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MYC inhibition increases PD-L1 expression induced by IFN-γ in hepatocellular carcinoma cells

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ABSTRACT

The effectiveness of immunotherapy targeting the immune checkpoint PD-L1/PD-1 pathway highlights importance of elucidating the regulatory mechanisms of PD-L1 expression in cancer cells. Previous studies demonstrate that oncogene MYC up-regulates PD-L1 expression in lymphomas. In the present study, we investigated the regulatory role of MYC in the PD-L1 expression induced by IFN-γ in HCC cells. Unexpectedly, knockdown of MYC expression using siRNA assay increased the inducible expression of PD-L1 both at mRNA and protein levels. Mechanistically, the inhibition of MYC elevated expression of STAT1, a critical component of IFN-γ signaling pathway, leading to the elevation of PD-L1 expression in HCC cells exposed to IFN-γ. These results suggest that MYC may down-regulate PD-L1 expression in the context of HCC. This study implicates that a combination therapy targeting MYC function and PD-L1/PD-1 pathway might be effective for treatment of HCC.

1. Introduction

The programmed cell death-ligand (PD-L) 1 / programmed cell death protein (PD)-1 pathway is an immune checkpoint responsible for tumor immune escape and has emerged as a major focus of immunotherapy (Chen and Han, 2015). The PD-L1 gene is normally expressed on immune cells or in immune privileged tissues, but its expression is found in multiple cancers, including hepatocellular carcinomas (HCC) (Calderaro et al., 2016). The PD-L1 molecule expressed on malignant cells binds to its receptor PD-1 expressed on the cytotoxic T lymphocytes (CTL) specific to the malignancy, limiting the anti-tumor function of the CTL (Zou et al., 2016). The immunotherapy using antibodies to block the PD-L1 / PD-1 pathway achieves a great success in the preclinical and clinical treatment of multiple cancers, including HCC. For instances, anti-PD-L1 treatment extends survival in mice with an aggressive transgenic HCC (Morales-Kastresana et al., 2013). Anti-PD-L1 cooperating with sorafenib elicits potent immune responses resulting in complete eradication or significant reduction of tumor growth in tumor-bearing mice (Wang et al., 2015). In addition to these evidences from animal models, the immunotherapy targeting the PD-L1 / PD-1 checkpoint was tested in an HCC-specific clinical trial. The efficacy was encouraging with an overall objective response rate of 19% (Harding et al., 2016). The blockade of PD-L1 / PD-1 immunotherapy also shows a dramatic response in human metastatic HCC with decrease in tumor size and drop in alfa-fetoprotein (Truong et al., 2016). The success of the antibody-based immunotherapy targeting the PD-L1/PD-1 pathway highlights a critical role of PD-L1 in the tumor immune evasion. It is intriguing to research the mechanisms underlying the PD-L1 expression in HCC cells. Precise understanding of how PD-L1 expression is controlled will allow development of new effective approaches to enhance the anti-tumor immunity.

The expression of PD-L1 in cancer cells can be classified into constitutive expression and inducible expression (Ritprajak and Azuma, 2015). The constitutive expression of PD-L1 is attributed to alteration of oncogenes or tumor suppressive genes in malignant cells. For instances, a mutation of oncogene RAS is cancer-driving and increases tumor cell-intrinsic PD-L1 expression (Coelho et al., 2017; Sumimoto et al., 2016). An activating mutation of the epidermal growth factor receptor (EGFR) gene contributes to PD-L1 overexpression in lung cancer cells (Azuma et al., 2014). Function loss of PTEN, a critical tumor suppressor,

Abbreviations: CTL, cytotoxic T lymphocytes; EGFR, epidermal growth factor receptor; FACS, fluorescence-activated cell sorting; HCC, hepatocellular carcinomas; Inr, initiator; PD-1, programmed cell death protein-1; PD-L1, programmed cell death-ligand; T-ALL, T cell acute lymphoblastic leukemia

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causally links to increased expression of PD-L1 in gliomas (Parsa et al., 2007) and lung cancers (Xu et al., 2014). On the other hand, the inducible expression of PD-L1 is attributed to extrinsic signals, such as inflammatory cytokines, of which IFN-γ is the most potent (Concha-Benavente et al., 2016; Doi et al., 2017; Tumeh et al., 2014).

Although the constitutive expression of PD-L1 is common in a variety of cancers, the inducible expression of PD-L1 is dominant in HCC (Sanmamed and Chen, 2014). PD-L1 is not constitutively expressed in majority of cultured HCC cell lines. By contrast, the expression of PD-L1 can be detected in HCC surgical and biopsy specimens. This implicates that the extrinsic factors in tumor microenvironment is mainly responsible for the expression of PD-L1 in HCC tissues (Xie et al., 2016). IFN-γ is a primary extrinsic factor released by the activated CD8 + T cells infiltrating in the HCC tissues (Chia et al., 2002; Nagao et al., 2000; Qiu et al., 2007). It can induce PD-L1 expression in HCC cell lines. Moreover, PD-L1 expression correlates to expression of IFN-γ in human HCC tissues (Xie et al., 2016). However, the regulatory mechanisms for the inducible expression of PD-L1 are not completely understood.

The oncogene MYC codes for a transcription factor that is overexpressed in many human cancers. Previous studies show that the overexpressed MYC protein binds to the promoter of PD-L1 gene to cause elevated expression of PD-L1 in T cell acute lymphoblastic leukemia (T-ALL) (Casey et al., 2016). Besides, MYC also promotes the expression of PD-L1 in neuroblastoma cells both in vitro and in vivo (Melaiu et al., 2017). These studies not only provide novel insights into the mechanisms whereby MYC maintains the malignancy, but also implicate that the therapies suppressing MYC expression and activity may restore an immune response against human cancers. In the case of HCC, the overexpression of MYC is caused by genomic amplification at 8q24.1 and presents in up to 70% of HCC. Moreover, the MYC overexpression indicates poor prognosis in liver cancer and is associated with metastatic or recurrent HCC (Zheng et al., 2017). Nevertheless, it is unclear whether MYC regulates the inducible expression of PD-L1 in HCC cells.

Since MYC is a driver gene in HCC and has potential in regulation of PD-L1 expression, we asked whether MYC regulates the PD-L1 expression induced by IFN-γ in HCC cells. Unexpectedly, we found that knockdown of MYC expression enhanced PD-L1 expression induced by IFN-γ in HCC cells. Mechanistically, the down-regulation of MYC increased expression of STAT1, resulting in elevated activation of IFN-γ receptor signaling. These findings imply that suppression of MYC activity might promote immune evasion mediated by PD-L1 molecule HCC.

2. Materials and methods

2.1. Cancer cells and treatments

HCC cell lines (SMMC-7721, BEL-7402, and Hep3B) were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Science, Shanghai, China. All the cancer cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 ºC with 5% CO_2.

A recombinant expressing plasmid pRK5-MYC was constructed from a commercial plasmid pEnter-MYC-flag plasmid (Vigene Biosciences, Shandong, China). All siRNAs were purchased from Sigma-Aldrich. Two siRNAs targeting the MYC gene were designated as siMYC #1 (sense: 5'-GCUUGUACCUGCAGGAUCUdTdT-3'; anti-sense5'-AGAUCCUGCAGGUACAAGCdTdT-3') and siMYC #2 (sense: 5'-CGUCCAAGCAGAGGAGCAAdTdT-3; anti-sense: 5'-UUGCUCCUCUGCUUGGACUdTdT-3'). The siNC represents the negative control siRNA: (sense: 5'-UUCUCCGAACGUGUCACGUTT-3'; anti-sense: 5'-ACGUGACACGUUCGGAGAATT-3'). Transfection reagent Interferrin (PolyPlus, France) was used to transfect siRNA into HCC cells while Lipofectamine 2000 (Invitrogen, USA) was used for transfection of plasmids.

After 24 h or 48 h of transfection with siRNA, the HCC cells were treated with 50 ng/ml of IFN-γ (Peprotech, UK) for indicted time periods. Then, the cells were harvested for quantitative RT-PCR, western blot or flow cytometry.

As for overexpression experiments, the HCC cells were transfected with siNC or siMYC for 24 h. Subsequently, these cells were subjected to transfection with pRK5 or pRK5-MYC for 24 h. Then, these cells were exposed to IFN-γ(50 ng/ml)for 6 h. The cells were harvested for quantitative RT-PCR.

2.2. Quantitative RT-PCR

The HCC cells were harvested and subjected to RNA extraction using RN07-EASYspin (Aidlab Biotechnologies, China). The cDNA for each sample was acquired using ReverTra Ace qPCR RT Kit (Toyobo, Japan). Quantitative PCR was performed using 2 x SYBR Green qPCR Mix (Aidlab Biotech, China) with primers specific for the PD-L1 gene (forward: 5'-TGTGGCATCCAAGATACAAACTCAAAG-3', reverse: 5'-TCCTCCTCTGCTTTCGCCAGGTTC-3'), the MYC gene (forward: 5'-TCTCTCCGTCCTCGGATTCT-3', reverse: 5'-TTGTTCCTCCTCAGAGTCGCT-3'), the STAT1 gene (forward: 5'-TGCGCGCAGAAAAGTTTCATTTG-3', reverse: 5'-CAGCTGTGACAGGAGGTCAT-3'), or the β-actin gene (forward: 5'-GACTACCTCATGAAGATCCTCACC-3', reverse: 5'-TCTCCTTAATGTCACGCACGATT-3'). The β-actin gene served as an internal control.

2.3. Western blot assay

The total protein was extracted and subjected to electrophoresis. Then, the protein was transferred onto a polyvinylidene difluoride membrane and probed with primary antibodies and HRP-conjugated secondary antibodies. The expression level of each protein was detected by enhanced chemiluminescence system (Beyotime Biotechnology, China). The density of the protein bands was quantified through software Imagine J 1.42q. The primary antibodies included anti-MYC (ab32072, 1:1000 dilution, Abcam, UK), anti-STAT1 (9175s, 1:1000 dilution, CST, USA), anti- p-STAT1 (AF2212, 1:1000 dilution, Beyotime Biotechnology, China) and anti-β-actin (PR-0255, 1:2000 dilution, ZSGB-BIO, China). The secondary antibodies were goat anti-rabbit IgG antibody or goat anti-mouse IgG antibody (ZB-2301 or ZB-2305, 1:2000 dilution, ZSGB-BIO, China).

2.4. Flow cytometry

The HCC cells were harvested and washed twice with ice-cold fluorescence-activated cell sorting (FACS) buffer (0.5% of FBS in PBS). Then, the cells were resuspended in 100ul of FACS buffer and stained with antibodies conjugated with fluorescein for 40 min at 4 ºC. Anti-human-PD-L1 PE (329706, Biolegend, USA) was used to detect the expression of PD-L1. Mouse IgG1 PE (12-4714-42, eBioscience, USA) was used as an isotype control. After 40 min, the cells were prepared for detection using flow cytometry. The data were analyzed by Flowjo Software.

2.5. Dual-luciferase reporter assay

The promoter region (from −2122bp to +94bp) of PD-L1 gene was amplified from DNA of human peripheral blood mononuclear cells and constructed into a luciferase reporter plasmid pGL3-Basic (Promega, USA). The recombinant luciferase reporter plasmid was designated as pGL3-PD-L1. Renilla luciferase plasmid pRL-TK served as internal control. BEL-7402 cells were transfected with siNC or siMYC for 24 h, then, co-transfected with a mixture of pGL3-PD-L1 and pRL-TK. After 24 h, cells were stimulated with human IFN-γ (50 ng/ml) for 6 h. At last, the cells were lysed and the luciferase activities were measured with Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacture's instruction.