

Original Article

The clinical value of T lymphocyte subsets and NK cells in peripheral blood of patients with high-risk HPV infection and different cervical lesions

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Abstract: Objective: To investigate the clinical value of T lymphocyte subsets and NK cells in peripheral blood of patients with high-risk HPV infection and different cervical lesions. Methods: CD3⁺, CD4⁺, CD8⁺ lymphocyte subsets and CD16⁺CD56⁺ cells in specimens of peripheral blood of 212 healthy control and 2167 patients with high-risk HPV infection and different cervical lesions were detected by flow cytometry. Results: Different patients with high-risk HPV infection in cervical lesions, CD4⁺, CD16⁺CD56⁺ cells and the numbers of CD4⁺/CD8⁺ ratio were lower than those in the healthy control group, and with the severity of lesions increment to gradually reduce ($P < 0.01$), while CD8⁺ cell numbers were higher than those in the healthy control group, and with increasing severity lesions gradually increased ($P < 0.01$). The cellular immune function of all patients with cervical squamous cell carcinoma were significantly lower than those in healthy control group, and with the severity of lesions increment to gradually reduce. The cellular immune function of patients of cervical squamous cell carcinoma with lymph node metastasis were significantly lower than that in patients without lymph node metastasis ($P < 0.01$), and in patients with well differentiated, poorly differentiated and moderately differentiated of cervical squamous cell carcinoma group were no difference between them ($P > 0.05$). But CD4⁺, CD8⁺ cells and CD4⁺/CD8⁺ ratio of patients with low differentiation are significantly lower than patients with well-differentiated. The number of cells of CD4⁺, CD16⁺CD56⁺ reducing and CD4⁺/CD8⁺ ratio decreased were negative correlated to clinical stages of cervical squamous cell carcinoma, namely were decreased with the increasing of clinical stages ($P < 0.01$). The number of CD8⁺ cell was positive correlated with clinical stages of cervical squamous cell carcinoma ($P < 0.01$). Conclusion: The cellular immune function of the patients with high-risk HPV infection and different cervical lesions is lower and especially even worse in patients with cervical squamous cell carcinoma and with advanced clinical stages. Detecting T lymphocyte subsets and NK immune cells can be used to monitor cellular immune function of patients with cervical squamous cell carcinoma and to estimate prognosis and provide reference for clinical treatment.

Keywords: T lymphocyte subsets, NK cell, CIN, cervical squamous cell carcinoma, HPV

Introduction

Global multi-center large sample epidemiological and experimental researches have shown that high-risk human papillomavirus (HPV) infection is essential for cervical squamous cell carcinoma [1, 2]. Viral infection often causes cell mutations, which can be removed by immune defense, thereby maintaining a relatively stable internal environment. Only one type of high-risk HPV infections leads to long-term sustainability of cervical squamous cell carcinoma, which is a continuous gradual pathological pro-

cess taking about 5-15 years from precancerous lesion, namely cervical intraepithelial neoplasia (cervical intraepithelial neoplasia, CIN) [3]. These evidences indicate that cervical cancer is the result of interactions between high-risk HPV infection and immune function, where viral infection is only an external factor, and loss or reduce of cellular immune surveillance function is the internal factor of cervical cancer. Therefore, to explore the relationship between cellular immune function in patients with different cervical lesions helps to understand the pathogenesis of cervical squamous cell carci-

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noma, which provides a theoretical basis for the prevention and treatment of cervical squamous cell carcinoma.

Materials

Subjects

A total of 2167 cases of married women were enrolled in this study with visits, hospitalizations, opportunistic screening, physical examination, and the "two cancer" screening at Shaanxi Provincial Tumor Hospital from September 2007 to June 2015, aged 21 years to 69 years with an average of 42.68 years, in which 212 cases are normal women aged 21 years to 63 years with an average of 42.23 years, whose HPV genotyping assay is negative, smooth cervical without inflammation or other symptoms and medical history, and with no history of drug use within three months. In 1,955 cases of various cervical lesions, 443 cases of CIN (CIN I 86 cases, CIN II 156 cases, CIN III 201 cases), aged 23 years to 60 years with an average age of 38.94 years; 1512 cases of cervical squamous cell carcinoma patients, aged 24 years to 69 years with an average of 42.87 years (261 cases of cervical squamous cell carcinoma I, 379 cases of IIa, 337 cases of IIb, 252 cases of IIIa, 191 cases of IIIb, 92 cases of IV. All patients were confirmed by pathological diagnosis. Pathological diagnosis shows 354 cases of well-differentiated, 762 cases of middle-differentiated, and 396 cases of poorly-differentiated. In these patients, 919 cases have no lymph node metastasis, while 593 cases have lymph node metastasis. Patients with negative HPV genotyping, low-risk HPV infection, adeno-squamous, or autoimmune diseases, and those who had chemotherapy, immunotherapy or taking any patient care products within three months were excluded.

Reagents and instruments

Reagents: mouse anti-human leukocyte grade antigen detection kit CD4-FITC, CD8-PE, CD3-PC5, mouse anti-human grade antigen detection kit CD3-FITC, CD16/CD56-PE, mouse anti-human IgG1-PC5/IgG1-FITC/IgG-PE, control lymphocyte, hemolytic agents, cleaning fluid and diluent were purchased from Beckman Coulter company. CD3⁺, CD4⁺ and CD8⁺T were used for labeling lymphocytes, CD16⁺CD56⁺ for labeling NK cells. Reagents for HPV genotyping were from Shenzhen-Biological Technology Co., Ltd.

Instruments: PAS dual laser Flow cytometry (FCM) was from PARTEC (Germany), and HPV genotyping system was from Shenzhen-Bio Technology Co., Ltd.

Methods

Sampling

Fasting blood samples (2 ml) were collected in EDTA anticoagulated collection tube and kept at 4°C and testing within 1 h.

Detection

The outer anticoagulant periphery blood was adjusted to lymphocyte concentration of approximately 5×10^6 , trypan blue staining, viable cell count should be greater than 85%; the determination of each tube and control tube was added 100 μ L whole blood was added PBS diluted to 200 μ L, was added to the monoclonal antibody 20 μ L, mix gently; incubated in the dark at room temperature 20 min; hemolytic agent added 1.5 ml, mix gently, destruction of red blood cells, and incubated at room temperature in the dark 15 min to complete transparency, join PBS wash, 1500 rpm for 5 min the supernatant was discarded, repeated three times; 0.1% formaldehyde solution 1 ml fixed, 2 h inside the machine detected. FCM laser as Asian cold laser, wavelength 488 nm, using the flow-check to adjust the optical path, using the same type of control tune voltage to SSC (Side Scatter) of the horizontal axis, FSC (forward scatter) as ordinate, Construction scatter plot on the scatter plot analysis using gating lymphocyte populations CD3⁺, CD4⁺, CD8⁺ and CD16⁺CD56⁺ antigen expression, each specimen count 1×10^5 cells.

The collection and test methods of HPV genotyping followed reference [4], HPV high-risk subtypes include: 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82 and 83.

Cells immune function was measured during the same time of HPV genotyping.

Statistical analysis

Measurement data were expressed as mean \pm standard deviation ($\bar{x} \pm s$) and analyzed by statistical software SPSS 19.0. The difference between two samples was compared using t

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Table 1. The numbers of lymphocyte subsets and NK cells in peripheral blood of patients with cervical lesions and with high-risk HPV infection ($X \pm s$)

Group	n	CD3 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁺ /CD8 ⁺	CD16 ⁺ CD56 ⁺
Normal control	212	68.13±8.37	36.59±7.34	23.53±5.17	1.56±0.57	16.48±5.79
CIN I	86	67.87±8.25	36.18±7.11	24.14±5.33	1.50±0.55	16.26±4.85
CIN II	156	67.41±8.23	35.86±6.72	24.79±5.52	1.45±0.51	15.91±4.57
CIN III	201	66.72±8.18	35.29±6.55	25.54±5.83	1.38±0.48*	15.43±4.47
Cervical cancer I	261	65.84±8.12	34.78±6.33	26.27±5.66	1.32±0.45*	14.66±4.11
Cervical cancer IIa	379	65.27±8.03	34.21±6.25	26.55±6.08*	1.29±0.41*	13.53±3.72
Cervical cancer IIb	337	64.11±7.62	32.34±6.12*	27.61±6.16*	1.17±0.39**	12.84±3.58*
Cervical cancer IIIa	252	62.74±7.46	30.95±5.68*	28.59±6.32*	1.08±0.37**	11.51±3.46**
Cervical cancer IIIb	191	61.86±7.41*	30.28±5.37**	29.67±6.75**	1.02±0.34**	10.82±3.38**
Cervical cancer IV	92	60.81±7.31**	28.96±5.31**	30.43±7.28**	0.95±0.33**	10.25±3.22**
F		1.726	5.648	7.153	17.849	13.741
P		>0.05	<0.01	<0.01	<0.01	<0.01

Compared with normal control group: *P<0.05, **P<0.01.

Table 2. The number of lymphocyte subsets and NK cells in peripheral blood of patients with cervical cancer of different histopathological differentiation and with high-risk HPV infection ($X \pm s$)

Differentiation	n	CD3 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁺ /CD8 ⁺	CD16 ⁺ CD56 ⁺
Well-differentiated	354	64.93±8.11	33.99±6.07	26.41±6.05	1.29±0.45	12.96±3.75
Middle-differentiated	762	64.47±7.78	32.84±6.02	27.55±6.23	1.19±0.39*	12.86±3.63
Poorly-differentiated	396	62.24±7.34	30.61±5.96*	29.17±6.36*	1.05±0.34**	12.12±3.56
F		0.000	0.852	0.731	2.784	0.000
P		>0.05	>0.05	>0.05	<0.05	>0.05

Compared with well-differentiated group: *P<0.05, **P<0.01.

test, and that among multiple samples was compared using ANOVA, followed by q test for post hoc compare. Spearman rank correlation analysis was used for the correlation between the cervical squamous cell carcinoma stage and the number of T lymphocyte subsets and NK cells. P<0.05 was considered statistically significant.

Results

The number of lymphocyte subsets and NK cells in peripheral blood of patients with cervical lesions and with high-risk HPV infection were shown in **Table 1**. From which, the number of CD4⁺, CD16⁺CD56⁺ and CD4⁺/CD8⁺ ratio decreased according to the severity of cervical lesions, while CD8⁺ cell count was gradually increased according to the severity of cervical lesions, with statistically significant difference (P<0.01). CD3⁺ was decreased with the severity of cervical lesions, with no significant statistical difference (P>0.05).

The number of lymphocyte subsets and NK cells in peripheral blood of patients with cervical cancer of different histopathological differentiation and with high-risk HPV infection was shown in **Table 2**. From which, the number of CD3⁺, CD4⁺, CD4⁺/CD8⁺, CD16⁺CD56⁺ were gradually decreased with the poor differentiation, CD8⁺ was gradually increased with it but no significant difference (P>0.05). Further q tests found, CD4⁺, CD8⁺ and CD4⁺/CD8⁺ ratio were statistically significant different between poorly-differentiated group and well-differentiated group.

The number of lymphocyte subsets and NK cells in peripheral blood of patients with and lymph node metastasis or not and with high-risk HPV infection were shown in **Table 3**. From which, the number of CD4⁺, CD4⁺/CD8⁺ and CD16⁺CD56⁺ were significantly lower in patients with lymph node metastasis than those in patients without lymph node metastasis, with significant differences (P<0.01); and on the

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Table 3. The number of lymphocyte subsets and NK cells in peripheral blood of patients with cervical cancer and lymph node metastasis or not and with high-risk HPV infection ($X\pm s$)

Lymph node metastasis	n	CD3 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁺ /CD8 ⁺	CD16 ⁺ CD56 ⁺
Negative	919	65.79±7.93	34.51±6.11	25.70±6.03	1.34±0.40	14.28±3.72
Positive	593	61.21±7.45	29.45±5.75	30.82±6.51	0.96±0.37	10.22±3.52
t		1.428	3.765	3.831	17.946	4.519
P		>0.05	<0.01	<0.01	<0.01	<0.01

contrary, the number of CD8⁺ was significantly higher in patients with lymph node metastasis than those in patients without lymph node metastasis, with significant differences ($P<0.01$). However, the number of CD3⁺ cells between the two groups was not significant difference ($P>0.05$).

Correlation analysis between cervical cancer stage and the numbers of T lymphocyte subsets and NK cell. The peripheral blood T lymphocyte subsets and NK cell numbers was correlated with cervical squamous cell carcinoma stage, in which, CD4⁺, CD16⁺/CD56⁺ and CD4⁺/CD8⁺ ratio was negative correlated with the stage (CD4⁺: $r=-0.532$, $P<0.01$; CD16⁺/CD56⁺: $r=-0.617$, $P<0.01$; CD4⁺/CD8⁺: $r=-0.745$, $P<0.01$); CD8⁺ cell number was positively correlated with the stage of cervical squamous cell carcinoma ($r=0.596$, $P<0.01$).

Discussion

In recent years, the incidence of cervical cancer is gradually increased and with an increase trend in younger population and it has become one of the most common gynecologic cancer after breast cancer in most developing countries [5]. According to studies, the new cervical cancer case around the world is about 500,000 each year, in which 130,000 from China, and it is a serious threat to the health and safety of Chinese women [6]. Although the possibility of cervical lesions after infection with high-risk HPV to develop into cervical cancer increased significantly, over 90% of patients with normal immune HPV infection naturally heal after two years. So the vast majority of women with normal immune function had rarely persistent HPV infection and cervical cancer.

T lymphocytes by function and leukocyte grade antigen can be divided into suppressor T lymphocytes, namely CD8⁺T cells, and helper T lymphocytes, namely CD4⁺T cells, from lymphoid stem cells in the thymus and is the largest number of lymphocytes with the most complex func-

tion. All CD3 molecules in human T lymphocytes, T lymphocytes are important identification mark. In the peripheral blood, CD4⁺ represents a killing effect of T lymphocytes by secreting cytokines to assist and enhance the ability of immune cells to kill tumor cells, and has the function of immune memory and direct killing tumor cells; CD8⁺T lymphocytes represents inhibition cells, plays a negative role in the regulation of immune responses; these two cells often coordinate in killing tumor, mutual restraint, to participate in regulation of the body's immune response Only a proper ratio of the two types of cells effectively play normal anti-tumor effect. CD16⁺/CD56⁺ cells are cells involved in killing target cells that does not need antibodies or a pre-sensitized antigen in lymphocyte, it can be quickly activated, suppress and kill tumor cells, and it will has stronger killing effect after activated by lymphatic factor. In the anti-tumor process, CD16⁺/CD56⁺ cells play an important roles, it is mainly presented in the peripheral blood, spleen and bone marrow, while participating in the innate immune response and the adaptive immune response [7]. Previous studies showed that tumor development and the immune status of the host cell are closely related, and the anti-tumor immunity of host plays the dominant effect. In particular, imbalance of CD4⁺ and CD8⁺ cells, changes in CD16⁺CD56⁺ function are significant associated with tumor cell development, proliferation [8]. The anti-tumor mechanism of body mainly depends on the specific killing effect of T lymphocyte and the non-specific effect of natural killer cells, so that the number of T cell subsets and NK cells is an important index of cellular immune function.

Previous study have reported about patients with cervical cancer in peripheral blood T lymphocyte subsets and NK cell [9, 10], but there is lack of study of about the cells immune function in patients with high-risk HPV infection and different cervix lesion. This study found that the number of CD3⁺, CD4⁺, CD16⁺CD56⁺ and CD4⁺/CD8⁺ ratio were lower in patients with high-risk

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HPV infection than those in the control group, which were progressively reduced with the increasing of severity; and CD8⁺ cell number was higher than the control group, and was gradually increased with the increasing of severity, with statistically significant difference, except CD3 (P<0.01). These results indicated that the increases in cervical disease severity in patients with high-risk HPV infection leads to the gradually decreases of cellular immune function. The pair wise comparison results with the control group showed that, the differences of CD3⁺, CD4⁺, CD8⁺, CD16⁺CD56⁺ and CD4⁺/CD8⁺ ratio were in cervical squamous cell IIIb, IIb, IIa, I and CIN III were statistically significant (**Table 1**), while the other groups were not statistically significant. CD4⁺/CD8⁺ ratio is the most sensitive indicator of the cellular immune function in patients with high-risk HPV infection and severe cervical lesions.

A total of 443 cases were in CIN group, the cell numbers of CD3⁺, CD4⁺, CD8⁺, CD4⁺/CD8⁺ and CD16⁺/CD56⁺ were 67.19±8.21, 35.66±6.72, 25.01±5.62, 1.43±0.49, 15.76±4.58, respectively; and there was no control group statistically significant (P>0.05). These results showed that the cells immune function in patients with high-risk HPV infection had no significant change. At this point if conducting local treatment but also active immunotherapy, enhance or improve cellular immune function in patients, it is possible to prevent further development of disease. A total of 1512 cases of cervical squamous cell carcinoma, the cell numbers of CD3⁺, CD4⁺, CD8⁺, CD4⁺/CD8⁺ and CD16⁺/CD56⁺ were 63.99±7.74, 32.53±5.97, 27.71±6.22, 1.18±0.39, 12.69±3.64, statistically significant different from those in control group, except CD3⁺ (P<0.05), indicating that the immune cell function in patients with cervical squamous cell carcinoma has been gradually declining. Further analysis revealed that CD3⁺ and CD4⁺ was not statistically significant different between CIN group and cancer group (P>0.05); and CD8⁺, CD4⁺/CD8⁺ and CD16⁺/CD56⁺ ratio were statistically significant different between the two groups (P<0.05); indicating that patients with cervical cancer group with high-risk HPV infection had significantly lower cellular immune function, particularly in advanced squamous cell carcinoma. These results indicated that once high-risk HPV infection causes squamous cell carcinoma, local treatment should take together with immunotherapy, which may help to

prevent the development or rehabilitation of disease.

The results of T lymphocytes and NK cells in patients with high-risk HPV infection and cervical lymph node metastasis showed that patients with lymph node metastasis had significantly lower cellular immune function than those patients without lymph node metastasis. This may also indicates they may reinforce each other, namely the development of cancer can significantly reduce or inhibit the cellular immune function of patients, while reduced cellular immune function cause lymph node metastasis. It is suggested that when performing local or systemic treatment of tumors, combined with immunotherapy helps to achieve a more favorable therapeutic effect. For high-risk HPV in cervical squamous cell carcinoma patients infected with different tissue grade lymphocyte subsets and NK cells were found between the groups was not statistically significant (P>0.05). It means that cellular immune function has no significant correlation with the grade of tumor. However, CD4⁺, CD8⁺ and their ratio were significant differences in well-differentiated group and poorly-differentiated group (P<0.05). These results indicated that the cellular immune function in patients with poorly-differentiated cervical squamous cell carcinoma than those in control group. This may be due to that low-differentiated grade cervical squamous cell carcinoma has higher metastasis activity, and further lead to the decrease of the immune function of host.

The correlation between peripheral blood T lymphocyte subsets and NK cell numbers and cervical squamous cell carcinoma stage showed that, the reduce of CD4⁺, CD16⁺CD56⁺ cells and CD4⁺/CD8⁺ ratio was negatively correlated with the cervical squamous cell carcinoma stage (P<0.01); while CD8⁺ cells was positively correlated the cervical squamous cell carcinoma stage (P<0.01). It suggests that patients with worse cellular immune function have more severe disease and more rapid development. And the possibility of lymph node metastasis or distant metastasis is also higher, and should arouse the attention of clinicians. Secondly, for patients with worse cellular immune function, the clinical treatment should focus on adapting or strengthen cellular immune function, which will leads better treatment.

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In summary, it is a simple method to analyze the cell immune function in patients with cervical cancer using FCM, not only helps to understand the cellular immune function in patients, but also helps to determine the severity of the disease. In particular, the changes CD4⁺/CD8⁺ ratio can provide valuable reference for clinical treatment, thereby enhancing the therapeutic effect of cervical cancer. However, it needs further study to determine whether reduction of cellular immune function in patients is the cause or the result of the occurrence and development of tumor.

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Disclosure of conflict of interest

None.

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