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Investigation of PD-1H in DEN-induced mouse liver cancer model

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Abstract. – OBJECTIVE: Immune therapy has recently become a novel strategy for treating liver cancer, making it of critical importance to identify novel targets for treatment. Programmed death-1 homology (PD-1H) is one newly discovered negative co-stimulating molecule, and plays important regulatory roles in suppressing T cell activation. However, the expression or function of PD-1H in liver tumors has not been reported.

MATERIALS AND METHODS: Liver cancer tissues were collected from The Cancer Genome Atlas (TCGA) (http://tcga-data.nci-nih.gov). This study then utilized diethylnitrosamine (DEN) induced liver cancer mice, on which PD-1H monoclonal antibody and PD-1H extra-cellular Fc domain fusion protein were injected intraperitoneal. General status, gross morphology of liver tissues was examined, followed by hematoxylin-eosin (HE) staining and plotting survival curve.

RESULTS: Among TCGA samples, PD-1H expression was significantly elevated. Induced liver cancer mice showed depressed mental status, early onset of hepatitis and liver cirrhosis. Five mice dead in model group (mortality=33.33%). No natural death occurred in control group. Injection of PD-1H-Fc-Ig fusion and PD-1H monoclonal antibody improved the condition to certain extents, with morality at about 20%. Comparing to DEN group, combined treatment group showed significantly fewer tumor lesion on liver surface, with increased body weight and lower liver-body weight ratio. HE staining showed significantly elevated ratio of normal cells in combined treatment group, although large amounts of cancer cells still existed.

CONCLUSIONS: Blocking of PD-1H signal pathway could suppress liver cancer cell growth, decrease mouse mortality, indicating promising application of PD-1H in tumor immune therapy.

Key Words:
Liver cancer, Programmed cell death-1 homology, Diethylnitrosamine.

Introduction

Liver cancer is one common malignant tumor in China, and is frequently occurred in Southeast coast region[1]. Currently neither pathogenesis nor ideology of liver cancer has been confirmed. Current knowledge believe it is one complicated process involved multiple factors and steps, under the influence of both genetics and environment[2]. Epidemiology study showed that hepatitis type B virus (HBV), hepatitis type C virus (HCV) infection, aflatoxin, drinking water contamination, alcohol abuse, liver cirrhosis, sex hormones, nitrosamine substances or trace elements all might be correlated with liver cancer pathogenesis[3,4], although precise pathogenesis is still unclear. Immune system plays a crucial role in liver cancer pathogenesis[5]. Co-stimulating signals including both positive and negative co-stimulating molecules are critical for activating T cell-induced tumor-specific immunity. The abnormal expression of co-stimulating signals may lead to the activation of anti-tumor immune response[6]. Currently, co-stimulating molecules can be divided into three families: B7 family, tumor necrosis factor (TNF) and cytokine family. With further research, a series of novel co-stimulating molecules have been discovered, and have been confirmed to have extremely important regulatory roles in tumor immunity, with promising clinical application perspectives[7]. For examples, anti-PD-1 antibody has obtained satisfactory efficacy in treating various tumors, and monoclonal antibody related drugs nivolumab and lambrolizumab have been approved for clinical use in 2014 under a fast track[8,9]. The application of co-stimulating molecule antibody in tumor treatment has brought new promise for immune therapy against cancer[10]. Therefore, the identification of novel co-stimulating molecu-

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les for T cell activation can help to further reveal the molecular mechanism of immune escape, to activate immune response of body anti-tumor immunity, and to provide novel molecular targets for immune treatment against tumors.

PD-1H is one newly discovered negative co-stimulating molecule, and has been shown to exert important regulatory roles in suppressing T cell activation[11]. However, the expression and role of PD-1H in liver tumors have not been reported yet.

This study thus aimed to use diethylnitrosamine (DEN)-induced primary liver cancer mouse, on which the expression level and potential role of PD-1H in liver tumors were investigated.

Materials and Methods

Major Reagent

DEN was purchased from Huacheng Industrial (Japan). HE staining kit was purchased from Kangcheng Biotech (Beijing, China). PD-1H monoclonal antibody was purchased from Abcam Biotech. (Cambridge, MA, USA). Other common reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Major Equipment

Stereotaxis microscope was purchased from Thermo Scientific Pierce (Rockford, IL, USA). Light field microscope was purchased from Leica (Frankfurt, Germany).

PD-1H Expression in Liver Cancer Samples

A total of 425 liver cancer samples (50 from tumor adjacent tissues and 375 from tumor tissues) were collected from The Cancer Genome Atlas (TCGA) based on gene sequencing data (TCGA, http://tcga-data-nci.nih.gov). R code was used to homogenize all sequencing data for analyzing the expression of PD-1H in liver cancer.

Animals

Specific-pathology-free (SPF) grade male C57 mice (19-23 g) were purchased from Charles River Biotech (Beijing, China). All mice were randomly assigned into groups for feeding. After 14 d acclimation, animals were randomly divided into normal control, liver cancer, PD-1H monoclonal antibody and PD-1H-Fc-Ig fusion protein treatment group (n=15 each). This study was approved by Ethical Committee of the First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China.

Generation of Liver Cancer Model

15 days after birth, C57 mice were acclimated for 14 d. Each animal was weighted, and 1 ml DEN (25 mg/kg) was infused into the peritoneal cavity. 12 h later, 0.15% DEN solution was given to animals for 4-week feeding. 1 mL DEN (25 mg/kg) was injected into the peritoneal cavity for a second time. During the feeding, 0.15% DEN solution was applied for constructing spontaneously induced liver cancer model. 4 months later, the occurrence of liver tumor was observed. Equal volume of saline was infused into the peritoneal cavity of the controlled mice, which received water *ad libitum*. Based on grouping conditions, PD-1H monoclonal antibody or PD-1H-Fc-Ig fusion protein was applied into the peritoneal cavity at the time of first DEN injection.

Generation Condition of Mice

Mouse mental status, gross activity, fur color and tumor growth were observed. Mouse survival time was recorded. 4 months later, mice were sacrificed, and the abdominal cavity was opened to observe and record the morphology, color and texture of livers.

Hematoxylin-Eosin (HE) Staining

Rat liver tissues were harvested and were fixed in 4% paraformaldehyde. HE staining was used to measure pathology change of liver tissues. HE pathology staining followed routine protocols. In brief, tissue blocks were fixed, dehydrated, embedded in paraffin, and were prepared into 4 μm slices, which were attached onto the glass slide. All sections were de-waxed in xylene, processed in HE staining, and were observed in light field microscope.

Construction of PD-1H-Fc-Ig Fusion Protein

Routine PCR approach was used to amplify human IgG1 (Fc) constant fragment domain and mouse PD-1H extracellular coding gene from recombinant plasmid pGEZ-Term/B7-H1/Fx and pIRES2-EGFP/HVEM. Human IgG1 (Fc) constant fragment and eukaryotic expression vector pIRES2-EGFP were digested by XhoI and BamHI enzymes. Digesting products were ligated to make pIRES2-EGFP/Fc. Amplified mouse PD-1H extracellular fragment gene was digested by XhoI and Nhe enzymes, and were inserted into pIRES2-EGPPF/Fc to generate pIRES2-EGFP/PD-1H-Fc recombinant plasmid. Using liposome transformation, the plasmid was introduced into

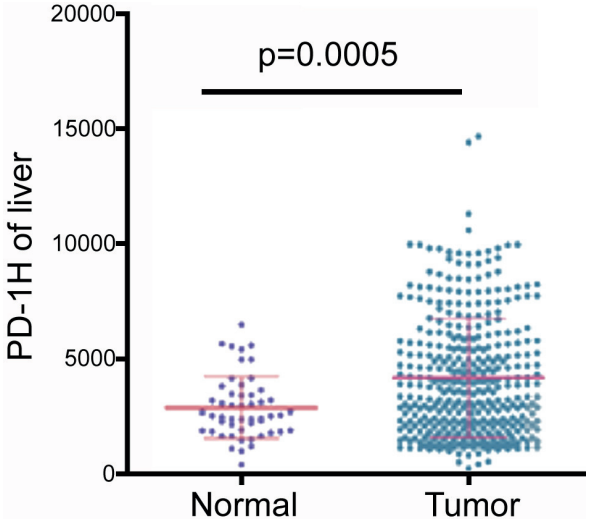

Figure 1. Expression of PD-1H in liver cancer tissues.

primary cultured tumor cells. Those cells containing fusion proteins were collected for the supernatant, which was filtered by Protein G affinity column for purification.

Statistical Analysis

SPSS 19.0 was used to process all collected data (SPSS, IBM, Armonk, NY, USA). Measurement data were presented by mean ± standard deviation (SD), and were tested for normality and equal variance. The Student's *t*-test was used to compare the differences between two groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. $p<0.05$ was considered as statistical significance.

Results

Expression of PD-1H in Liver Cancer

A total of 425 liver cancer samples (50 of tumor adjacent tissues, and 375 tumor tissues) were collected from TCGA. Based on gene sequencing data (https://tcga-data.nci.nih.gov). R language was used to homogenize gene-sequencing data for analyzing expression of PD-1H in liver cancer. As shown in Figure 1, comparing to normal liver tissues, PD-1H expression level was significantly elevated in liver tumor cells, with statistical significance ($p<0.05$).

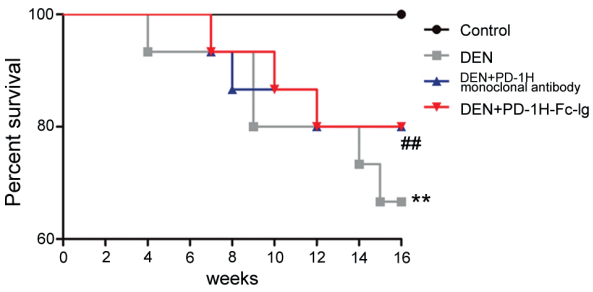

Figure 2. Survival curve of all mice. $^{**}p<0.05$ compared to control group. $^{\#\#}p<0.05$ compared to DEN group.

General Conditions of Mice

Three weeks after cancer induction, model group presented decreased appetite, gray furs with loss of shining, depressed mental status, immobility and indigestion. Liver cancer model mice showed death cases since week 4, and were diagnosed as acute hepatitis. On week 9, two mice were dead from liver cancer. At the end of 4 month, a total of 5 mice dead (33.3% mortality). Control group showed normal status without natural death, nor did any pathological change of liver morphology. Mice treated with PD-1H-Fc-Ig fusion protein and PH-1H monoclonal antibody showed minor improvement, with survival curves plotted in Figure 2.

Mouse Liver Pathological Changes

Normal mice showed no nodules on liver surface, which was presented as evenly distributed pink color. Liver cancer model group showed significantly enlarged liver volume, with rough surface, darkening color, and sparsely distributed gray-white nodules in multiple numbers and regular round-shape. PD-1H monoclonal antibody group presented similar change as those in liver cancer group, but with alleviated symptoms (Figure 3).

Liver Surface Nodules, body wWeight and Liver Weight

To investigate the role of PD-1H in DEN-induced mouse liver cancer, we further recorded the number of nodules on liver surface, body weight and liver weight from all groups of mice. As shown in Table I, comparing to control group, DEN model group showed large amounts of cancer-like nodules on liver surface, accompanied with significantly decreased body weight and higher liver weight, plus elevated liver/body weight ratio. The application of PD-1H monoclonal antibody or fusion protein decreased the number of tumor nodules by around one third, accompanied with minor increase of body weight and lower liver weight, all of which had significant difference against DEN group. Moreover, liver/body weight

Table I. Tumor nodule number, body weight, liver weight. and liver/body weight ratio.

Group	No.	Tumor nodule number	Body weight/g	Liver weight/g	Body/liver mass ratio (%)
Control group	15	0	45.03±2.12	1.15±0.46	2.55
DEN group	15	31. 23±2.13**	35.82±1.86**	2.29±1.64**	6.39
PD-1H monoclonal antibody group	15	22.29±2.87##	41.23±2.18##	1.89±0.64##	4.58
PD-1H-Fc-Ig group	15	20.15±1.28##	38.28±1.46##	1.78±1.22##	4.65

**$p<0.05$ comparing to normal control group. ##$p<0.05$ comparing to liver cancer model.

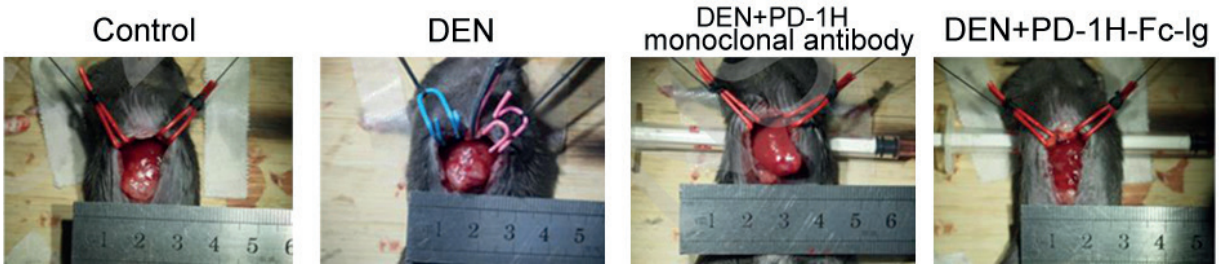

Figure 3. Gross morphology of mouse liver from all groups.

ratio was remarkably decreased compared to DEN group. These data suggested that blocking PD-1H signal pathway could inhibit tumor growth, indicating a wide range of application perspectives of PD-1H in tumor immune therapy.

Liver Pathology of Mouse

By HE staining, normal controlled mice showed clear boundary between cells, with similar cell size, homogenized nucleus. Liver cancer group showed prominent distribution of cancer cells, which presented as round or oval shape around nucleus without chromatin aggregation. Cells arranged in thread or adeno-shapes, plus infiltration towards peripheral tissues. In PD-1H-Fc-Ig fusion protein and PD-1H monoclonal antibody group also presented large amounts of liver cancer cells, with few normal hepatocytes plus tissue infiltration. The overall condition showed minor improvement (Figure 4).

Discussion

Epidemiology survey showed that liver cancer was now the fourth popular malignant tumor in China, and its mortality locates on the third place, thus severely threatening the public health[12]. Majority of liver cancer patients are not suitable for surgical resection, and current drug choice was still limited. In recent years, immune therapy has become the new frontiers in liver cancer treatment[13]. T cell activation is the development of tumor-specific immunity. CD8+ T cells and CD4+

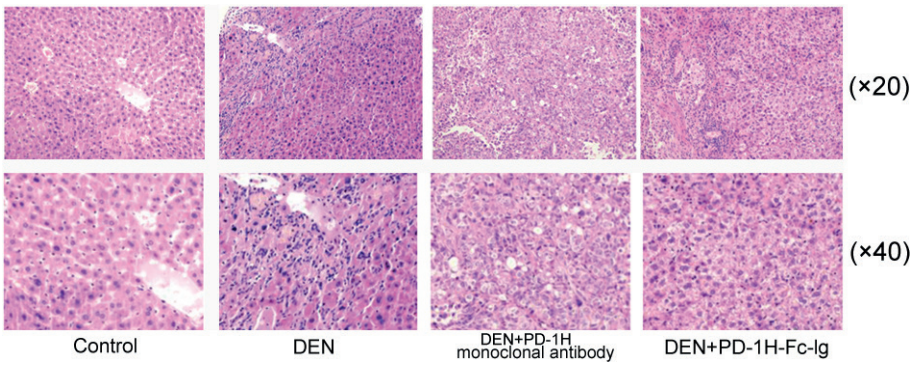

Figure 4. HE staining of mouse liver tissues (20 ×).

T cells are major effector cells. Under the co-stimulation of antigen presented by tumor cell surface MHC type I molecules (primary signal), and co-stimulating factors such as B7-1 and B7-2 (secondary signal), CD8+ T cells can be effectively activated to differentiate into CD8+ cytotoxic lymphocyte (CTL) cells to initiate tumor-specific immune response[14,15]. On tumor cell surface, negative co-stimulating molecules such as PD-L1 or B7-H4 were frequently over-expressed, suppressing T cell activation, thus causing tumor cell immune escape[16]. Therefore, further investigation and identification of tumor cell surface co-stimulating molecules and their regulatory roles in T cell activation can help to further reveal the molecular mechanism of T cell activation or suppressing in tumor-specific immunity. It can help to find molecular targets that can effectively activate T cells, and better activate body anti-tumor immune response.

DEN-induced liver cancer model is the most widely studied induced liver cancer model, with moderate model preparation time and high successful rate for cancer induction. During model preparation, there were about three stages including hepatocyte injury, hepatocyte hyperplasia/cirrhosis, and cancer formation stage, which can effectively mimic human liver cancer pathogenesis and making it an ideal model for studying human liver cancer[17-19]. In this report, we used DEN-induced liver cancer model to study the role of PD-1H.

As one newly discovered B7 family co-stimulating molecule in 2011, PD-1H is mainly expressed in myeloid-derived mononuclear cells and the surface of CD4+ T cells and CD8+ T cells[19,20]. PD-1H is one type I transmembrane glycoprotein containing 309 amino acids. The unique structure of PD-1H predicted its specific structure and functions[11]. PD-1H is widely expressed in various tissues, and is highly expressed in immune cells including myeloid-derived monocytes and CD4+ and CD8+ T cells plus Treg cells[21]. PD-1H expression is significantly elevated in allergy disease, human immunodeficiency virus (HIV) carrying population[20,21]. In this work, we collected 425 liver cancer samples from TCGA for analyzing the genomic sequencing data. Results showed significantly elevated PD-1H expression in liver cancer tissues, as similar with previous findings from squamous carcinoma[22]. Currently few researches have been performed about PD-1H. Current studies showed that PD-1H on APC surface could suppress CD4+ T cell-induced immune response, and the knockout of PD-1H or the application of PD-1H antibody could significantly enhance immune response against immunogen of the body[11]. Other investigations found that PD-1H signal pathway significantly inhibited T cell activation or proliferation, and is one typical negative co-stimulating molecule that probably had wide perspectives in tumor immunity[23]. In this study, we utilized DEN-induced primary liver cancer mouse model, on which PD-1H monoclonal antibody and PD-1H-Fc-Ig fusion protein was used to block PD-1H signaling pathway, and confirmed the role of blocking PD-1H signal for suppressing liver cancer formation and pathogenesis.

It is worth noticing that this paper illustrated the expressional profile in liver cancer population, and the protective role of PD-1H signal in protecting against DEN-induced primary liver cancer in mouse model, illustrating that blocking PD-1H signal may work as one novel target for tumor immune therapy. However, whether PD-1H was expressed consistently in various tumors. The expression of PD-1H on the surface across various immune cells, or its detailed mechanisms for T cell activation still require further future studies.

Conclusions

For the first time we utilized DEN-induced mouse liver cancer model to investigate the effect of PD-1H-Fc-Ig and PD-1H monoclonal antibody on tumor growth and survival of mice. Our results provided evidences for novel immune therapy strategy of tumors, and had potential application values in future.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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