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The research on the influences of hyperthermal perfusion chemotherapy combined with immunologic therapy on the immunologic function and levels of circulating tumor cells of the advanced colorectal cancer patients with liver metastasis

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Abstract. – OBJECTIVE: To investigated the influence of hyperthermal perfusion chemotherapy combined with immunologic therapy on the immunologic function and levels of circulating tumor cells of the advanced colorectal cancer patients with liver metastasis.

PATIENTS AND METHODS: We enrolled 98 advanced colorectal cancer patients with liver metastasis that were admitted to this hospital for treatment and were randomly divided into two groups, the observation group (n = 49) and the control group (n = 49). We administered systemic vein chemotherapy for patients in the control group, and hyperthermal perfusion chemotherapy for the patients in the observation group in order to compare the subgroup levels of T lymphocytes, NK cells and immunoglobulin (IgG, IgA, and IgM) in the immune system of patients in both groups. We also assayed the circulating tumor cells (CTC) in the peripheral blood of patients in both groups using the cell search method, and compared the efficacy using response evaluation criteria in solid tumors and the survival rates of patients in both groups using the Kaplan-Meier method.

RESULTS: After two treatment courses, the levels of CD3+, CD4+ and CD4+/CD8+ of the patients in the observation group were significantly higher than those of the control group, but the levels of CD8+ of patients in the observation group was lower than that in the control group ($p < 0.05$). The levels of immunoglobulins (IgG, IgA, and IgM) in the observation group were higher than the control group ($p < 0.05$). The levels of NK cell cells were significantly lower than the control group ($p < 0.05$). The objective response rate, as well as the disease control rate of the observation group, were remarkably higher than those of the control group ($p < 0.05$). Compared to the control group, the observation group enjoyed a prolonged survival time, higher survival rate and significantly lower positive rate of CTC ($p < 0.05$).

CONCLUSIONS: Better efficacy and tolerance, fewer toxic and side effects, improvement in the immunologic functions of patients for the indirect anti-tumor effect, a significant decrease in CTC of patients, and a higher long-term survival rate have been achieved in the treatment with hyperthermal perfusion chemotherapy combined with immunologic therapy for the advanced colorectal cancer patients with liver metastasis. Thus, it can serve as the preferable drug for the treatment of advanced colorectal cancer with liver metastasis.

Key Words:
Hyperthermal perfusion chemotherapy, Immunologic therapy, Advanced colorectal cancer patients with liver metastasis, Immunologic function, Circulating tumor cells.

Introduction

As a common malignant tumor in the digestive system, colorectal cancer, due to its atypical clinical symptoms at an early stage, is apt to be neglected, leading to a delay in diagnosis and treatment. Thus, colorectal cancer is usually diagnosed in the mid- or late-stage. Liver is the most

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frequently susceptible site to the distant metastasis of colorectal cancer, and almost 20% of patients are found with liver metastasis at the time of surgery for the primary tumor lesion and about 50% of the patients have been found with liver metastasis 5 years after the surgery with poor prognosis[1,2]. Therefore, increasing the survival rate and prophylaxis for local recurrence as well as liver metastasis has become a major challenge in clinical practice. Generally, advanced colorectal cancer patients with liver metastasis have been clinically treated with comprehensive therapies, such as surgery combined with chemotherapy or immunologic therapy[3]. For advanced colorectal cancer patients with liver metastasis, anti-tumor drugs are not strongly irritable due to their poor physical conditions as well as the decreased immunological functions; hence, hyperthermal perfusion chemotherapy combined with immunologic therapy has been frequently adopted for such patients[4]. Circulating tumor cells (CTC) refer to tumor cells that are released by solid tumors into the blood circles due to various reasons during the occurrence and development of tumors. The research on CTC, firstly formulated in the 1960s, contributes further to the understanding of the metastasis mechanism of the tumor, which can be taken as a new theoretical basis for the treatment of tumor and reduction of the recurrence and metastasis[5,6]. In this study, we acquired better efficacy in the treatment of hyperthermal perfusion chemotherapy combined with immunologic therapy for the advanced colorectal cancer patients with liver metastasis and the relevant information is reported as follows.

Patients and Methods

Sample Selection

We enrolled 98 advanced colorectal cancer patients that were admitted to and treated at our hospital and that were randomly divided into the control group (n = 49) and the observation group (n = 49). Inclusion criteria were as follows: a) patients who were diagnosed with advanced colorectal cancer according to pathological examination and CT scan, b) patients with liver metastasis and c) patients who had signed the written informed consent. This study was approved by the Ethics Committee of Shengli Hospital of Shengli Petroleum Administration of Sinopec Group. Signed written informed consents were obtained from the patients. The general materials of the patients in both groups are shown in Table I.

Methods

Treatment

For patients in the control group, we administered intravenous chemotherapy through the following procedures: for the first day, injection of standard dosage (130 mg/m^2) of oxaliplatin was administered; for the period between the 1st day and 21st day, a standard dosage of capecitabine (1 g/m^2/d) was administered twice a day for 21 days, which was a chemotherapy cycle.

For patients in the observation group, we adopted the accurate hyperthermal perfusion chemotherapy combined with immunologic therapy due to the following procedures: the oxaliplatin (dosage: 130 mg/m^2) was dissolved in 3000 mL of 9% sodium chloride, and the solution was perfused into a bag to be heated. The heated solution was perfused into the abdominal cavity through the extracorporeal circulation (Temperature: 43°C; speed: 500 ml/min; time: 90 min) for treatment. At the 8th and 15th days, ammonium pyrrolydine dithiocarbamate (APDC) (1×10^7) was sequentially perfused through the vein for immunologic treatment, and 21 days were set as a chemotherapy cycle. All patients in both groups underwent two treatment cycles, and we determined the efficacy as well as the relevant indicators.

Assay of the Indicators

After 2 cycles of treatment, the serum was separated from 3-5 mL of fasting venous blood collected from patients in both groups. Then, monocytes were isolated by the Ficoll method. The sample was incubated with the antibodies of CD3, CD4, CD8, CD16 and CD56 (manufactured by Shanghai Yantuo Biotechnology Co., Ltd, Shanghai, China) for 30 min at 4°C in a dark environment. The ratio of CD^{4+}/CD^{8+}werecalculated with the results of detection using flow cytometry (BD Biosciences, San Jose, CA, USA). The levels of immunoglobulins (IgG, IgA, and IgM) of patients were assayed using immunoturbidimetry strictly under the manufacturer's instructions (Weifang Guarantee Biological Technology Co., Ltd, Weifang, Shandong Province). The content of IgG, IgA, and IgM in the samples was calculated using the turbidity of the reaction liquid with the standards as the reference. The assay of CTC was performed using the CellTracks® AutoPrep® CTC detector (Johnson & Johnson, New Brunswick, NJ, USA) with the kits of CTC (Johnson & Johnson, New Brunswick, NJ, USA) in following procedures: epithelial cells expressing the EpCAM were

Table I. General information of the subjects.

Item	The control group (n = 49)	The observation group (n = 49)	t/X^2	*p*
Gender (M/F)	21/28	19/30	0.042	0.837
Age (Year)	40-70	40-75		
Average age (Year)	56.36±7.49	55.85±7.58	0.335	0.738
Differentiation degree [n(%)]				
Highly differentiated adenocarcinoma	23 (46.93)	20 (40.81)	0.408	0.815
Moderately differentiated adenocarcinoma	15 (30.61)	16 (32.65)		
Poorly differentiated adenocarcinoma	11 (22.44)	13 (26.53)		
Primary lesion site of tumor [n(%)]				
Colon	29 (59.18)	27 (58.33)	0.459	0.795
Rectum	13 (26.53)	16 (32.65)		
Juncture between the rectum and sigmoid	7 (14.28)	6 (12.24)		
Size of the metastasis lesion in liver (cm)	3.68±1.59	3.85±1.68	0.514	0.608
Quantity of the metastasis lesions in liver (n)				
≤3	29 (59.18)	31 (63.26)	0.043	0.835
>3	20 (40.82)	18 (36.74)		

collected using magnetic activated cell sorting (MACs). Fixation and permeabilization were performed for the collected epithelial cells followed by the immunostaining with the antibodies of CD45 and cytokerins. Then, the cell nucleus was identified using DAPI staining. Thereafter, the stained cells were analyzed and counted under the fluorescence microscope, in which we defined all of the CK+DAPI+CD45- cells as CTC.

Evaluation Criterion

After 2 courses of treatment, the serum was separated from 3-5 mL of the fasting venous blood collected from patients in both groups. Then, the levels of the subgroups of T lymphocytes (CD^{3+}, CD^{4+}, CD^{8+}, and CD^{4+}/CD^{8+}) as well as the NK cells were assayed using flow cytometry (BD, USA); and the levels of immunoglobulins (IgG, IgA and IgM) were tested using immunoturbidimetry.

CTCs in peripheral blood of patients were measured using the cellsearch method. Cells that met the following criteria were categorized as CTCs: a) the marker of epithelial cells was positive, and most of the epithelial cells were oval or round shaped, occasionally slender shaped or polygon, and were intact with a length of greater than 4 μm; b) positive results were obtained through the staining (DAPI) of the nucleus; the area of the nucleus was smaller than that of the cytoplasm and not less than 50% of the nucleus was inside the cytoplasm; c) the marker of white blood cell was (CD45/APC) negative and the blank channel was also negative. The criteria for determining the positive and negative CTCs include: a) the peripheral blood containing CTC≥2/7.5 ml was positive; and b) the peripheral blood containing CTC<2 was negative.

For efficacy evaluation, we refer to the evaluation criteria of efficacy for solid tumors, which were divided into four degrees, i.e. complete remission (CR), partial remission (PR), stable disease (SD) and progression of disease (PD). The objective response rate (ORR) = (the cases of CR + the cases of PR)/total cases; the disease control rate (DCR) = (the cases of CR + the cases of PR + the cases of SD)/total cases.

Statistical Analysis

We used the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) for data processing. The data related to the subgroup levels of T lymphocytes, NK cells, and immunoglobulins (IgG, IgA, and IgM) were presented as $X \pm s$ and analyzed using the *t*-test. The condition of CTC, as well as the efficacy, were presented as % and analyzed using the chi-square test. The analysis of the survival of patients was performed using Kaplan-Meier. $p < 0.05$ suggested that differences were statistically significant.

Results

Through conducting the comparison of the subgroup levels of T lymphocytes of patients in both groups after two courses of treatment, we found that the levels of CD^{3+}, CD^{4+} and CD^{4+}/CD^{8+} in the observation group were higher than those in the control group, and the level of CD^{8+} in the observation group was lower than that in the con-

Table II. Comparison of the subgroup levels of T lymphocyte as well as the NK cells of patients in both groups

Group	Cases	CD3+	CD4+	CD8+	CD4+/CD8+	NK
The observation group	49	70.53±5.56	40.83±5.46	24.38±3.67	1.68±0.47	12.46±3.38
The control group	49	64.48±5.32	37.47±5.72	26.35±3.65	1.43±0.38	24.65±3.47
t		5.503	2.974	2.664	2.895	17.615
p		<0.0001	0.003	0.009	0.004	<0.0001

Table III. Comparison of the immunologic indexes of patients in both group.

Group	Cases	IgA	IgG	IgM
The observation group	49	1.23±0.42	8.15±2.64	0.59±0.26
The control group	49	0.68±0.36	6.62±2.43	0.81±0.47
t		6.960	2.985	2.867
p		<0.0001	0.003	0.005

Table IV. Comparison of the positive and negative expression of CTCs between the two groups

Group	N (cases)	CTC expression	Negative expression (cases)	Positive (cases) expression
The observation group	49	41	8	16.32%
The control group	49	31	18	36.73%
X^2			4.240	
p			0.039	

trol group. The levels of NK cells in the observation group were significantly lower than that in the control group; differences in these intergroup comparisons were statistically significant ($p < 0.05$) (Table II and Figure 1).

In the comparison of immunologic indexes of patients in both groups after two courses of treatment, we found that the levels of immunoglobulin indexes (IgA, IgG and IgM) in the observation group were significantly higher than those in the control group with statistically significant differences ($p < 0.05$) (Table III).

In the comparison of the CTC levels in both groups, the positive expression rate (71.05%) of the observation group was significantly higher than that of the control group (36.84%) with a statistically significant difference ($p < 0.05$) (Table IV and Figure 2).

When comparing the efficacy of patients in both groups, the ORR and DCR in the observation group was 67.34% and 87.76%, respectively, which was significantly higher than those in the control group at 36.72% and 65.07%, respectively (X^2=11.383, p=0.009) (Table V).

For the analysis of the survival rate of patients in both groups using Kaplan-Meier analysis, the survival time of patients in the observation group were significantly higher than that in the control group (X^2=4.789, p=0.027) (Figure 3).

Discussion

Most patients with colorectal cancer are diagnosed at the mid- or advanced stage at the time of diagnosis due to non-specific symptoms at the

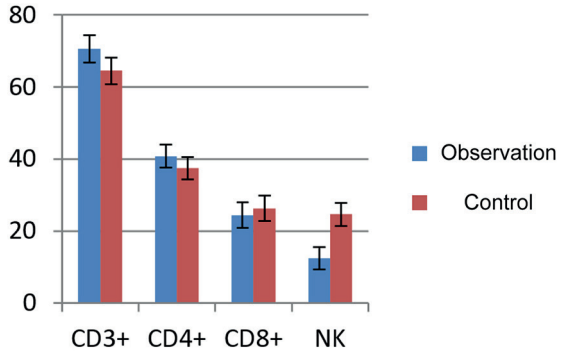

Figure 1. Standard deviation figure of the subgroup levels of T lymphocyte as well as the NK cells of patients in both groups.

Table V. Comparison of the efficacy of patients in both groups.

Group	Cases	CR	PR	SD	PD
The observation group	49	18 (36.73)	15 (30.61)	10 (20.41)	6 (12.24)
The control group	49	7 (14.28)	11 (22.44)	14 (28.57)	17 (34.93)

early stage[7]. Relevant research has revealed that liver metastasis was identified in 10% or more of the patients in the first diagnosis of colorectal cancer, the incidence of liver metastasis remains as high as 20% in patients who have accepted the radical resection, and about 25-50% of patients suffer from the recurrence of colorectal cancer due to liver metastasis even after the surgery[8-11]. The potential pathogenesis of liver metastasis involves residual micro-lesions and injured lymphatic vessels that accumulate in the abdominal cavity of patients and are released into the blood or lymph, leading to the distant metastasis of the tumor cells[12].

The Value of Hyperthermal Perfusion Chemotherapy Combined with Immunologic Therapy

Currently, the hyperthermal perfusion chemotherapy, as a type of promising comprehensive treatment, can increase the local dosage of drugs and exert a direct effect inside the abdominal cavity of patients, which can be applied to rapid and effective prophylaxis and treatment for the local recurrence and metastasis. The drugs are delivered through the veins of parietal peritoneum into the post-caval veins, resulting in a drug concentration in the liver that is much higher than that in the regular venous chemotherapy; this can effectively control the liver metastasis of tumor[13]. However, the temperature should be accurately regulated in hyperthermal perfusion chemotherapy. For instance, the cytotoxicity of some chemotherapeutics (Platinum drugs or anthracyclines) can be increased at a temperature higher than 45°C, which can bring irreversible damage to normal tissues. While at a temperature lower than 41°C, the hyperthermal perfusion chemotherapy shows poor efficacy. According to relevant studies, it has been confirmed that at a temperature of 42-44°C, drugs are more easily absorbed and can induce the apoptosis of tumor cells by activating the release of lysosomes[14]. The immunologic therapy based on the dendritic cells (DC) can be used for the treatment of tumors by its regulatory effects on the autonomous anti-tumor immunology in order to enhance the original anti-tumor mechanism, which has become a hotspot in the research of biotherapy[15]. Currently, the DC is known as the most powerful and effective antigen-presenting cell in the body, which is also the sole factor for activating native T cells for the ability to activate the CD^{4+} and CD^{8+}[15]. According to relevant research, it has been proven that not only can the hyperthermal perfusion chemotherapy, combined

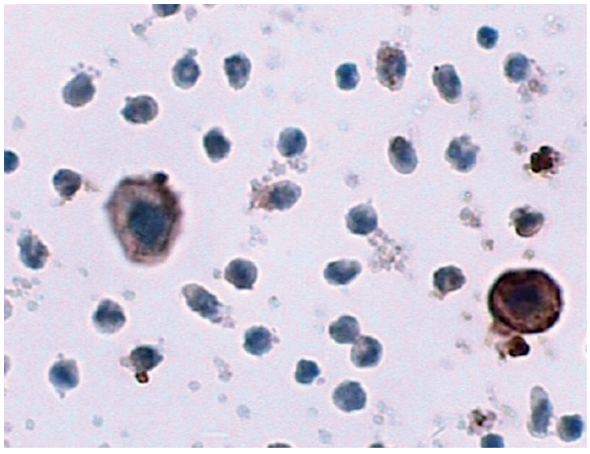

Figure 2. Positive expression of CTC in peripheral blood of the patients.

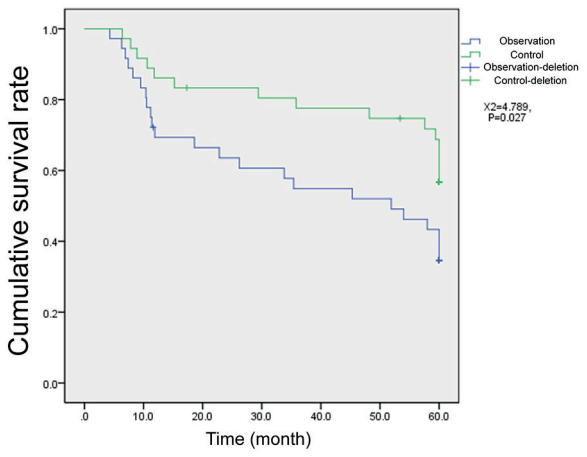

Figure 3. Kaplan-Meier survival analysis of the patients in both groups.

with immunologic therapy, effectively regulate and enhance the autonomous anti-tumor immunologic reactions of the body, but also reduce the drug-tolerance of patients and the toxicity of chemotherapy, thereby achieving a synergistic therapeutic effect much better than the sole application of immunologic therapy or hyperthermal perfusion chemotherapy[16,17]. The results of this study reveal that the ORR and DCR in the observation group were significantly higher than those in the control group, in which the patients only accepted the venous chemotherapy ($p < 0.05$).

Immunologic Function of the Colorectal Cancer Patients with liver Metastasis

The occurrence and development of colorectal cancer is closely related to immunologic functions. The results of various studies show that the subgroup levels of the T lymphocytes are in a deep connection with the occurrence, development and metastasis of tumors; i.e., patients with a weakened immunologic function are more susceptible to the postoperative recurrence and metastasis of the tumors. These patients usually suffer from an imbalanced immunologic functions with the manifestations of disturbance of cellular immunologic functions, that are mainly characterized by the alternations in the levels of the subgroups of T lymphocytes, in which the CD^{3+} represents all of the T lymphocytes, CD^{4+} represents the inductive subgroup of T cells positively regulating the immunologic function, CD^{8+} represents the inhibitory subgroup of T cells which, in their active state, can damage and kill the infectious cells and show an inhibitory effect on the CD^{4+}, resulting in the disturbance of immunologic functions. Thus, the ratio of CD^{4+}/CD^{8+} can reflect the cellular immunity state and the immunologic response of the body to some degree[18,19]. Among the immunoglobulins that exist in the body fluid, IgG is a type of anti-bacteria and anti-virus antibody, which plays a major role in the anti-infection reaction. IgA, together with the surrounding cells, can constitute a local immunologic system to protect the body from infection. IgM is a type of highly efficient antibody, which acts as an important role in the early defense of body with the ability of bacteriolysis, aggravation and phagocytosis[20]. The results of this study indicate that the subgroup levels of the T lymphocytes, the levels of the NK cells and the improvement of immunoglobulins of the patients in the observation group were significantly higher than those of the control group ($p < 0.05$), suggesting that the patients enjoyed an optimized immunologic function, decreased postoperative recurrence rate and improved survival quality. Moreover, the 5-year survival rate of patients in the observation group was also significantly higher than those in the control group.

The Significance of CTC Assay for the Advanced Colorectal Cancer Patients with Liver Metastasis

Most of the tumor originates from epithelial cells. Tumor cells in the blood system are able to be recognized and detected due to the differences between epithelial cells characteristics and those of the circulating cells. With the continuous improvement of CTC assay technology, the value of CTC is increasingly important to the clinical practice. Various studies[21] have revealed that the correlation between the levels of CTC and clinical pathological characteristics of advanced colorectal cancer makes CTC a marker for the tumor. Thus, the measurement of CTC in the peripheral blood of advanced colorectal cancer patients with liver metastasis can assist the physicians to perform a diagnosis and treatment for the patients, and the expression of CTC can also be served as the theoretical reference for the selection of the therapy options.

Conclusions

For advanced colorectal cancer patients with liver metastasis, the hyperthermal perfusion chemotherapy, combined with immunologic therapy, can effectively improve their immunologic functions and the efficacy in the treatment of cancer and prolong the survival time with characteristics such as safety, simplicity and efficiency. Thus, it is an effective and comprehensive method for the treatment of advanced colorectal cancer with liver metastasis. However, due to the limited sample size in this study, further studies have focused on the long-term efficacy if this method is still necessary.

Conflict of interest

The authors declare no conflicts of interest.

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