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Association Between Activation of the Programmed Cell Death-1 (PD-1)/Programmed Death-Ligand 1 (PD-L1) Pathway and Pain in Patients with Cancer

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Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABC 1 **Jian Zhang**
DFG 2 **Huali Zhang**
CDE 3,4 **Yongli Luo**

1 Department of Pain Management, The Third Hospital Affiliated to Hebei Medical University, Shijiazhuang, Hebei, P.R. China
2 Department of Laboratory, The First Hospital of Shijiazhuang, Shijiazhuang, Hebei, P.R. China
3 Department of Palliative Care, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, P.R. China
4 Department of Oncology, The First Affiliated Hospital of Chengdu Medical College, Chengdu, Sichuan, P.R. China

Corresponding Author: Yongli Luo, e-mail: yongliluoyx@163.com

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Background: The aim of this study was to investigate the clinical correlation between sPD-1 (soluble programmed cell death-1) and PD-1 (programmed cell death-1) expression and cancer pain.

Material/Methods: sPD-1 content in peripheral blood was determined by enzyme-linked immunosorbent assay (ELISA). T cell surface-positive rate was determined by flow cytometry, and the correlation of clinical characteristics of patients with cancer pain was analyzed.

Results: The positive expression rate of PD-1 in sPD-1 and T cells of patients with cancer pain was higher than that in normal patients. There was a significant correlation between sPD-1 and PD-1 positivity on T cell surface with tumor type, differentiation degree, and VAS scores of patients with cancer pain ($P < 0.05$). Peripheral blood sPD-1 level and PD-1 positivity in patients with liver cancer and melanoma cancer were higher than those in patients with renal cell carcinoma and breast cancer. In addition, peripheral blood sPD-1 level and PD-1 positivity in patients with poorly-differentiated cancer pain were higher than those in patients with intermediately- to well-differentiated cancer. The sPD-1 content was lower and PD-1 positivity rate was higher in cancer pain patients with low VAS scores.

Conclusions: The positive expression rate of sPD-1 and PD-1 in patients with cancer pain is higher than that in normal people. The activation rate of the PD-1/PD-L1 pathway was mediated by sPD-1 and PD-1 positive expression, age, tumor type, and differentiation. There are correlations between clinical characteristics such as degree and pain level as shown by VAS score.

MeSH Keywords: **Adrenal Cortex Neoplasms • Antigens, Differentiation, B-Lymphocyte • Disorders of Sex Development**

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Background

Cancer is a major disease that negatively affects human survival and health. Cancer pain, a common symptom of mid- and advanced-stage cancer, imposes tremendous physical and psychological burdens on patients and seriously affects a patient's quality of life [1]. With an increase in the number of in-depth studies on cancer pain, cancer pain has become an area of focus for clinicians. Epidemiological surveys show that more than 50% of cancer patients experience cancer pain, and the incidence of cancer pain is as high as 80% in patients with intermediate and advanced cancer. Cancer pain is usually moderate or severe, and 50–80% of this pain cannot be effectively controlled [2]. Cancer pain may impair bodily function and disease resistance, and greatly affects the outcomes of surgery, radiotherapy, chemotherapy, and other cancer treatments. During its development, cancer may release pain factors such as vascular endothelial growth factor, nerve growth factor, proteases, prostaglandins, endothelins, and bradykinin, which may further aggravate cancer pain. However, most cancer patients do not have pain in the early stages of disease [3]. Therefore, scholars believe that different pain-related factors may be produced in different stages of cancer. The body may produce pain factors and analgesic factors at the same time. These factors mutually act on one another to positively and negatively regulate a patient's sensitivity to pain [4].

As an analgesic factor, PD-L1 has attracted the attention of researchers. PD-L1 is a type I transmembrane protein mainly expressed on antigen-presenting cells, B cells, T cells, epithelial cells, myocytes, endothelial cells, and various tumor cells, and is involved in tumor-related immune response [5]. PD-1 and PD-L1 constitute the PD-1/PD-L1 signaling pathway, which inhibits growth factor production and cell proliferation, and plays an important role in activating T cells and regulating immune responses. In addition, PD-L1 can inhibit T cell-mediated immune response and help tumor cells avoid identification and killing by the immune system. In a healthy body, activation of the PD-1/PD-L1 signaling pathway reduces the damage of the immune response to surrounding tissue and prevents autoimmune disease [6]. Conversely, activation of this pathway can reduce the immune effect of T cells in the local tumor micro-environment, mediate tumor immune escape, and promote cancer progression [7]. Related studies have shown that exogenous administration of PD-L1 produces analgesic effects in normal mice, but blocking endogenous PD-L1 and PD-1 induces pain. Primary nociceptors and immune cells have some similarities and can communicate with immune cells. Nociceptors express major immune modulators such as cytokines, chemokines, and Toll-like receptors (TLRs) [8]. sPD-1, a blocker of the PD-1/PD-L1 signaling pathway, inhibits PD-1 expression. sPD-1 can block PD-L on tumor cells, which promotes tumor immunity, and sPD-1 is closely related to the immune function

of the body. Therefore, the abnormal expression of sPD-1 may be involved in the occurrence and development of various diseases, including tumors. sPD-1 may be useful as a treatment for cancer pain. However, it is unclear whether the expression of sPD-1 and PD-1 in cancer pain patients is related to the type of cancer, patient age and sex, tumor differentiation type, decreased expression of sPD-1 and PD-1 in patients with advanced cancer, and the severity of cancer pain. The present study explored the clinical relevance of sPD-1 and PD-1 in patients with cancer pain.

Material and Methods

Subjects

A total of 516 patients with cancer pain admitted to the First Affiliated Hospital of Chengdu Medical College were included in this study. All the patients met the following inclusion criteria: (1) Voluntary participation in this study; (2) Diagnosis of cancer according to the diagnostic criteria for cancer in the *Regulations on Diagnosis and Treatment of Common Malignant Tumors*; (3) Diagnosis of cancer pain (cancer pain generally refers to pain caused directly by the tumor and tumors, which can invade or oppress nerve roots, nerve trunks, nerve plexuses, and nerves, as well as invading the brain and spinal cord, periosteum or bone, and essential and hollow organs, and can invade or block the vasculature, and cause local necrosis, ulcers, and inflammation, causing severe pain in these situations, and the pain caused during the treatment of cancer is also considered to be cancer pain), with a VAS score (Visual Analogue Scale/Score) >4 points; (4) Expected survival >1 month; and (5) Informed consent from the patient and family, and active cooperation in this study. Exclusion criteria were: (1) Serious complications in heart, liver, or kidney function; (2) Serious mental disorders; (3) Pregnant or lactating women; (4) Allergy to opioids; and (5) Refusal to participate in this clinical study. The study was approved by the ethics Committee of the Fourth Hospital of Hebei Medical University.

Methods

Specimens

Peripheral venous blood was collected from the 516 patients with cancer pain and 500 normal people (serum was used in the detection of sPD-1 and PD-1 positive rate on T lymphoid surfaces).

Test instruments and reagents

Anti-human CD8-FITC/PD1-PE fluorescent-labeled monoclonal antibody was purchased from Becton Dickinson; human

peripheral blood T lymphocyte separation solution was purchased from Stem Cell. The enzyme-linked immunosorbent assay (ELISA) kits were purchased from Becton Dickinson. The centrifuge was purchased from HITACHI Japan (model: 05PR-22). The ELX800 microplate reader was purchased from BioTech, USA.

Analysis of serum sPD-1 content by ELISA

We collected 2-mL samples of peripheral venous blood from the 516 patients (group A) and 500 normal people (group B) after fasting for 12 h. Blood was allowed to stand for 30 min, then centrifuged (HITACHI, Japan, model: 05PR-22) at 2000 g for 5 min). After centrifugation, the upper serum layer was stored at -80°C . Expression of PD-1 and sPD-1 cytokines was evaluated using the ELISA kit (Becton Dickinson). All steps were performed in strict accordance with the instructions provided by the manufacturer of the kit. To calculate the linear regression equation, a standard curve was generated according to the concentration of a standard substance and the corresponding absorbance (A) value, and the corresponding sample concentration was calculated according to the A value of the samples. The measurement was repeated 3 times for each specimen, and the mean of the results was regarded as the final measured value to reduce experimental error. Absorbance values were read on an ELX800 microplate reader (BioTech, USA) and the values were compared between group A and B.

Analysis of T lymphocyte PD-1 expression levels with flow cytometry

EDTA-K2 anticoagulant tubes were used to collect 2 mL of venous blood, which was thoroughly mixed with the same volume of PBS solution. Subsequently, a dropper was used to slowly add the mixture to 5 mL of lymphocyte stratified fluid along the wall of the tube, followed by horizontal centrifugation at room temperature at 2000 rpm (centrifuge radius $r=13.5$ cm) for 20 min. The mononuclear cell layer fluid (middle layer) was then carefully aspirated, washed 3 times with PBS, and re-suspended, then horizontally centrifuged at room temperature at 1500 rpm for 10 min. The supernatant was discarded, the pellet was washed with PBS, re-suspended, centrifuged at 1500 rpm for 10 min, and then the resulting sediment was suspended in buffer to count the mononuclear cells. One microliter of CD8-FITC/PD1-PE fluorescent-labeled monoclonal antibody reagent was added to 100 μL of mononuclear cell suspension ($10^6/\text{mL}$) and the mixture was incubated in the dark at 4°C for 30 min before being examined by flow cytometry. MACSQuantify was used to analyze and obtain related data and to analyze PD-1 expression levels on T cell surfaces. The data were compared between group A and B.

Table 1. Clinical characteristics of patients with cancer pain.

Baseline characteristic	n=516
Sex	
Female	262
Male	254
Age (years)	
<40	60
40–60	226
>60	230
Cancer types	
Hepatocellular carcinoma (HCC)	133
Bladder cancer	46
Melanoma	76
Lung cancer	107
Gastric cancer	88
Renal cell carcinoma (RCC)	19
Breast cancer	47
The degree of tumor differentiation	
Poor	164
Intermediate	235
Well	117
VAS score	
4–6	105
6–8	267
8–10	144

Statistical methods

SPSS19.0 software was used for statistical analysis. A two-sample *t* test was performed for the test group and control group. The relationship between variables is presented as the mean \pm SD. $P<0.05$ was considered statistically significant.

Results

General conditions of patients

Patients with cancer pain ($n=516$) admitted to our hospital from January 2017 to December 2017 were included in this study. The clinical pathological characteristics of the patients are shown in Table 1. There were 254 men and 262 women included, age 34–86 years, mean age 61 years, including 133

Table 2. The content of sPD-1 in serum of cancer patients compared with that of normal control group.

Group	n	sPD-1	t	p
Cancer pain group	516	128.24±11.13	0.341	0.023
Control group	100	89.07±32.54		

Table 3. Correlation between serum sPD-1 levels and clinicopathological parameters in patients with cancer pain.

Baseline characteristic	n=516	sPD-1	p
Sex			
Female	262	128.82±22.17	P=0.867
Male	254	132.33±27.87	
Age (years)			
<40	60	129.22±39.45	p=0.039
40–60	226	162.33±38.44	
>60	230	210.65±36.94	
Cancer types			
Hepatocellular carcinoma (HCC)	133	145.89±10.89	p=0.011
Bladder cancer	46	139.24±16.23	
Melanoma	76	144.25±18.63	
Lung cancer	107	160.36±20.13	
Gastric cancer	88	139.68±23.22	
Renal cell carcinoma (RCC)	19	152.11±22.01	
Breast cancer	47	176.23±23.66	
The degree of tumor differentiation			
Poor	164	263.98±88.25	p=0.023
Intermediate	235	172.3±78.21	
Well	117	104.56±99.27	
VAS score			
4–6	105	109.49±67.32	p=0.031
6–8	267	132.42±68.01	
8–10	144	180.22±70.96	

cases of liver cancer, 46 cases of bladder cancer, 76 cases of melanoma, 107 cases of lung cancer, 88 cases of gastric cancer, 19 cases of renal cell carcinoma, and 47 cases of breast cancer. There were 164 patients with poorly-differentiated cancer, 235 patients had intermediately-differentiated cancer, and 117 patients had well-differentiated cancer (Table 1).

Serum sPD-1 level in patients with cancer pain versus patients in the normal control group

The serum expression levels of sPD-1 in 516 patients with cancer pain and 500 healthy volunteers during the same

period were evaluated using ELISA. The results of the ELISA showed that serum sPD-1 levels in patients with cancer pain (128.24±11.13) pg/mL were significantly higher than those of the normal control group (88.63±23.44) pg/mL, $P<0.05$ (Table 2).

Correlation between serum sPD-1 level and clinicopathological parameters of patients with cancer pain

As shown in Table 3, serum sPD-1 level was correlated with age, tumor type, tumor differentiation degree, and VAS pain score of patients with cancer pain ($P<0.05$), but not with the sex of patients with cancer pain ($P>0.05$) (Table 3).

Table 4. Correlation between the expression of PD-1 in peripheral blood T cells and clinicopathological parameters in patients with cancer pain.

Baseline characteristic	n=516	Positive expression rate (%)	p
Sex			
Female	262	62.31	p=0.131
Male	254	64.94	
Age (years)			
<40	60	60.34	p=0.049
40–60	226	76.33	
>60	230	67.23	
Cancer types			
Liver cancer	133	56.31	p=0.009
Bladder cancer	46	79.32	
Melanoma	76	81.33	
Lung cancer	107	75.44	
Gastric cancer	88	70.32	
Renal cell carcinoma	19	76.09	
Breast cancer	47	92.31	
The degree of tumor differentiation			
Poor	164	83.143	p=0.029
Intermediate	235	68.43	
Well	117	65.79	
VAS score			
4–6	105	79.98	p=0.031
6–8	267	58.69	
8–10	144	65.79	

Correlation between the expression of PD-1 in peripheral blood T cells and clinicopathological parameters in patients with cancer pain

As shown in Table 4, the expression of PD-1 in peripheral blood T cells was correlated with age, tumor type, tumor differentiation degree, and VAS pain score of patients with cancer pain ($P<0.05$), but not with the sex of patients with cancer pain ($P>0.05$) (Table 4).

Statistical analysis showed that the expression levels of sPD-1 and PD-1 were significantly correlated with patient age and cancer pain. In the pairwise comparisons of the Q test, the expression levels of sPD-1 and PD-1 in patients older than 60 years significantly differed from those in patients younger than 40 years, while PD-1 content gradually increased with age (Figure 1).

Statistical analysis by one-way ANOVA showed that the expression levels of sPD-1 and PD-1 significantly differed between cancer pain patients with different types of cancer (i.e., the expression levels of sPD-1 and PD-1 were different between different cancers). In the pairwise comparisons of the Q test, the expression levels of sPD-1 and PD-1 in patients with liver cancer and melanoma significantly differed from those with other types of cancer (Figure 2).

Statistical analysis by one-way ANOVA showed that the sPD-1 and PD-1 expression levels significantly differed between cancer pain patients with different differentiation degrees of cancer. In the pairwise comparisons of the Q test, the expression levels of sPD-1 and PD-1 significantly differed between cancer pain patients with poorly-differentiated cancer and those with intermediately- or well-differentiated cancer (i.e., the expression levels of sPD-1 and PD-1 in patients with poorly-differentiated cancer were significantly higher) (Figure 3).

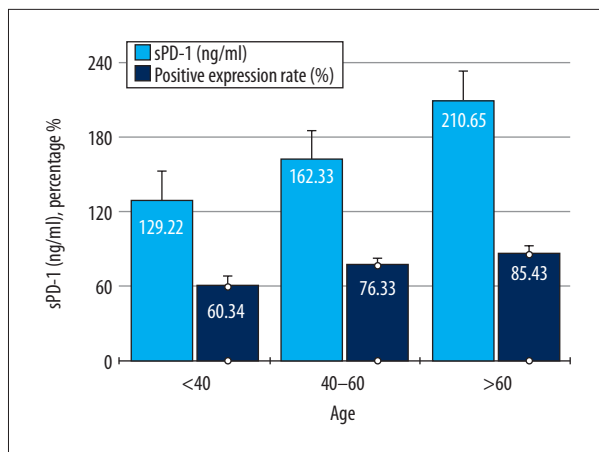


Figure 1. sPD-1 and positive expression rate of PD-1 on T cell surface in cancer pain patients at different ages. (■) The level of sPD-1; (■) The positive expression rate of pd-1 on T cell surface.

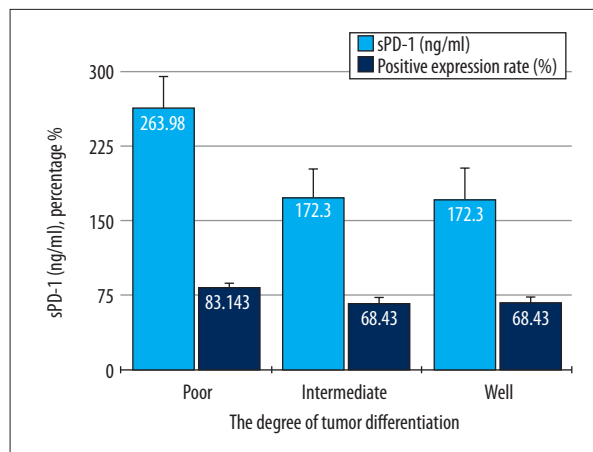


Figure 3. sPD-1 and positive expression rate of PD-1 on T cell surface in cancer pain patients with different differentiation degrees of cancer. (■) The level of sPD-1; (■) The positive expression rate of pd-1 on T cell surface.

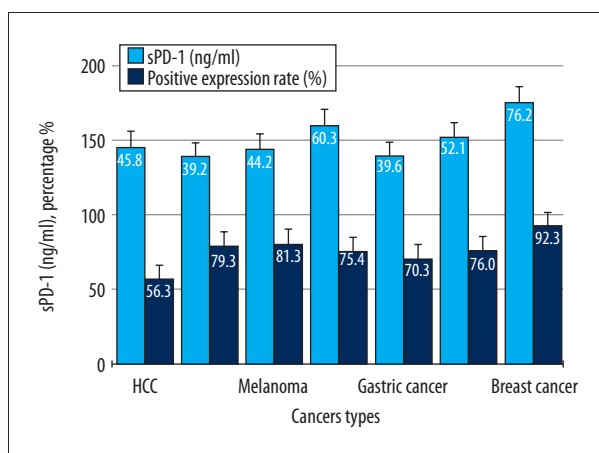


Figure 2. sPD-1 and positive expression rate of PD-1 on T cell surface in cancer pain patients with different types of cancer. (■) The level of sPD-1; (■) The positive expression rate of pd-1 on T cell surface.

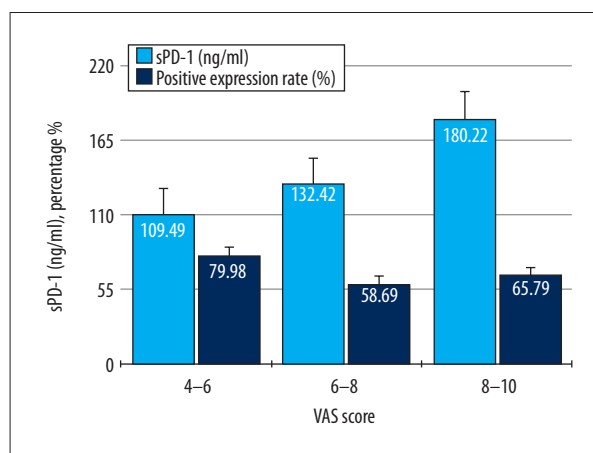


Figure 4. sPD-1 and positive expression rate of PD-1 on T cell surface in cancer pain patients with different pain degrees. (■) The level of sPD-1; (■) The positive expression rate of pd-1 on T cell surface.

Statistical analysis by one-way ANOVA showed that the expression levels of sPD-1 and PD-1 significantly differed between cancer patients with different degrees of pain. In the pairwise comparisons of the Q test, the expression levels of sPD-1 and PD-1 in patients with 4 < VAS < 6 significantly differed from those in other patients (i.e., the expression levels of sPD-1 and PD-1 were higher in patients with milder pain (Figure 4).

Discussion

Cancer pain is a common complication of advanced cancer that imposes a significant burden on patients and seriously affects patient quality of life. The release of a large number of pain factors is one of the major mechanisms of advanced cancer

pain [9]. Common pain factors include bradykinin, growth factors, endothelin, protons, prostaglandins, and proteases [10]. Although the pain-causing mediators produced by tumors have long been of concern, some cancer patients do not experience pain until their cancer metastasizes to the bone. For example, patients with liver cancer and melanoma do not experience pain in the early stages of cancer [11]. Therefore, Esin reported that different pain-related mediators may be produced by different cancers, and even by the same cancer at different growth stages, and these mediators can positively or negatively regulate pain sensitivity [12]. The negative co-stimulatory molecule PD-1 was discovered by researchers in 1992. It is one of the important members of the immunoglobulin CD28 superfamily. It plays an important role in regulating the activation,

proliferation, and self-recognition of T lymphocytes in the body, and can be induced and expressed on a variety of immune cells [13]. However, it is unclear how sPD-1 in the body participates in regulating immune cells in cancer pain, and whether its expression level is correlated with sex, age, differentiation degree, or other clinical characteristics of patients.

Currently, sPD-1 is known to be transcribed from PD-1 Δ ex3, one of the isoforms of the PD-1-encoding gene. Chen et al. analyzed the expression level of this soluble factor in healthy volunteers using a self-developed sPD-1 ELISA kit. The results showed that peripheral sPD-1 expression level was low in children, and there was no significant difference in the serum content of this factor between adults aged 20–30 years and those older than 50 years [14]. However, D’Incecco et al. analyzed the rate of PD-1 receptor positivity on T cell surfaces in patients of different ages and found that PD-1 receptor positivity differed between patients of different ages, and was higher in elderly patients [15]. Shimada et al. analyzed PD-1 expression in T lymphocytes of elderly mice, and found similar results, indicating that the weakened function of T lymphocytes in the mice was closely related to high PD-1 expression [16]. PD-1 is a negative co-stimulatory molecule expressed on immune effector cells. It is involved in programmed cell apoptosis and negatively regulates activated T lymphocytes. It is therefore considered an important cause of immunosuppression [17].

In the present study, ELISA results showed that PD-1 expression levels were evaluated. Flow cytometry was used to assess the positive rate of PD-1 receptor on T cells, showing that sPD-1 expression level and the PD-1 positivity rate on T cells significantly differed between cancer pain patients of different ages, and increased with age. This likely occurred because the capacity of the body’s immune cells to produce sPD-1 is related to frequency and duration of external antigen stimulation in the immune system, and the number of resulting memory cells and activated cells. The external stimulation to the body increases with age, which may explain why the expression level of sPD-1 became higher, and might be related to reduced pain sensitivity in the elderly.

Kim [18] examined whether the rate of PD-1/PD-L1 positivity differed in patients with different types of malignant tumors. The PD-1/PD-L1 positive rate in liver cancer was significantly higher than that in breast cancer. Sunshine found that the number of PD-1⁺ T cells in gastric cancer, breast cancer, kidney cancer, melanoma, and other malignant tumors was significantly higher than that in healthy patients, and was positively correlated with tumor progression [8]. Swaika found that the expression level of sPD-1 protein was significantly increased in melanoma, as shown by ELISA [19]. Chen found that plantar injection of sPD-1 to mice quickly induced spontaneous pain and mechanical tactile allodynia and led to conditioned

place preference, but the immunity indexes were not affected by sPD-1 [20]. PD-1 expression is sufficient to mask the cancer pain resulting from melanoma. After intravenous injection of nivolumab and RMP1-14, mice with melanoma developed spontaneous pain and mechanical tactile allodynia [21]. Blocking downstream signals of PD-1 also caused spontaneous pain in mice with melanoma [22]. These findings suggest that PD-L1 masks the cancer pain resulting from non-metastatic melanoma via PD-1 [23]. In the present study, the rate of PD-1 positivity on T cells in patients with liver cancer and melanoma was significantly higher than that in breast cancer patients. In patients with liver cancer and melanoma, there was no obvious pain sensation in the early stage, which may be related to the high expression of PD-L1 in liver cancer and melanoma that markedly inhibited and alleviated pain via the PD-1/PD-L1 pathway.

Hoang analyzed the rates of PD-1/PD-L1 positivity on T cell surfaces in patients with liver cancer at different degrees of differentiation, and found that PD-1/PD-L1 positivity on T cell surfaces in poorly-differentiated liver cancer was high [24]. In the present study, the rate of PD-1/PD-L1 was the highest in poorly-differentiated tumors compared with that in intermediately- and well-differentiated tumors, which was consistent with the results of the above study. Peripheral sPD-1 content was the highest in poorly-differentiated tumors compared with that in intermediately- and well-differentiated tumors. This topic had not been the subject of study thus far. It appears that the high degree of differentiation and weak antigenicity of intermediately- and well-differentiated tumor cells stimulate the body to produce less PD-1.

It was shown that PD-L1 inhibited the activity of nociceptor neurons and alleviated acute and chronic pain through the PD-1 receptor [23]. However, there is no study on the correlation between degree of pain and expression of PD-1 on T cell surface or peripheral sPD-1 content. The results of the present study show that expression of PD-1 on T cell surfaces decreased and peripheral sPD content increased with increasing degree of cancer pain. The specific mechanism is unclear, but may be as follows: abnormal increases of sPD-1 result in excessive binding with PD-L1 and reduce the analgesic effect of the PD-L1 pathway. In addition, the frequency and probability of interaction between PD-1 on the surface of T and B cell membranes and PD-L1 on the surface of vascular endothelial cells, tissue cells, and monocytes decreased significantly, so that the activated T cells could not promptly obtain negative regulatory signals with sufficient intensity, resulting in excessive activation and proliferation of T cells, continuous immune response to autologous antigens, damage to autologous tissue, production of many inflammatory factors, and aggravation of pain.

Conclusions

The positive expression rate of sPD-1 and PD-1 in patients with cancer pain is higher than that in normal people. Activation of the PD-1/PD-L1 pathway in cancer patients is associated with clinical characteristics such as age, tumor type, degree of differentiation, and pain level of VAS score. This information can be used for the development of cancer pain and analgesic drugs targeting the PD-1/PD-L1 pathway. In fact, the nerve-endocrine-immune network regulation indicates the inseparable relationship between pain and immune function. With further studies on PD-1/PD-L1 in molecular-targeted drugs, some scholars have gradually shifted their attention to the analgesic effects. Traditional anti-cancer drugs and treatments aggravate cancer

pain when killing tumor cells; therefore, PD-1/PD-L1 can be regarded as a breakthrough point for developing new anti-cancer and analgesic drugs to alleviate cancer pain and improve the quality of life of patients with cancer pain.

There are several limitations in the present study. This was a single-center study with a small sample size, and the study did not include all types of cancer. Future studies should include more patients and have a mixture of cancer types.

Conflict of interests

None.

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