tumor cell line irradiation

To determine whether GLUT-1 is a key factor involved in the mediation of tumor immune mircoenvironment by radiotherapy, we inhibited GLUT-1 expression using GLUT-1 siRNA. GLUT-1 siRNA was purchased from GenePharma Co. Ltd. (Shanghai, China). The sequences were: sense, 5'-GGAAUUCAAUGCUGAUGAUTT-3'; antisense, 5'-AUCAUCAGCAUUGAAUUCCTT-3'. We performed the GLUT-1-siRNA transfection when the cells reached 50% confluence. The FaDu cell and siRNA-GLUT-1 FaDu cell were both seeded at a density of 10,000–20,000 per 25 cm^2 and were divided into 4 goups respectively: control group, 24h, 48h, 96h group. Tumor cells were subjected to radiation after resting overnight except the control group. RT was taken using an X-ray generator (22.7 mA, 120 kV, variable time; GE Inspection Technologies, Germany) with a single dose of 10 Gray on day 1. The tumor cells of 24h, 48h, 96h group were harvested at 24h, 48h and 96h after the radiotherapy respectively, PD-L1 and GLUT-1 expression on tumor cells were analyzed. The control group tumor cells were harvested and analyzed on day2.

Reverse transcription polymerase chain reaction (RT-PCR)

The PCR primers used were as follows: GLUT-1 sense, 5'-GTCAACACGGCC TTCACTG-3', GLUT-1 antisense, 5'-GGTCATGAGTATGGCACAACC-3' (111 bp), PD-L1 sense, 5'-TTACAGCAGCCAGACGATCA-3', PD-L1 antisense, 5'-CCCTGC AGTAGGTTTCTGCT-3'(233 bp). GAPDH sense, 5'-TGTTGCCATCAATGACCCCTT-3', GAPDH antisense, 5'-CTCCACGACGTACTCAGCG-3' (202bp). The specific steps are the same as described previously[14]. To calculation differential gene expression, the 2-ΔΔCt formula was used.

Western blotting

Tumor cells were lysed in Radio Immunoprecipitation Assay (RIPA) lysis solution and were separated by gel electrophoresis and transferred to membranes. We blocked the membranes with 5% non-fat dry milk in TBST and soaked in the primary antibody buffer at 4°C overnight (PD-L1 1:800 dilution(Proteintech, Chicago, IL, USA, Art No: 66248-1-1g), (GLUT-1 1:800 dilution (Proteintech, Chicago, IL, USA, Art no: 20960-1-AP). We soaked the membranes in secondary antibody buffer and incubated at room temperature for for 2h. Enhanced chemiluminescence was used to visualize the proteins and then the proteins were exposed to X-ray film. Protein expression was analyzed semi-quantitatively using the ChemiDoc XRS+ System (Bio-RAD, USA).

Statistical analysis

SPSS software (ver. 22.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. Categorical variables were assessed by $\chi 2$ or Fisher's exact tests. Correlation analyses were performed using Spearman's rank analysis. Changes in PD-L1, Glut-1 expression and CD4+, CD8+ T cells before and after RT were tested with Student's t-test. The Kaplan-Meier method and log-rank test were used to calculate survival curves and compare the results. The Cox proportional hazards regression model was used for multivariate analysis. P values <0.05 were considered to indicate statistical significance.

Results

PD-L1, Glut-1 expression and CD4+, CD8 + T cells infiltration in hypopharyngeal cancer at baseline (pre-RT).

PD-L1 expressed mainly on membranes (cell surface) and partially in the cytoplasm of tumor cells, and PD-L1 expression exhibited heterogeneity in the specimens, a cutoff of 10% was used to define "low" vs. "high" expression. The percentage of stained tumor cells in the patient population ranged from 3.7–23.1% before RT. 53.2% (25/47) patients were considered with high PD-L1 expression. Glut-1 was expressed on cell membranes in tumor cells, and its expression exhibited positive in 51.1% (24/47) patients. Based on the median number of CD4 + and CD8 + cells respectively, a cutoff of 8% and 25% were used to define "low" vs "high" CD4 + and CD8 + T cells infiltration respectively. The percentage of CD4 + T cells in the tumor tissues ranged from 2.18–28.0% before RT, 46.8% (22/47) patients were considered with high CD4 + T cell infiltration, while the percentage of CD8 + T cells in the tumor tissues ranged from 11.9–48.9% before RT, 40.4% (19/47) patients were considered with high CD8 + T cell infiltration(Fig. 1).

Association between PD-L1, Glut-1 expression, CD4+, CD8 + T cells infiltration and clinicopathological factors in hypopharyngeal cancer

Of the 47 patients with hypopharyngeal cancer, all patients were male. The median age of these patients was 64 years old (range, 4488 years old) at diagnosis. The clinical stage was classified according to the National Comprehensive Cancer Network (NCCN) guidelines, 23 patients were clinical stage III and 24 patients were clinical stage IV. 17 patients underwent surgery and postoperative (chemo)radiotherapy(50-70Gy), 11 of 17 patients received chemotherapy with cis-platinum or nedaplatin. 5 patients only taken radiotherapy following the biopsy, the time interval between the surgery and RT was 30.6 days, While the other 25 patients were given biopsy and adjuvant radiotherapy(30-50Gy), followed by surgery, the time interval between the end of RT and cancer resection was 62.8 days.The follow-up time was 6—59 months (median: 27.5 months). 25(25/47, 53.2%) patients died during the follow-up time. The median OS time was 35.3 months [95% confidence interval (CI), 29.541.0 months]. The other clinicopathological characteristics of patients are summarized in Table 1.

Table 1
Patient characteristics

Patient characteristics	Number
Age >60 years <60 years	31 16
Tumor location Pyriform sinus carcinoma Posterior pharyngeal wall carcinoma Postcricoid area carcinoma	38 6 3
Histologic grade Well Moderate poor	12 26 9
Clinical stage III IV	23 24
Treatment method Surgery+(chemo)radiotherapy Biopsy + Adjuvant Radiotherapy + Surgery Biopsy + Radiotherapy	17 25 5

The expression of PD-L1 was not associated with the clinicopathological factors, including age, clinical stages, histologic grade and the tumor locations of patients. Furthermore, the Glut-1 expression and CD4+, CD8 + T cells in the tumor tissue had no significant association with these clinicopathological factors too.

PDL1 expression, CD4 + T cells infiltration and clinical stages were associated with the OS of patients with hypopharyngeal cancer

The OS of hypopharyngeal cancer patients based on PDL1, Glut-1 expression and CD4+, CD8 + T cells infiltration on baseline (Pre-RT) were assessed.

The OS time of high PDL1 expression patients was 41.9 months (95% CI, 33.650.2 months), whereas the OS time of low PDL1 expression patients was 25.5 months (95% CI, 21.129.9 months). The OS was significantly different between the patients with high and low PDL1 expression as presented by the KaplanMeier analysis (P = 0.0025, Figure. 2A).

The OS time of high Glut-1 expression patients was 37.9 months (95% CI, 35.740.6 months), whereas the OS time of low Glut-1 expression patients was 36.2 months (95% CI, 31.4.241.2 months). The OS of the two groups of patients was not significantly different as assessed by KaplanMeier analysis (P = 0.45).

For patients with high CD4 + T cell, the OS time was 45.8 months (95% CI, 36.655.0 months), whereas for patients with low CD4 + T cell infiltration, the OS time was 26.0 months (95% CI, 21.630.4 months). The OS of high CD4 + T cell patients was significantly longer than those with low CD4 + T cells infiltration (P < 0.001, Figure. 2B).

The OS time of high CD8 + T cell patients was 39.2 months (95% CI, 30.448.1 months), whereas the OS time of low CD8 + T cell patients was 35.7 months (95% CI, 28.1–43.3 months). There was no significant difference between them (P = 0.07, Figure. 2C). However, there was a tendency for high CD8 + T cell patients to have a longer OS compared with low CD8 + T cell patients.

The OS of hypopharyngeal cancer patients with clinical stages III was 43.0 months (95% CI, 34.0-51.9 months) versus 29.7 months (95% CI, 22.5–36.9 months) for the patients with clinical stages IV, Kaplan-Meier curve analysis showed that patients with clinical stages III had a higher OS than patinets with clinical stages IV (P = 0.038; Fig. 2D). However, age (p = 0.929), histologic grade(p = 0.249), tumor location(p = 0.334) showed no correlation with OS of HNSCC patients.

Multivariate analyses of factors affecting OS in hypopharyngeal cancer patients

As presented in Table 2, Multivariate Cox regression hazards analysis demonstrated that low PD-L1 expression (HR, 3.48; 95% CI, 1.111.2; P = 0.037), low CD4 + T cell infiltration(HR, 4.30; 95% CI, 1.3114.7; P = 0.016) and advanced clinical stage (HR, 3.33; 95% CI, 1.229.09; P = 0.019) were independent prognostic factors in hypopharyngeal cancer patients. Patients with low PD-L1 expression, low CD4 + T cell infiltration and advanced clinical stage (stage IV) had poorer survival than patients with high PD-L1 expression, high CD4 + T cell infiltration and early clincial stage (clinical stage III). However, Glut-1 expression (HR, 0.95; 95% CI, 0.35–2.57; P = 0.927) and CD8 + T cell infiltration status (HR, 2.48; 95% CI, 0.72–8.55; P = 0.149) were not significant independent prognostic factors.

Table 2
Multivariate analysis of overall survival in patients with hypopharyngeal cancer

Variable	Overall survival
	Hazard ratio (95% CI) P-value
PD-L1 expression (ref. low)	3.483 (1.080-11.235) 0.037
CD4 + T cell infiltration (ref. low)	4.299 (1.313–14.072) 0.016
CD8 + T cell infiltration (ref. low)	2.483 (0.721–8.550) 0.149
Glut-1 expression (ref. low)	0.955 (0.354–2.574) 0.927
Age(ref. <60 years)	1.772 (0.625–5.022) 0.282
Clinical stage(ref. Stage IV)	3.32 (1.22–9.09) 0.019
Histologic grade(ref.moderate)	0.848 (0.186–3.873) 0.832
Histologic grade(ref.high)	0.883 (0.220–3.541) 0.860
Location (ref. Posterior pharyngeal wall carcinoma)	4.586 (0.369–57.002) 0.236
Location (ref. Postcricoid area carcinoma)	1.739 (0.105–28.866) 0.700

PD-L1 expression and CD4+, CD8 + T cell increased after RT, RT influenced tumor immunity

PD-L1 expression of hypopharyngeal cancer was evaluated in every patient pre-RT and(or) after RT. 47 pre-RT biopsies or surgical specimens and 25 post-RT specimens were available. The PD-L1 expression in tumor cells increased obviously after RT with statistical signifificance. In the pre-RT specimens, the proportion of high PD-L1 expression tumor cells was 11.4% only. It increased obviously after RT reaching 19.8%($p = 0.003$) (Fig. 1B, Fig. 3A).

Glut-1 expression, CD4 + and CD8 + T cell infiltration were also studied in pre-RT and post-RT specimens. CD4 + T cell infiltration in tumor tissues increased obviously after RT with statistical signifificance. for the pre-RT specimens, the proportion of CD4 + T cells was only 8.6%, it increased remarkably after RT reaching 21.0%($p < 0.001$) (Fig. 3B). Meanwhile, CD8 + T cell infiltration in tumor tissues also increased obviously after RT with statistical signifificance. The proportion of CD8 + T cells in pre-RT specimens was only 24.9%, it increased clearly after RT reaching 35.7%($p = 0.006$) (Fig. 3C). However, Glut-1 expression only had an increased tendency in post-RT specimens compared with pre-RT specimens($p = 0.097$).

Furthermore, PD-L1 expression was correlated with Glut-1 expression in pre-RT and had tendency of correlation with the expression of Glut-1 after-RT ($P = 0.002$, $P = 0.051$ respectively) (Fig. 3D, Fig. 3E). However, CD4 + and CD8 + T cell infiltration status were not correlated with the expression of Glut-1 neither in pre-RT nor after-RT.

RT induced PD-L1 upregulation on FaDu cells in vitro, and GLUT-1 might be a key factor of the mechanism

To study the influence of RT on PD-L1 expression, FaDu cells were treated with a single fraction of 10 Gy in vitro. PD-L1 mRNA expression of FaDu cells was upregulated after 10 Gy of RT compared with the control group, the upregulation was most significant at 96 h after RT ($p < 0.001$) (Fig. 4A). The PD-L1 protein expression of FaDu cells was also upregulated after RT, and at 96 h after RT it was significantly higher than the control group($p < 0.001$) (Fig. 4B).

After RT the GLUT-1 mRNA expression of FaDu cells decreased firstly, and then increased. It decreased at 48 h after RT compared with the control group($p < 0.01$), and it increased significantly at 96 h after RT compared with 48 h after RT ($p < 0.001$) (Fig. 4C). The alteration of Glut-1 protein expression was mainly the same as GLUT-1 mRNA expression. Glut-1 protein expression of FaDu cells decreased at 48 h after RT compared with the control group and the 24 h after RT group ($p < 0.01$, $p < 0.05$ respectively), and at 96 h after RT it increased significantly compared with 48 h after RT($p < 0.001$) (Fig. 4D).

we used GLUT-1 siRNA to inhibit GLUT-1 expression (Fig. 5A, 5B). At the siRNA-GLUT-1 FaDu cells, the PD-L1 mRNA and protein expression were also upregulated significantly at 96 h after RT compared with the control group ($p < 0.01$, $P < 0.001$ respectively). More importantly, at 96 h after RT the PD-L1 mRNA and protein expression of siRNA-GLUT-1 FaDu cells was significantly decreased when compared with that of FaDu cells as presented by the two-way analysis of variance ($p < 0.001$, $P < 0.001$ respectively) (Fig. 5C, 5D). This result demonstrated that inhibiting the expression of GLUT-1 could interfere the increasing of PD-L1 expression after RT.

Discussion

In 2016, the FDA approved the application of anti-PD-1 monoclonal antibodies pembrolizumab and nivolumab to the treatment of HNSCC, which opened a new page in HNSCC treatment. Now FDA has approved anti-PD-1/PD-L1 drugs for head and neck cancers and there are many clinical trials worldwide. However, the dark side of this therapy has emerged with the time going, including drug resistance. Therefore, combination therapy have been adopted to improve the efficacy[15].

Diverse studies have examined the expression of the PD-L1 in HNSCC and found that the expression levels of PD-L1 was between 46% and 100% depending on the fixation, staining method and site[16–18], however, there is very little research on hypopharyngeal cancer. In our study, we found that the PD-L1 expressed in 100% patients of hypopharyngeal cancer, the percentage of stained tumor cells in the patient population ranged from 3.7–23.1% before RT.

In our study, PD-L1 expression and CD4 + T cell infiltration in tumor tissue were related with OS of the hypopharyngeal cancer patients. In the Multivariate analysis we found high PD-L1 expression, high CD4 + T cell infiltration and early clinical stage were all independent prognostic factors with favourable OS in hypopharyngeal cancer patients. According to our finding, Fukushima Y et al demonstrated that in

oropharyngeal squamous cell carcinoma, patients with high PD-L1 expression in tumor cells had a favourable outcome than patients with low PD-L1 expression for both progression-free survival (PFS) and OS[19]. Vassilakopoulou M et al showed that TILs density was associated with PD-L1 protein expression on tumor cells in laryngeal squamous cell carcinoma patients. Both high TILs and high PD-L1 expression was independently associated with better OS and disease-free survival in multivariate analysis[20]. Liu YJ et al showed that low PD-L1 expression on tumor cells significantly correlates with local recurrence in EBV-positive nasopharyngeal carcinoma patients after RT[21]. Combined low PD-L1 expression on inflammatory cells and tumor cells is an independent negative prognostic factor for OS in rectal adenocarcinoma [22]. Adversely, Lin YM et al found that high PD-L1 expression was associated with poor outcome and metastasis in Oral Squamous Cell Carcinoma[23]. Furthermore, several clinical reports suggested that a high density of tumor-infiltrating lymphocytes was associated with favorable prognosis in patients with locally advanced non-small cell lung cancer[24], and brease cancer[25].

As mentioned above, high PD-L1 expression was correlated with favourable and unfavourable prognosis in different tumors of different studies. The cause of this may be the use of different cutoffs to determine PD-L1 positivity and the heterogeneity of the clinical and pathological features in diverse tumors. In the future studies, we should develop a standardized protocol with which PD-L1 expression can be evaluated and a larger number of patients should be included in the study.

In addition to cytotoxic effect, RT could induce anti-tumor immune effect[26]. The effects of RT on tumor microenvironment and its interaction with tumor immunity is a complex balance of suppressing and activating signals[27]. Sufficient evidence has shown that RT might enhanced the therapeutic effects of anti-PD-1/PD-L1 agents in HNSCC[28], however, many questions remain regarding how RT affects tumor immunity in hypopharyngeal cancer.

In fact, RT plays a role in the recruitment of T cells in the tumor microenvironment[29] and increases PD-L1 expression in tumors[30]. However, the knowledge about upregulation of PD-L1 expression and TILs recruitment in tumor cell after exposure to RT in HNSCC especially in hypopharyngeal cancer is scarce. Our study demonstrated that PD-L1 expression was increased after RT in hypopharyngeal cancer patients, and in the vitro experiment we found PD-L1 expression was increased after RT at 24,48,96 hours, at 96 h after RT the expression of PD-L1 is the highest. For cytotoxic T lymphocytes, we found the CD4+, CD8 + TILs increased after RT in hypopharyngeal cancer patients, RT could transform the tumor microenvironment. The increase in TILs after RT demonstrated that RT can activate the immune system. Thus, our data suggest that RT induces favorable changes within the tumor microenvironment in hypopharyngeal cancer for additional blockade of PD-1/PD-L1 axis.

Similar to our study, other preclinical studies have demonstrated that RT could upregulate PD-L1 expression on tumor cells, RT and PD-L1 blockade had the synergistically antitumor effect on MC38 colon adenocarcinoma and TUBO mammary carcinoma[30, 31]. Herter-Sprie GS observed that the CD8+/Treg ratio increased 96 hours after RT in Kras-mutant lung cancer[32]. In clinical study, some data also demonstrated that radiotherapy is correlated with an increased PD-L1 expression in rectal

adenocarcinoma[22] and locally advanced esophageal adenocarcinoma[33]. Similar to our results, Keung EZ found that the PD-L1 expression in undifferentiated pleomorphic sarcoma of the extremity and trunk (ET-UPS) increased after radiation and an increase in median number of CD4+, CD8 + T cells after RT was also recognized, moreover, although PD-L1 was not expressed at baseline, positive PD-L1 expression was observed in 21% (3/14) of ET-UPS tumor cells after RT[34].

Oweida A et al demonstrated that RT could transform the immune landscape of tumors and render poorly immunogenic murine orthotopic HNSCC sensitive to anti-PD-L1 drugs[35]. There were indeed a number of reports in the preclinical and clinical studies to show that radiotherapy and immunotherapy have the synergistic anti-tumor effect in other tumors[6, 30, 36].

Therefore, the changes in the immune microenvironment after radiotherapy make the tumor more sensitive to immunotherapy, but the mechanism of how radiotherapy interferes with the tumor immune microenvironment is still unclear and needs further studies.

The alteration of PD-1/PD-L1 expression after RT is dependent on multiple factors such as the signaling cascades, individual genetic background, general somatic mutation prevalence, tumor mircoenvironment and it cannot be generalized. Radiotherapy kills tumor cells by free radical-induced DNA damage, and also promotes metabolic changes in tumor cells, ie, metabolic reprogramming. Metabolic reprogramming means the activity and expression of enzymes and their regulators involved in the metabolic activities of tumor cells altered, involving multiple metabolic pathways, the most important is the glycolysis pathway. Aerobic glycolysis and immune escape are two major features of tumors[37]. The dependence of immune cell proliferation and activity on cell metabolism has received increasing attention[38].

GLUT is an important energy transporter that mediates the Warburg effect. The glucose transporter is a protein that mediates the transmembrane transport of glucose, which is a major reason for the increased glucose metabolism seen in malignant tumor cells. GLUT-1 is a representative protein of the GLUT family and is widely expressed in cells of many body tissues. Our study demonstrated that Glut-1 expression increased in post-RT specimens compared with pre-RT, but not significantly, and the expression of Glut-1 was correlated with the expression of PD-L1 in pre-RT, after RT Glut-1 expression had a tendency of correlation with PD-L1 expression. Furthermore, in our vitro experiment we detected a significant decline in Glut-1 expression 48 hours after RT, and it began to increase significantly at 96 hours after RT. PD-L1 expression increased at 96 h after RT, however, when the GLUT-1 expression was inhibited, the PD-L1 expression of siRNA-GLUT-1 FaDu cells significantly decreased at 96 h after RT compared with the FaDu cells. This result demonstrated that inhibiting the expression of GLUT-1 could interfere the increasing of PD-L1 expression after RT. Therefore, we speculate that changes in PD-L1 expression after RT are related to tumor glucose metabolism, ie, metabolic reprogramming after RT.

There are currently few researches on the interaction between glucose metabolism and tumor immunity after tumor radiotherapy. Li D et al analyzed the checkpoint receptors expression levels on T cell populations at multiple post-RT time points ranging from 1 to 4 weeks in mice receiving a single fraction of 1 or 4 Gy. Their results showed that RT resulted in significant increased expression of PD-1 in both

CD8 + and CD4 + populations. They also studied the metabolic reprogramming parameters and found that the expression of Glut1 and HK-2 decreased in activated T cells after RT compared with unirradiated controls[12]. Li HH et al also found that RT influence T cell activation via metabolic reprogramming[39].

Previous studies showed that tumor immune evasion and tumor glycolysis are interdependent[40, 41]. Metabolic reprogramming of tumor cells is an important biochemical basis for tumor immune escape. Enhanced tumor glycolysis attenuates the clearance of tumor cells by immune cells[41]. It is increasingly appreciated that immune cell proliferation and function is dependent on cellular metabolism[38]. A recent study reported that PD-L1 expression is regulated by GLUT-1 in clear cell renal cell carcinoma[42], in pulmonary pleomorphic carcinoma [43]. Chang CH et al found that the metabolic competition between tumor cells and immune cells may induce to tumor immunosuppression, the competition for glucose in tumor microenvironment could drive cancer progression, it would occur when tumors surpass T-cells for glucose supply, impeded their IFN-γ production, which is critical for anti-tumor activity [40].

Glycolytic activity was a more consistent and stronger predictor for immune signatures in a number of cancers. Glycolytic activity increases anti-PD-1/PD-L1 immunotherapy effects via enhancing PD-L1 expression on tumor cells. Thus, glycolytic activity of the tumor cells could be a predictive factor for immunotherapy response in diverse cancers[11]. Many metabolic mechanisms are thought to be related to tumor immune escape and could serve as co-targets in immunotherapy[44].

Therefore, we hypothesized that RT can change the tumor immunity, increase PD-L1 expression and content of CD4+, CD8 + T cells in hypopharyngeal cancer, the mechanism may be that RT interferes with cell glycolysis, alleviates competition for glucose with T cells, metabolic reprogramming after radiotherapy is one of the causes of tumor immune changes. But this still needs further research.

The present study had some limitations. Firstly, this was a single-institution retrospective study with a small sample size, not a trial-based correlative study, there was bias due to the small sample size and its retrospective design. The second limitation is that PD-L1 immunohistochemistry was conducted using only one cut-off value and one antibody, the cutoffs used for high PDL1expression in the present study was inconsistent with some other studies, and there was no unified standard for PDL1 expression positivity. Finally, as no standardized PDL1 immunohistochemistry assay is available currently, caution should be taken in the interpretation of these results.

Conclusion

The present study demonstrates for the first time that high PDL1 expression and CD4 + T cell infiltration were associated with a favorable OS in hypopharyngeal cancer patients, RT could increase the PD-L1 expression and CD4+, CD8 + T cell infiltration level. Our data thus suggest that RT induces favorable changes within the tumor microenvironment in hypopharyngeal cancer for additional blockade of PD-L1. Furthermore, PD-L1 expression was associated with the Glut-1 expression, and in vitro experiment, inhibiting GLUT-1 expression could interfere the increasing of PD-L1 expression after RT, so we speculate that RT interferes with tumor cell glycolysis, alleviates competition for glucose with T cells, GLUT-1 is a

key factor involved in the upregulated PD-L1 expression after radiotherapy. But this still needs further research.

Abbreviations

RT, radiotherapy; PD-L1, programmed cell death ligand 1; PD-1, programmed cell death 1; GLUT-1, glucose transporter 1; OS, overall survival; HNSCC, Head and neck squamous cell carcinoma; HK-2, hexokinase 2; ESCC, esophageal squamous cell carcinoma; TILs, Tumor Infiltrating Lymphocytes; MRI, magnetic resonance imaging; RT-PCR, Reverse transcription polymerase chain reaction; RIPA, Radio Immunoprecipitation Assay; NCCN, National Comprehensive Cancer Network.

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The institutional review board of The First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, Zhejiang, China) approved the present study. Informed consent was obtained from all individual participants included in the study.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no conflict of interest.

Funding

This study was supported by the National Natural Science Foundation of China (No. 81372903 and 81172562).

Authors' contributions

Shui-Hong Zhou and Yu Guo analyzed and interpreted the patient data regarding hypopharyngeal cancer. Li-Fang Shen performed the histological examination of the hypopharyngeal cancer, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We acknowledge everyone who assisted in this research.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin. 2019;69(1):7–34.
2. Miller KD, Siegel RL, Lin CC, Mariotto AB, Kramer JL, Rowland JH, et al. Cancer treatment and survivorship statistics, 2016. CA Cancer J Clin. 2016;66(4):271–89.
3. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. Nature. 2011;480(7378):480–9.
4. Ferris RL, Blumenschein G Jr, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab for Recurrent Squamous-Cell Carcinoma of the Head and Neck. N Engl J Med. 2016;375(19):1856–67.
5. Chang YL, Yang CY, Huang YL, Wu CT, Yang PC. High PD-L1 expression is associated with stage IV disease and poorer overall survival in 186 cases of small cell lung cancers. Oncotarget. 2017;8(11):18021–30.
6. Filatenkov A, Baker J, Mueller AM, Kenkel J, Ahn GO, Dutt S, et al. Ablative Tumor Radiation Can Change the Tumor Immune Cell Microenvironment to Induce Durable Complete Remissions. Clin Cancer Res. 2015;21(16):3727–39.
7. Takamori S, Toyokawa G, Takada K, Shoji F, Okamoto T, Maehara Y. Combination Therapy of Radiotherapy and Anti-PD-1/PD-L1 Treatment in Non-Small-cell Lung Cancer: A Mini-review. Clin Lung Cancer. 2018;19(1):12–6.
8. Schoenhals JE, Seyedin SN, Tang C, Cortez MA, Niknam S, Tsouko E, et al. Preclinical Rationale and Clinical Considerations for Radiotherapy Plus Immunotherapy: Going Beyond Local Control. Cancer J. 2016;22(2):130–7.
9. Verbrugge I, Hagekyriakou J, Sharp LL, Galli M, West A, McLaughlin NM, et al. Radiotherapy increases the permissiveness of established mammary tumors to rejection by immunomodulatory antibodies. Cancer Res. 2012;72(13):3163–74.
10. Ling DC, Bakkenist CJ, Ferris RL, Clump DA. Role of Immunotherapy in Head and Neck Cancer. Semin Radiat Oncol. 2018;28(1):12–6.
11. Jiang Z, Liu Z, Li M, Chen C, Wang X. Increased glycolysis correlates with elevated immune activity in tumor immune microenvironment. EBioMedicine. 2019;42:431–42.
12. Li D, Chen R, Wang YW, Fornace AJ Jr, Li HH. Prior irradiation results in elevated programmed cell death protein 1 (PD-1) in T cells. Int J Radiat Biol. 2018;94(5):488–94.
13. Jiang C, Zhu Y, Tang S, Zhang G, Lin Q, Xu Y, et al. High PD-L1 expression is associated with a favorable prognosis in patients with esophageal squamous cell carcinoma undergoing postoperative adjuvant radiotherapy. Oncol Lett. 2019;17(2):1626–34.

14. Shen LF, Zhao X, Zhou SH, Lu ZJ, Zhao K, Fan J, et al. In vivo evaluation of the effects of simultaneous inhibition of GLUT-1 and HIF-1alpha by antisense oligodeoxynucleotides on the radiosensitivity of laryngeal carcinoma using micro 18F-FDG PET/CT. Oncotarget. 2017;8(21):34709–26.
15. Yang B, Liu T, Qu Y, Liu H, Zheng SG, Cheng B, et al. Progresses and Perspectives of Anti-PD-1/PD-L1 Antibody Therapy in Head and Neck Cancers. Front Oncol. 2018;8:563.
16. Ukpo OC, Thorstad WL, Lewis JS. Jr. B7-H1 expression model for immune evasion in human papillomavirus-related oropharyngeal squamous cell carcinoma. Head Neck Pathol. 2013;7(2):113–21.
17. Hsu MC, Hsiao JR, Chang KC, Wu YH, Su IJ, Jin YT, et al. Increase of programmed death-1-expressing intratumoral CD8 T cells predicts a poor prognosis for nasopharyngeal carcinoma. Mod Pathol. 2010;23(10):1393–403.
18. Zandberg DP, Strome SE. The role of the PD-L1:PD-1 pathway in squamous cell carcinoma of the head and neck. Oral Oncol. 2014;50(7):627–32.
19. Fukushima Y, Someya M, Nakata K, Hori M, Kitagawa M, Hasegawa T, et al. Influence of PD-L1 expression in immune cells on the response to radiation therapy in patients with oropharyngeal squamous cell carcinoma. Radiother Oncol. 2018;129(2):409–14.
20. Vassilakopoulou M, Avgeris M, Velcheti V, Kotoula V, Rampias T, Chatzopoulos K, et al. Evaluation of PD-L1 Expression and Associated Tumor-Infiltrating Lymphocytes in Laryngeal Squamous Cell Carcinoma. Clin Cancer Res. 2016;22(3):704–13.
21. Liu YJ, Tsang NM, Hsueh C, Yeh CJ, Ueng SH, Wang TH, et al. Low PD-L1 Expression Strongly Correlates with Local Recurrence in Epstein-Barr Virus-Positive Nasopharyngeal Carcinoma after Radiation-Based Therapy. Cancers (Basel). 2018;10(10).
22. Hecht M, Buttner-Herold M, Erlenbach-Wunsch K, Haderlein M, Croner R, Grutzmann R, et al. PD-L1 is upregulated by radiochemotherapy in rectal adenocarcinoma patients and associated with a favourable prognosis. Eur J Cancer. 2016;65:52–60.
23. Lin YM, Sung WW, Hsieh MJ, Tsai SC, Lai HW, Yang SM, et al. High PD-L1 Expression Correlates with Metastasis and Poor Prognosis in Oral Squamous Cell Carcinoma. PLoS One. 2015;10(11):e0142656.
24. Tokito T, Azuma K, Kawahara A, Ishii H, Yamada K, Matsuo N, et al. Predictive relevance of PD-L1 expression combined with CD8 + TIL density in stage III non-small cell lung cancer patients receiving concurrent chemoradiotherapy. Eur J Cancer. 2016;55:7–14.
25. Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, et al. Tumor-infiltrating CD8 + lymphocytes predict clinical outcome in breast cancer. J Clin Oncol. 2011;29(15):1949–55.
26. Twyman-Saint Victor C, Rech AJ, Maity A, Rengan R, Pauken KE, Stelekati E, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. Nature. 2015;520(7547):373–7.

27. Formenti SC, Demaria S. Combining radiotherapy and cancer immunotherapy: a paradigm shift. J Natl Cancer Inst. 2013;105(4):256–65.
28. Kikuchi M, Clump DA, Srivastava RM, Sun L, Zeng D, Diaz-Perez JA, et al. Preclinical immunoPET/CT imaging using Zr-89-labeled anti-PD-L1 monoclonal antibody for assessing radiation-induced PD-L1 upregulation in head and neck cancer and melanoma. Oncoimmunology. 2017;6(7):e1329071.
29. Wu CY, Yang LH, Yang HY, Knoff J, Peng S, Lin YH, et al. Enhanced cancer radiotherapy through immunosuppressive stromal cell destruction in tumors. Clin Cancer Res. 2014;20(3):644–57.
30. Deng L, Liang H, Burnette B, Beckett M, Darga T, Weichselbaum RR, et al. Irradiation and anti-PD-L1 treatment synergistically promote antitumor immunity in mice. J Clin Invest. 2014;124(2):687–95.
31. Dovedi SJ, Cheadle EJ, Popple AL, Poon E, Morrow M, Stewart R, et al. Fractionated Radiation Therapy Stimulates Antitumor Immunity Mediated by Both Resident and Infiltrating Polyclonal T-cell Populations when Combined with PD-1 Blockade. Clin Cancer Res. 2017;23(18):5514–26.
32. Herter-Sprie GS, Koyama S, Korideck H, Hai J, Deng J, Li YY, et al. Synergy of radiotherapy and PD-1 blockade in Kras-mutant lung cancer. JCI Insight. 2016;1(9):e87415.
33. Kelly RJ, Zaidi AH, Smith MA, Omstead AN, Kosovec JE, Matsui D, et al. The Dynamic and Transient Immune Microenvironment in Locally Advanced Esophageal Adenocarcinoma Post Chemoradiation. Ann Surg. 2018;268(6):992–9.
34. Keung EZ, Tsai JW, Ali AM, Cormier JN, Bishop AJ, Guadagnolo BA, et al. Analysis of the immune infiltrate in undifferentiated pleomorphic sarcoma of the extremity and trunk in response to radiotherapy: Rationale for combination neoadjuvant immune checkpoint inhibition and radiotherapy. Oncoimmunology. 2018;7(2):e1385689.
35. Oweida A, Lennon S, Calame D, Korpela S, Bhatia S, Sharma J, et al. Ionizing radiation sensitizes tumors to PD-L1 immune checkpoint blockade in orthotopic murine head and neck squamous cell carcinoma. Oncoimmunology. 2017;6(10):e1356153.
36. Tang C, Wang X, Soh H, Seyedin S, Cortez MA, Krishnan S, et al. Combining radiation and immunotherapy: a new systemic therapy for solid tumors? Cancer Immunol Res. 2014;2(9):831–8.
37. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74.
38. Sabharwal SS, Rosen DB, Grein J, Tedesco D, Joyce-Shaikh B, Ueda R, et al. GITR Agonism Enhances Cellular Metabolism to Support CD8(+) T-cell Proliferation and Effector Cytokine Production in a Mouse Tumor Model. Cancer Immunol Res. 2018;6(10):1199–211.
39. Li HH, Wang YW, Chen R, Zhou B, Ashwell JD, Fornace AJ. Jr. Ionizing Radiation Impairs T Cell Activation by Affecting Metabolic Reprogramming. Int J Biol Sci. 2015;11(7):726–36.
40. Chang CH, Qiu J, O'Sullivan D, Buck MD, Noguchi T, Curtis JD, et al. Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. Cell. 2015;162(6):1229–41.
41. Cascone T, McKenzie JA, Mbofung RM, Punt S, Wang Z, Xu C, et al. Increased Tumor Glycolysis Characterizes Immune Resistance to Adoptive T Cell Therapy. Cell Metab. 2018;27(5):977–87.

42. Ruf M, Moch H, Schraml P. PD-L1 expression is regulated by hypoxia inducible factor in clear cell renal cell carcinoma. Int J Cancer. 2016;139(2):396–403.
43. Chang YL, Yang CY, Lin MW, Wu CT, Yang PC. High co-expression of PD-L1 and HIF-1alpha correlates with tumour necrosis in pulmonary pleomorphic carcinoma. Eur J Cancer. 2016;60:125–35.
44. Khalil DN, Smith EL, Brentjens RJ, Wolchok JD. The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. Nat Rev Clin Oncol. 2016;13(5):273–90.

Figures

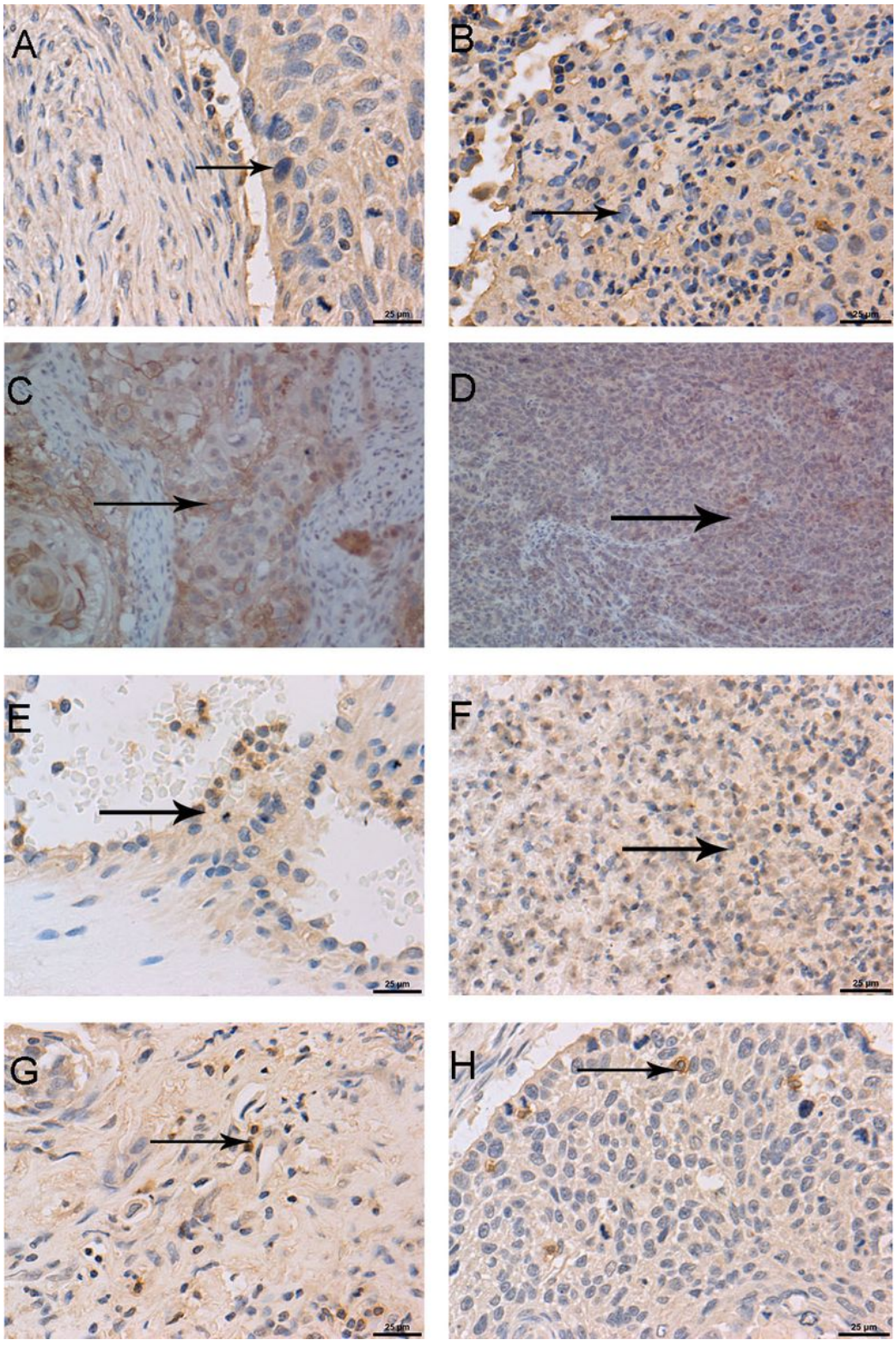

Figure 1

PD-L1, Glut-1 expression and CD4+, CD8+ T cells infiltration (the black arrows point) in HNSCC at baseline (pre-RT) and after RT (magnification x40). Positive expression of PD L1 pre-RT (A) and after RT (B); Positive expression of Glut 1 pre-RT (C) and after RT (D); CD4+ T cells infiltration in tumor tissues pre-RT (E) and after RT (F); CD8+ T cells infiltration in tumor tissues pre-RT (G) and after RT (H).

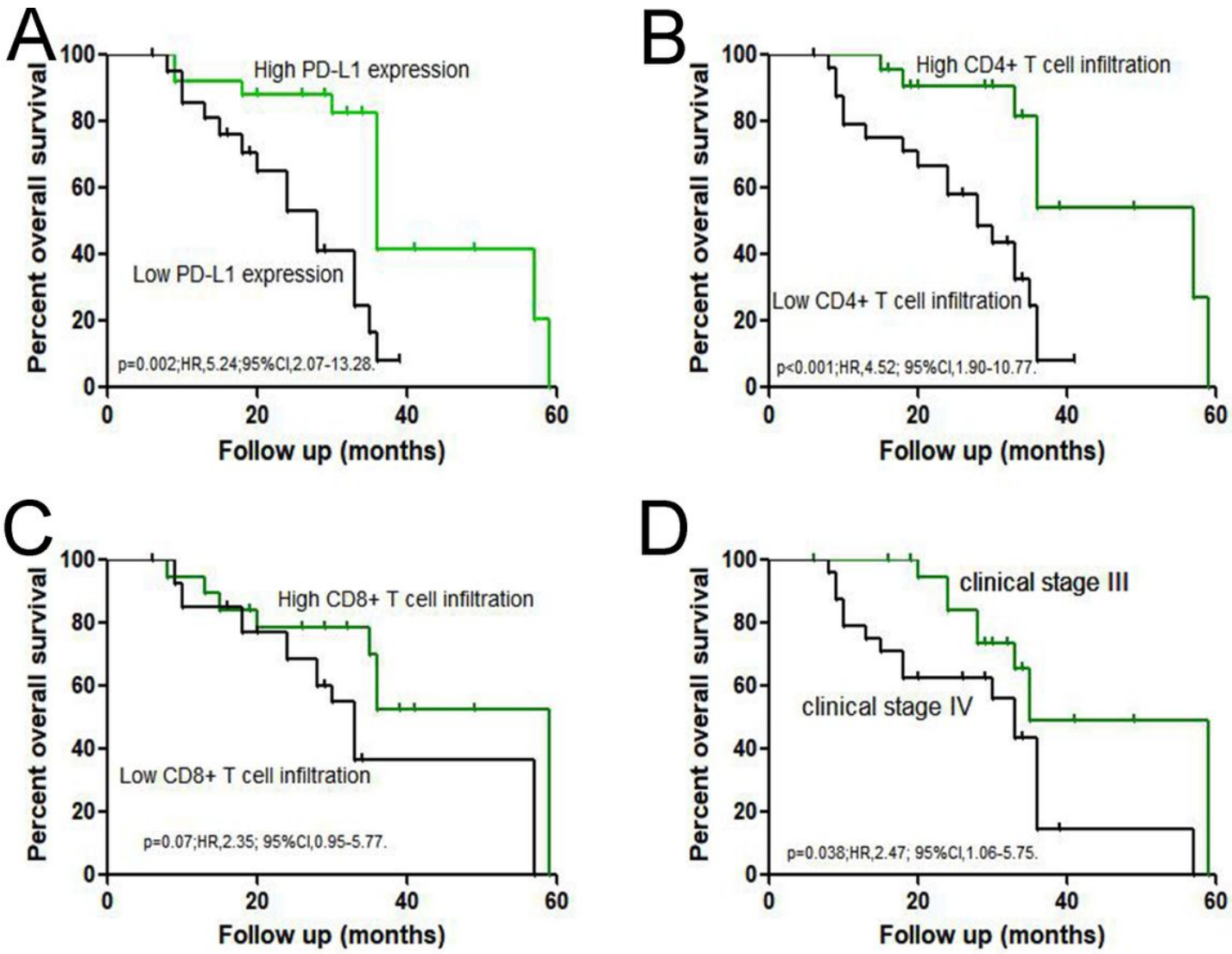

Figure 2

The survival analysis in patients with HNSCC. A: The survival analysis in patients with high PD-L1 expression and low PD-L1 expression; B: The survival analysis in patients with high CD4+ T cell infiltration and low CD4+ T cell infiltration; C: The survival analysis in patients with high CD8+ T cell infiltration and low CD8+ T cell infiltration; D: The survival analysis in patients of clinical stage III and clinical stage IV.

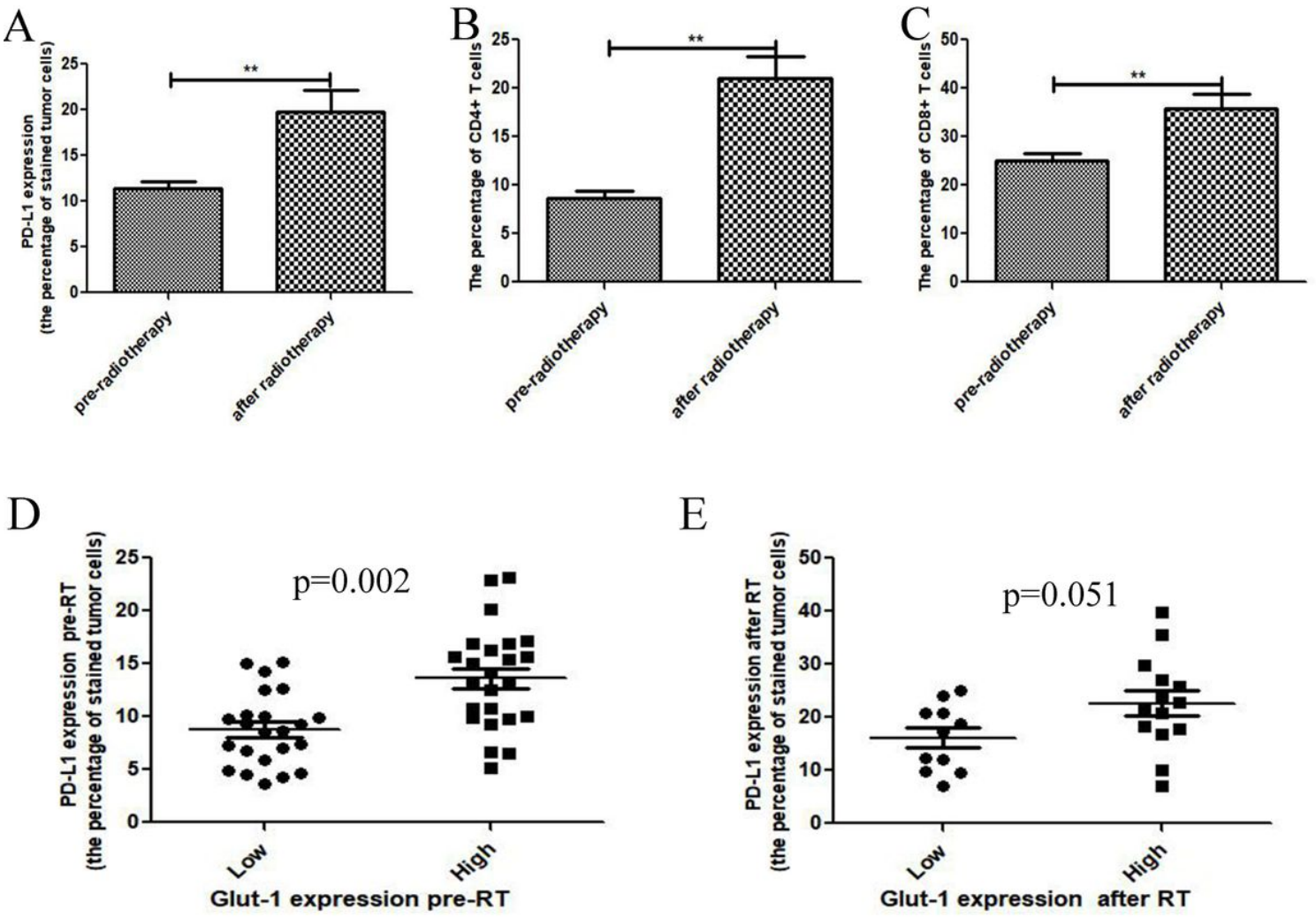

Figure 3

The alteration of PD-L1, CD4+ and CD8+ T cells after RT compared with pre-RT. A: The PD-L1 expression in tumor cells increased significantly after RT compared with pre-RT; B: CD4+ T cell infiltration increased after RT obviously; C: CD8+ T cell infiltration increased after RT obviously; D: The expression of PD-L1 was correlated with the expression of Glut-1 in pre-RT; E: PD-L1 expression had tendency of correlation with the expression of Glut-1 after-RT(**p<0.01).

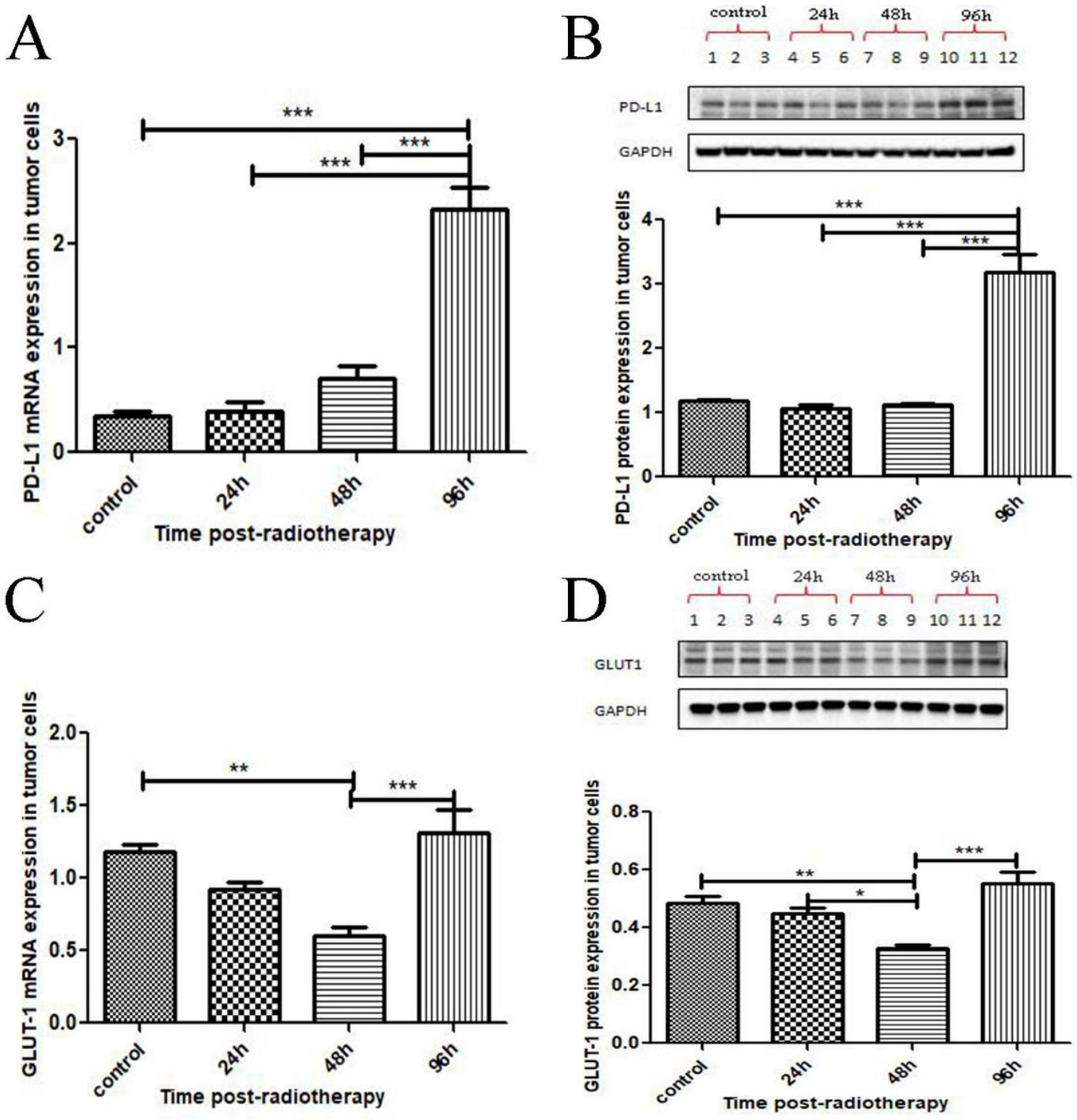

Figure 4

PD-L1 and GLUT-1 expression before and after radiotherapy. A: PD-L1 mRNA expression of FaDu cells was upregulated significantly at 96h after RT compared with the control group($p<0.001$); B: PD-L1 protein expression was also upregulated significantly at 96h after RT($p<0.001$); C: GLUT-1 mRNA expression decreased at 48h after RT compared with the control group($p<0.01$), and it increased significantly at 96h after RT compared with 48h after RT ($p<0.001$); D: Glut-1 protein expression decreased at 48h after RT

compared with the control group and the 24h after RT group ($p<0.01$, $p<0.05$ respectively), and at 96h after RT it increased significantly compared with 48h after RT($p<0.001$). (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

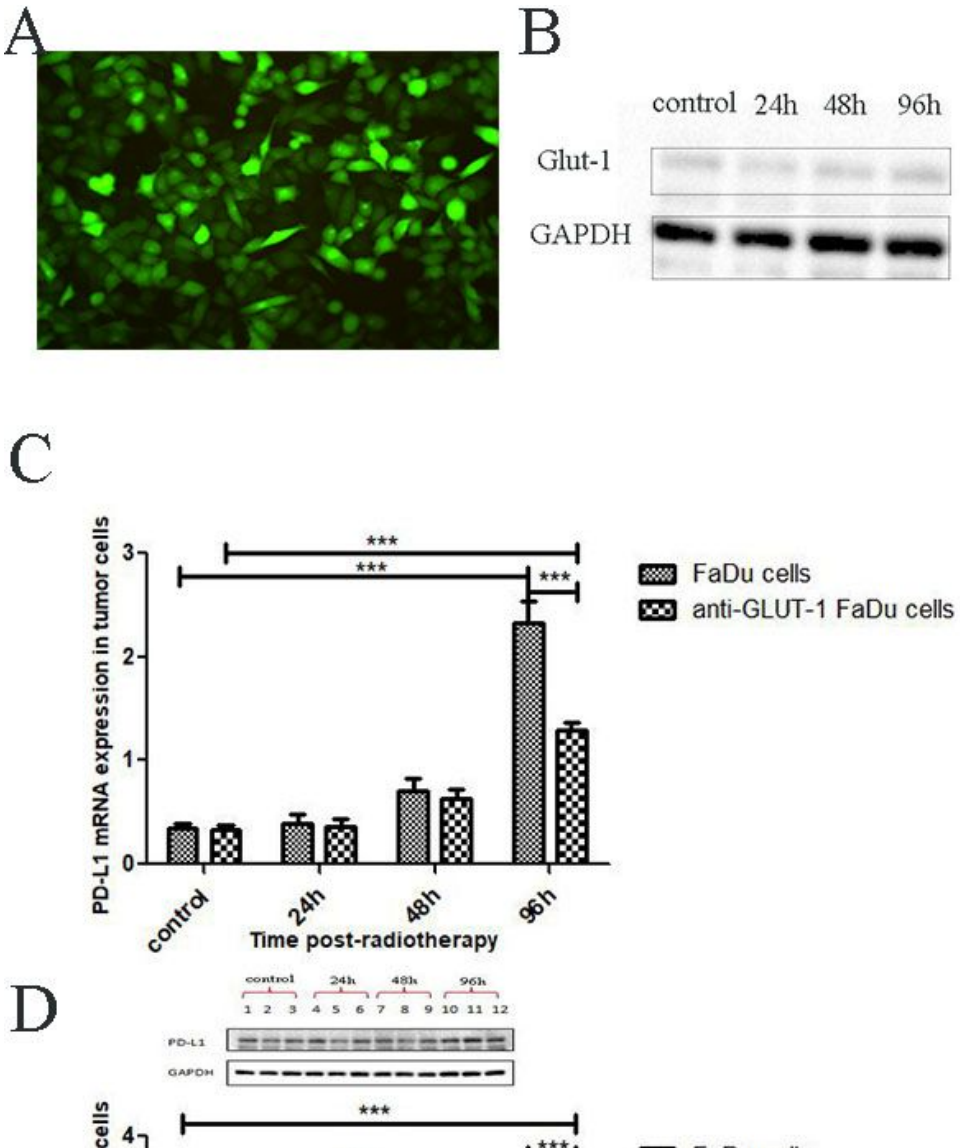

Figure 5

The PD-L1 expression before and after RT in siRNA-GLUT-1 FaDu cells. A：Transfection efficiency of GLUT-1 siRNA by fluorescence; B: The Glut-1 protein expression of siRNA-GLUT-1 FaDu cells before and after RT; C,D: PD-L1 mRNA and protein expression of siRNA-GLUT-1 FaDu cells at 96h after RT was

significantly decreased when compared with that of FaDu cells as presented by the two-way analysis of variance ($p<0.001$, $P<0.001$ respectively). (***$p<0.001$).