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Research Paper

Radiotherapy Upregulates Programmed Death Ligand-1 through the Pathways Downstream of Epidermal Growth Factor Receptor in Glioma

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ABSTRACT

Background: In the present study, we aimed to investigate the role of epidermal growth factor receptor (EGFR) pathway in the up-regulation of programmed death ligand-1 (PD-L1) caused by radiotherapy (RT).
Materials and Methods: Tissue microarrays (TMA) consisting of glioma cancer specimens from 64 patients were used to examine the correlation between PD-L1 and EGFR levels. Furthermore, we performed in vitro experiments to assess the role of EGFR pathway in RT-upregulated PD-L1 expression using human glioma cell lines U87 and U251.
Results: Our data demonstrated that the PD-L1 expression was significantly correlated with EGFR expression in glioma specimens ($\chi^2 = 5.00$, $P = 0.025$). The expressions of PD-L1 at the protein and mRNA levels were both significantly up-regulated by RT ($P < 0.05$). The expressions of phosphorylated EGFR and janus kinase 2 (JAK2) were also induced by RT ($P < 0.05$). Besides, inhibition of EGFR pathway could abrogate the RT-triggered PD-L1 up-regulation ($P > 0.05$). The combination of RT with EGFR inhibitor exhibited the same effect on antitumor immune response compared with the combination of RT with PD-L1 neutralizing antibody (Ab).
Conclusions: RT could up-regulate the PD-L1 expression through the pathways downstream of EGFR in glioma.

1. Introduction

Malignant glioma is a highly lethal and common central nervous system tumor with poor 5-year survival rate (Wu et al., 2015). According to the histological classification, glioma can be divided into three types as follows: anaplastic oligodendroglioma, anaplastic astrocytoma and glioblastoma (Wang and Jiang, 2013). At the moment, the main therapeutic strategy for glioma is multimodal therapy, which consists of radiotherapy (RT), surgical resection and systemic treatment with alkylating agents (Stupp et al., 2005; Wen and Kesari, 2008; DeAngelis, 2005). Previous studies have shown that radiotherapy can improve the median survival of glioma patients from 6 months to 1 year (Walker et al., 1978; Stupp et al., 2005). However, it is still urgently necessary to develop more effective treatments since most patients with glioma will eventually die of disease relapse (Vatner et al., 2014).

In recent years, immunotherapy targeting inhibitory checkpoint molecules has become a new treatment strategy for glioma (Song et al., 2016). Programmed death ligand-1 (PD-L1) is a representative inhibitory checkpoint, which is expressed in many types of cancers (Pardoll, 2012). When PD-L1 binds to its receptor named programmed death-1 (PD-1) which is expressed in $CD8^+$ cytotoxic T lymphocytes (CTLs), the function of activated CTLs is suppressed (Jie et al., 2013; Pardoll, 2012). Indeed, blocking the PD-L1/PD-1 pathway using antibodies could reduce the inhibition effect on the activated CTLs. Researchers have found that patients with high PD-L1 expression have better treatment response (Topalian et al., 2012b; Brahmer et al., 2012; Topalian et al., 2012a; Taube et al., 2014).

Benavente et al. have found that the PD-L1 expression in tumor cells is regulated by two major mechanisms (Concha-Benavente et al., 2016). First, an 'extrinsic' mechanism relies on interferon gamma (IFN-γ) produced by natural killer (NK) cells and $CD8^+$ CTLs, in which IFN-γ not only activates the antitumor cellular immune response but also in turn induces PD-L1 expression in tumor cells. Second, an 'intrinsic' mechanism independent of IFN-γ exists, in which epidermal growth factor receptor/janus kinase 2 (EGFR/JAK2) signaling pathways within the tumor cells lead to PD-L1 over-expression. A previous study has

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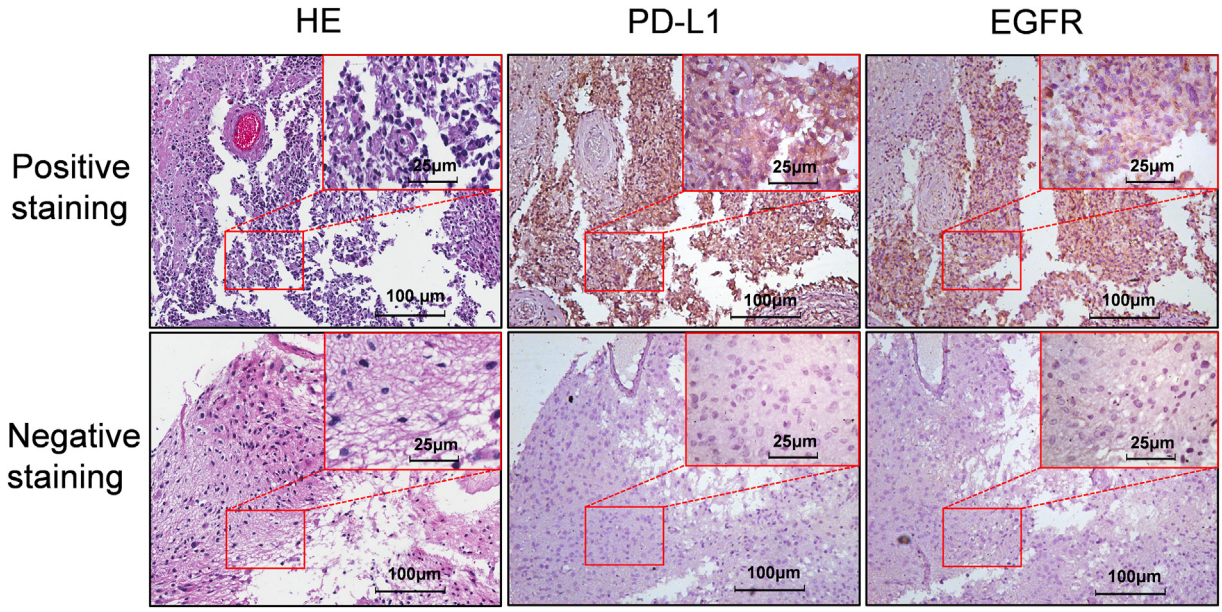

Fig. 1. IHC staining for PD-L1 and EGFR in glioma specimens. Representative images of HE staining, positive PD-L1 and EGFR staining on slides from a selected tumor specimen, and representative HE staining, negative PD-L1 and EGFR staining on slides from another tumor specimen, are shown. Three consecutive slices from the same patient were stained, and one was chosen as representative image for shown.

confirmed that radiotherapy can induce PD-L1 expression in tumor cells through the 'extrinsic' mechanism, but the effect of 'intrinsic' mechanism in this process remains ambiguous (Wang et al., 2017). Park et al. have found that radiotherapy can up-regulate the expression of phosphorylated EGFR to activate pathways downstream in glioblastoma (Park et al., 2006). Therefore, we hypothesized that the 'intrinsic' mechanism, especially pathways downstream of EGFR, also played an important role in up-regulating PD-L1 expression by radiotherapy in glioma. Moreover, we further investigated the effects of EGFR and PD-L1 inhibitors on tumor immune response in irradiated glioma cells.

2. Materials and Methods

2.1. Cell Culture and Reagents

Human glioma cell lines (U251 and U87) were obtained from the Shanghai Institutes of Biological Sciences Cell Bank and maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2.

Antibodies (Ab) against EGFR, JAK2, PD-L1 and beta-tubulin as well as phosphorylated forms of EGFR (Y1173) and JAK2 (Y1007 + Y1008) were purchased from Abcam (UK). Specific inhibitor of EGFR (AG490) was also supplied from Abcam. The PD-L1-blocking antibody (avelumab) was purchased from EMD Serono (Germany). Bound antibodies were detected with horseradish peroxidase-linked antibody against mouse (Abcam) or rabbit (Santa Cruz Technology, USA) immunoglobulin G, followed by enhanced chemiluminescence (ECL) detection (Amersham, USA). Lymphocyte separation medium and the CellTrace™ CFSE kit were purchased from Sigma (USA). AntiCD3/CD28 stimulation beads were purchased from Thermo Fisher Scientific Pierce (USA). $CD8^+$ T cell immunomagnetic beads positive selection kit was purchased from Stem-Cell Tech (USA). Annexin V/PI kit was purchased from Abcam.

2.2. Immunohistochemistry (IHC) and Staining Evaluation

Approved by the Ethics Committee of Soochow University, cancer specimens from 64 patients with glioma were selected to build tissue microarrays (TMA). The following clinical parameters of patients were collected: gender, age, histopathology and grade. The quality of the TMA slides was confirmed by the pathologist using HE-stained slides. The tissue section slides were deparaffinized and rehydrated, and then washed in phosphatebuffered saline (PBS) solution three times. For antigen retrieval, slides were immersed in 10 mM sodium citrate (pH 6.0) and autoclaved at 120 °C for 15 min. The sections were incubated in 0.3% hydrogen peroxidase in absolute methanol for 30 min to deactivate endogenous peroxidases. After nonspecific binding was blocked with 3% bovine serum albumin (Cell Signaling Technology, USA) in PBS, the specimens were incubated with primary antibodies at 4 °C overnight. Then the specimens were incubated with anti-mouse/rabbit secondary antibody at room temperature for 30 min (Abcam). Staining was carried out using diaminobenzidine (DAB) kit (Sigma).

The intensity of staining was evaluated according to the following scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining (Ikeda et al., 2016). The proportion of all staining tumor cells was determined and then multiplied by the staining intensity score to obtain a final semi-quantitative H score (maximum value of 300 corresponding to 100% of staining tumor cells with an overall staining intensity score of 3). The scores exhibiting <100 were classified

Table 1
Clinical and pathological characteristics associated with PD-L1 expression.

Variable	Cases	PD-L1 expression		χ^2	*P* value
		Group low	Group high		
Gender				1.02	0.313
Male	32	16	16		
Female	32	20	12		
Age				11.46	0.001
<55	29	23	6		
≥55	35	13	22		
Histopathology				3.00	0.083
Astrocytoma	26	18	8		
Glioblastoma	38	18	20		
Grade				7.11	0.008
I/II	8	8	0		
III/IV	56	28	28		
EGFR				5.00	0.025
Low	33	23	10		
High	31	13	18		

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