Variation of NK, NKT, CD4⁺ T, CD8⁺ T cells, and IL-17A by CalliSpheres® microspheres-transarterial chemoembolization in refractory liver metastases patients

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ABSTRACT

Immune environment plays an important role in the management of liver cancer. The current study aimed to explore the change of NK and NKT cells, IL-17A, CD4⁺ T and CD8⁺ T cells in refractory liver metastases patients before and after CalliSpheres® microspheres-transarterial chemoembolization (CSM-TACE). Peripheral blood (PB) samples from 35 refractory liver metastases patients were collected before CSM-TACE (baseline), 2 days (D2) and 5 days (D5) after CSM-TACE. Then, NK and NKT cells, IL-17A, CD4⁺ T and CD8⁺ T cells from PB samples were detected. All enrolled patients successfully completed CSM-TACE procedure and achieved disease control rate of 100% after 1 month. NK cells were increased from baseline to D2 and D5 [median (range): 5.88% (1.53%-12.05%) vs. 9.54% (5.19%-15.71%) vs. 7.12% (2.77%-13.29%)], NK cells were also enhanced from baseline to D2 and D5 [median (range): 14.35% (5.85%-20.52%) vs. 20.36% (15.88%-27.30%) vs. 30.82% (22.18%-37.72%)], while IL-17A was declined from baseline to D2 and D5 [median (range): 22.11 (9.46-39.18) pg/ml vs. 12.41 (3.24-26.84) pg/ml vs. 6.55 (1.11-20.98) pg/ml]. Furthermore, IL-17A was negatively correlated with the NK and NKT cells at baseline, D2 and D5 (all p < .05), respectively. Additionally, CD4⁺ T cells and CD4⁺ T/CD8⁺ T ratio were increased while CD8⁺ T cells were declined from baseline to D2 and D5 (all p < .05). NK cells, NKT cells, and CD4⁺ T cells are increased but IL-17A and CD8⁺ T cells are declined after CSM-TACE in refractory liver metastases.

Introduction

Liver is a common site of metastases in solid tumors [1,2]. The occurrence of liver metastases is far more than that of primary liver cancer, which is responsible for most tumor-related deaths [1–3]. In recent years, oligo liver metastasis is potentially curable; however, the treatment is limited for patients with refractory liver metastases whose tumor either is unresectable or progresses after treatment [4,5]. Currently, CalliSpheres® microspheres-transarterial chemoembolization (CSM-TACE) is effective and safe in treating refractory liver metastases, which is partly because CalliSpheres® presents impressive characteristics (including continuous chemotherapeutic drug-releasing property, great drug-loading ability, etc.) [6–8]. However, the survival profile of patients with refractory liver metastases after CSM-TACE is still unfavorable, with the median overall survival ranging from 20 months to 28 months [7–9]. Importantly, it has been reported that the immune environment plays an important role in the management of liver cancer [10]. Thus, the understanding of the variation of the immune environment by CSM-TACE might further help to improve the management of patients with refractory liver metastases.

Accumulating researches have reported that immune microenvironment plays an important role in liver cancer [11–15]. For instance, natural killer (NK) cells are important innate immune cells, whose activation and increment are able to suppress tumor growth and metastasis in liver cancer [11,12,15]; in the clinical field, both levels of CD4⁺ and CD8⁺ T cells are able to predict prognosis among patients with liver cancer [16,17]; moreover, patients with liver cancer possessing a higher level of IL-17A have a worse survival
Importantly, an interesting study has presented that regulatory T (Treg) cells and CD8⁺ T cells are decreased while CD4⁺ T cells are elevated in patients with liver cancer after TACE compared to its pre-treatment level, indicating immune microenvironment is restored after TACE [14]. Inspired by the above-mentioned data, we deduced that the immune microenvironment might also be changed among patients with refractory liver metastases after CSM-TACE, while related information is obscured.

Thus, the current study aimed to explore the variation of NK cells, NKT cells, IL-17A, CD4⁺ T, and CD8⁺ T cells among patients with refractory liver metastases receiving CSM-TACE.

Patients and methods

Patients

This study serially enrolled a total of 35 cancer patients with liver metastases who were about to receive CSM-TACE treatment in Minimally Invasive Tumor Treatment Center of our hospital from May 2020 to April 2021. The patients who met the following criteria were included: 1. confirmed as liver metastasis; 2. defined as refractory liver metastases if they met one of the following criteria: (i) experienced disease recurrence or progression after surgical resection or ablative therapy; (ii) the lesion was adjacent to gallbladder or hilum hepatitis, which was unable or unsuitable for surgical resection or ablative therapy; (iii) had more than three metastases in liver, or confirmed as diffuse liver metastases; (iv) was intolerance to systemic chemotherapy, or experienced disease progression after immunotherapy, or experienced disease progression after second-line chemotherapy; 3. within 18 to 85 years; 4. were about to receive CSM-TACE treatment; 5. Eastern Cooperative Oncology Group (ECOG) score 0 to 2. The exclusion criteria were set as: 1. had severe dysfunction of cardiopulmonary or liver, or had presence of severe infections; 2. pregnant and breast-feeding patients. The written informed consents were collected from all patients. The study was permitted by Ethics Committee.

Data collection

Clinical characteristics of all patients were obtained after enrollment, which included age, gender, ECOG score, Child-Pugh stage, type of hepatic metastasis, tumor number, tumor size, extrahepatic metastasis and primary cancer.

Sample collection and examination

Peripheral blood (PB) samples were collected from all eligible patients with heparin sodium anticoagulant blood collection tube before CSM-TACE, 2 days after CSM-TACE, and 5 days after CSM-TACE. Then, within 4 h after PB sample collection, sample detections were carried out. The levels of NK cells and NKT cells were detected using CD3 FITC/CD16 + CD56 PE/CD45 PerCP/CD19 APC with BD Trucount™ Tubes (BD, USA). The levels of CD4⁺ T cells and CD8⁺ T cells were examined using CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC with BD Trucount™ Tubes (BD, USA). Besides, the collected PB samples were used to isolate serum samples by centrifuge for 5 min, then the serum samples were applied for the detection of the level of interleukin-17A (IL-17A) by enzyme-linked immunosorbent assay (ELISA) using Human IL-17A DuoSet Kit (Bio-Technne China Co. Ltd., China). All experiments were operated in strict accordance with the manuals provided by manufactures.

Treatment and evaluation

All patients received computed tomography (CT) and magnetic resonance imaging (MRI) examinations for the evaluation of the targeted tumor according to the Milan criteria [18,19] (Figure 1(A)).

Before CSM-TACE operation, the chemoembolization materials were prepared: 1.0 g CalliSpheres® microspheres (Jiangsu Hengrui Medicine Co., Ltd., China) with a diameter of 100–300µm were mixed with 60 mg pirarubicin. The mixture was shaken up every 5 min for 30 min, then mixed with non-ionic contrast agent iodixanol injection by 1:1, followed by addition of gentamicin 80,000 units. Finally, the mixture was diluted 0–20 times for subsequent use.

After chemoembolization material preparation, CSM-TACE was performed, and the procedures were as follows: after puncture of femoral artery by Seldinger technique, the angiography for celiac trunk artery and common hepatic artery angiography were carried out under digital subtraction angiography (DSA) to detect tumor-supplying artery (Figure 1(B)). The intraoperative DSA images were compared to the preoperative images to confirm that all intrahepatic lesions and arteries were identified. Following that, super-selective catheterization was performed, and the prepared chemoembolization materials were slowly injected into the tumor-supplying artery (Figure 1(C)). The end point of embolization was the disappearance of tumor staining or the truncation of tumor-supplying artery (Figure 1(D)).

After CSM-TACE, CT or MRI examination were carried out again to dynamically observe the changes of targeted tumor in liver (Figure 1(E–F)). About 1 month after CSM-TACE, treatment response was evaluated as well according to the modified RECIST (mRECIST) [20]. For patients who were suitable for surgery after TACE treatment, surgery was operated and pathological examination was performed (Figure 1(G)), then CT or MRI examination were conducted again (Figure 1(H)).

Statistics

SPSS V.22.0 statistical (IBM Corp., USA) was used to complete statistical analysis, and GraphPad Prism V.6.0 (GraphPad Software Inc., USA) was applied to plot figures. Paired analysis was carried out using Wilcoxon signed-rank test. Correlation of two variables was determined using Spearman’s rank correlation test. p < .05 was considered statistically significant.
Results

Clinical characteristics

Among 35 patients, the mean age was 62.0 (ranging from 34.0 to 77.0) years. Meanwhile, there were 11 (31.4%) females and 24 (68.6%) males. Regarding Eastern Cooperative Oncology Group (ECOG) score, there were 25 (71.4%), 9 (25.7%), and 1 (2.9%) patient with a score of 0, 1, and 2, respectively. Furthermore, there were 10 (28.6%) patients with tumor numbers $\leq 3$ and 25 (71.4%) patients with tumor number $>3$. Besides, the tumor size of 13 (37.1%) patients was within 3-5 cm and that of 22 (62.9%) patients was $>5$ cm. In addition, 17 (48.6%) patients presented extrahepatic metastasis. More detailed characteristics of the patients were shown in Table 1.

Change of NKT cells, NK cells and IL-17A before and after CSM-TACE

The proportion of NKT cells was elevated on 2 days ($p < .001$) and 5 days ($p < .001$) after CSM-TACE compared to that before treatment [median (range): 5.88% (1.53%–12.05%) vs. 9.54% (5.19%–15.71%) vs. 7.12% (2.77%–13.29%)]; meanwhile, the proportion of NK cells was declined on 5 days after CSM-TACE compared to 2 days after CSM-TACE ($p = .026$) (Figure 2(A)). Furthermore, the proportion of NK cells was also enhanced on 2 days ($p < .001$) and 5 days ($p < .001$) after CSM-TACE compared to that before treatment [median (range): 14.35% (5.85%–20.52%) vs. 20.36% (15.88%–27.30%) vs. 30.82% (22.18%–37.72%)]; besides, the proportion of NK cells was increased on 5 days after CSM-TACE compared to 2 days after CSM-TACE ($p < .001$) (Figure 2(B)). However, IL-17A expression was decreased on 2 days ($p < .001$) and 5 days ($p < .001$) after CSM-TACE compared to that before treatment [median (range): 22.11 (9.46–39.18) ng/L vs. 12.41 (3.24–26.84) ng/L vs. 6.55 (1.11–20.98) ng/L]; meanwhile, IL-17A was declined on 5 days after CSM-TACE compared to 2 days after CSM-TACE ($p < .001$) (Figure 2(C)).

Correlation of IL-17A with NK and NKT cells

IL-17A level was negatively correlated with the proportion of NK cells before CSM-TACE ($r = -0.370, p = .028$) (Figure 3(A)), on 2 days after CSM-TACE ($r = -0.420, p = .013$)
(Figure 3(B)), and 5 days after CSM-TACE ($r = -0.370$, $p = 0.028$) (Figure 3(C)). Meanwhile, IL-17A was also negatively correlated with proportion of NKT cells before CSM-TACE ($r = -0.420$, $p = 0.011$) (Figure 3(D)), on 2 days after CSM-TACE ($r = -0.390$, $p = 0.020$) (Figure 3(E)), and 5 days after CSM-TACE ($r = -0.370$, $p = 0.028$) (Figure 3(F)).

**Change of CD4+ and CD8+ T cells before and after CSM-TACE**

The proportion of CD4$^+$ T cells was elevated on 2 days ($p < 0.001$) and 5 days ($p < 0.001$) after CSM-TACE compared to that before treatment. However, the proportion of CD8$^+$ T cells was decreased on 2 days ($p < 0.001$) and 5 days ($p = 0.010$) after CSM-TACE compared to that before treatment. Meanwhile, the ratio of CD4$^+$ T/CD8$^+$ T cells was increased on 2 days ($p < 0.001$) and 5 days ($p < 0.001$) after CSM-TACE compared to that before treatment (Table 2). Meanwhile, the ratio of CD4$^+$ T/CD8$^+$ T cells before treatment was not related to any tumor features (such as the child-pugh, metastasis etc.) (all $p > 0.05$) (Supplementary Table 1).

**Discussion**

In clinical field, accumulating studies have reported that immunotherapy has improved the treatment response and survival in liver cancer, indicating the alteration of the immune microenvironment plays a crucial role in liver cancer [21,22]; moreover, it has been reported that the immune microenvironment is involved in liver cancer and takes part in the progression of liver cancer [11–15]. Furthermore, NK cells, NKT cells, IL-17A, CD4$^+$ T and CD8$^+$ T cells have been illustrated to play important roles in the immune microenvironment of liver cancer [15,23–27]. For instance, NK cells are able to inhibit tumor growth in liver metastatic model mice [15]; moreover, it has been reported that increased NKT cells inhibit colorectal cancer liver metastases in mice [25]; in addition, IL-17A is a tumor-promoting cytokine in liver cancer, which accelerates angiogenesis in vivo and promote liver cancer cell invasion in vitro [23,24]; furthermore, CD4$^+$ T and CD8$^+$ T cells modulate tumor growth and metastases in liver cancer [26,27]. The above-mentioned data indicate that the exploration of change of these indexes after CSM-TACE might contribute to the understanding of the mechanism of CSM-TACE in patients with refractory liver metastases.

A previous study reports that Treg cells level is declined and the ratio of CD4$^+$ T/CD8$^+$ T cells is elevated among patients with liver cancer at 1-2 weeks and 3-5 weeks after TACE [14]; moreover, it also has been presented that TACE

**Table 1. Clinical characteristics.**

| Items                                      | Patients (N = 35) |
|--------------------------------------------|-------------------|
| Age (years), mean (range)                  | 62.0 (34.0–77.0)  |
| Gender, No. (%)                            |                   |
| Female                                     | 11 (31.4)         |
| Male                                       | 24 (68.6)         |
| ECOG score, No. (%)                        |                   |
| 0                                          | 25 (71.4)         |
| 1                                          | 9 (25.7)          |
| 2                                          | 1 (2.9)           |
| Child-Pugh class, No. (%)                  |                   |
| Class A                                    | 26 (74.3)         |
| Class B                                    | 9 (25.7)          |
| Type of hepatic metastasis, No. (%)        |                   |
| Simultaneous                               | 10 (28.6)         |
| Metachronal                                | 25 (71.4)         |
| Tumor number, No. (%)                      |                   |
| <3                                         | 10 (28.6)         |
| ≥3                                         | 25 (71.4)         |
| Tumor size, No. (%)                        |                   |
| 3-5 cm                                     | 13 (37.1)         |
| >5 cm                                      | 22 (62.9)         |
| Extrathepatic metastasis, No. (%)          |                   |
| Absent                                     | 18 (51.4)         |
| Present                                    | 17 (48.6)         |
| Primary cancer, No. (%)                    |                   |
| Colorectal cancer                          | 16 (45.7)         |
| Stomach cancer                             | 7 (20.0)          |
| Breast cancer                              | 4 (11.4)          |
| Pancreatic cancer                          | 3 (8.6)           |
| Ovarian cancer                             | 2 (5.7)           |
| Lung cancer                                | 2 (5.7)           |
| Hypopharyngeal cancer                      | 1 (2.9)           |

ECOG, Eastern Cooperative Oncology Group.

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**Figure 2.** Comparison of IL-17A level, the proportion of NKT cells and NK cells after CSM-TACE with their pre-treatment levels. Proportion of NKT cells (A), NK cells (B), and IL-17A level (C) before and after treatment. CSM-TACE, CalliSpheres® microspheres transarterial chemoembolization; NK, natural killer; IL-17A, interleukin-17A.
stimulates immune-exhausted T cells in liver cancer [28]; additionally, another interesting research has illustrated that the ratio of CD4\(^+\)/CD8\(^+\) T cells is obviously elevated while CD4\(^+\)CD25\(^+\) Treg cells are markedly declined following TACE in patients with liver cancer [29]. Inspired by above-mentioned data, we deduced that immune microenvironment might also be changed in patients with refractory liver metastases treated by CSM-TACE, while related information is obscured. To verify our deduction, we enrolled 35 patients with refractory liver metastases who received CSM-TACE and subsequently found that NK cells, NKT cells, CD4\(^+\) T cells, and ratio of CD4\(^+\)/CD8\(^+\) T cells were all elevated, while IL-17A and CD8\(^+\) T cells were declined after CSM-TACE. The possible explanation might be that after CSM releasing great chemotherapeutic drugs into tumor continuously, tumor cell necrosis is elevated obviously and tumor burden is alleviated (reflected by disease control rate of 100% among patients with refractory liver metastases in the current study), which might result in better immunostimulative effect on the body, consequently leading to increased NK cells, NKT cells, and CD4\(^+\) T cells but declined IL-17A and CD8\(^+\) T cells after treatment [6–8,14].

Until now, the data about the correlation of IL-17A with NK cells and NKT cells in refractory liver metastases is obscured. Only a previous study reports that IL-17A affects the production of NK cells in liver injury mice model [30]. In the current study, we found that IL-17A was negatively correlated with NK and NKT cells among patients with refractory liver metastases receiving CSM-TACE. The potential explanations might be that IL-17A could inhibit activity, maturation, and proliferation of NK cells via several mechanisms, such as restraining IL-15-responsible cell maturation through modulating suppressor of cytokine signaling 3, as well as janus kinase/signal transducer and activator of transcription pathway [30–33]. Thus, IL-17A was negatively correlated with NK and NKT cells among patients with refractory liver metastases receiving CSM-TACE.

Some aspects in the current study should be noticed as follows: the current study detected the changes of immunological indexes within in a short period (1 week) after CSM-TACE, the reason was that previous study had presented the change of immune microenvironment caused by TACE is obvious in a short period; thus it was reasonable to observe the variation of immune microenvironment within 1 week after CSM-TACE [34], indicating the timing for the application of immunotherapy with CSM-TACE in refractory liver metastases is remarkable. The current study indicated that anti-tumor immunity was enhanced in patients with refractory liver metastases after CSM-TACE, which
emphasized the pleiotropic effect of CSM-TACE on regulating the immune microenvironment and highlighted the rationale of combining immunotherapy and CSM-TACE to further improve the management of refractory liver metastases.

There still existed several limitations in the present study: (1) we discovered that NK cells, NKT cells, and CD4\(^+\) T cells were increased but IL-17A and CD8\(^+\) T cells were declined after CSM-TACE in refractory liver metastases, thus whether immunotherapy synergized with CSM-TACE was another interesting topic which could be explored in the future; (2) the underlying mechanism of CSM-TACE affecting immune microenvironment could be further explored; (3) the sample size could be enlarged in the further study to contribute to more generalized discoveries; (4) the survival of patients refractory liver metastases after CSM-TACE could be explored in the future.

In conclusion, anti-tumor immune environment is restored in patients with refractory liver metastases after CSM-TACE, indicating CSM-TACE has the potential to be combined with immunotherapy to promote the management of these patients.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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