Original Article

Efficacy analysis of triple-negative breast cancer patients treated with dendritic cell-cytokine-induced killer cell immunotherapy

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Abstract: Background: To examine the efficacy of dendritic cell-cytokine-induced killer (DC-CIK) cell immunotherapy combine with chemotherapy with or without radiotherapy for triple-negative breast cancer (TNBC) patients following surgery. Methods: Sixty-seven TNBC patients were selected between December 2008 and February 2012 for this retrospective study. Twenty-five patients who received an infusion of DC-CIK cells were placed in the DC-CIK group while an additional 42 patients were in the non-DC-CIK group. Results: The 3-year disease-free survival (DFS) rate was significantly improved in the DC-CIK group versus the non-DC-CIK group (P=0.021). The 5-year DFS rate was improved from 20.08% to 39.32% although it approached statistical significance (P=0.052). A subgroup analysis showed that the 3-year and 5-year DFS rate were significantly higher in the DC-CIK group, including advanced TNM stage TNBCs compared with the non-DC-CIK group (P=0.026, P=0.042, respectively). The 3-year OS rate for the advanced-stage subgroup in the DC-CIK group was significantly higher compared with the non-DC-CIK group (P=0.025). Although compared with the non-DC-CIK group, the improvement of 5-year OS rate with DC-CIK treatment in the advanced-stage subgroup approached statistical significance (P=0.051), the 5-year OS rate was still improved from 22.72% to 41.56%. Conclusions: Advanced TNM stage TNBC patients may benefit from an adjuvant infusion of DC-CIK cells concurrent with conventional therapy to prevent disease relapse and improve OS.

Keywords: Cytokine-induced killer cell, dendritic cell, adjuvant immunotherapy, triple-negative breast cancer

Introduction

Currently, breast cancer is the leading cancer affecting females worldwide. In 2012, nearly one million new cases were diagnosed, and among these cases, more than 170,000 involved triple-negative breast cancer (TNBC) [1]. TNBC is defined as being negative for estrogen receptor (ER), human epidermal growth factor receptor-2 (HER-2), and progesterone receptor (PR) expression in immunohistochemistry assays [2]. In general, TNBC represents 10-20% of all breast cancers and it exhibits biological behavior that is distinct from other breast cancers [3]. In particular, the clinical pathological features of TNBC include a more aggressive phenotype and a higher risk of metastatic recurrence compared with other types of breast cancer. It has been reported that up to 30% of TNBC cases progress to metastatic breast cancer [4].

When distant metastasis events are detected in TNBC patients, they mainly affect lung, liver, brain, bone, and skin tissues and the prognosis is very poor [5]. Due to the absence of endocrine and HER-2 targets, TNBC is less likely to respond to conventional therapies such as anti-HER-2 or hormone therapies, and chemotherapy is currently the primary treatment for TNBC. However, this therapy is only beneficial for approximately 20% of TNBC patients who are sensitive to chemotherapy [6]. In addition, the recurrence rate among patients with residual TNBC is high (30-40%) [7]. The greatest risk of recurrence is generally between 1 and 3 years after treatment, and a sharp decrease in survival is observed within the first five years after treatment [8]. Targeted therapies have been
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More recently, immunotherapy has been considered as a possible therapy when contraindications for surgery, radiotherapy, and chemotherapy exist [10]. Several previous studies have investigated the safety and efficacy of various immunotherapy approaches, as well as their potentially curative effects [11, 12]. In particular, dendritic cells (DCs) represent antigen-presenting cells which can induce naïve T cells to have a specific and cytotoxic response [13]. In addition, cytokine-induced killer (CIK) cells represent a heterogeneous population of cells which can be induced by multiple cytokines [10]. Several reports have described the benefits of DC-CIK cell immunotherapy [14, 15]. Moreover, the advantages of DC-CIK cultures include enhanced cell proliferation and cytokine secretion and high tumoricidal activity. Thus, the infusion of DC-CIK cells currently represents a novel and effective treatment for cancer. Our own work and that of other laboratories have demonstrated that DC-CIK immunotherapy enhances immune function and the microenvironment of breast cancer patients that are otherwise compromised by the ability of tumor cells to evade detection [16, 17]. However, limited data are available regarding the effects of DC-CIK immunotherapy on TNBC patients [18, 19]. Therefore, a retrospective analysis of the effect of adjuvant DC-CIK immunotherapy combined with chemotherapy with or without radiotherapy on TNBC patients after surgery (n=25) was conducted and clinical outcome was evaluated.

Methods

Patients’ clinical features

Approval for this study was obtained from the Ethics Committee of our institute and written consent was obtained from each participating patient. Between December 1, 2008 and February 28, 2012, 67 TNBC patients who underwent surgery and received chemotherapy with or without radiotherapy at Fujian Cancer Hospital were enrolled in this study. TNBC was confirmed with PR and ER nuclear staining <1% and HER-2 staining scored as 0 to 2+ at our pathology department. Twenty-five patients underwent adjuvant DC-CIK therapy while the remaining 43 patients did not (the DC-CIK group and control group, respectively) (Table 1).

Chemotherapy with or without radiotherapy treatment

All of the TNBC patients underwent four 21-day cycles of anthracyline and paclitaxel-based chemotherapy after surgery. Intravenous administration of epirubicin (60 mg/m²) was performed on day 1, followed by a 3-h intravenous infusion of 175 mg/m² paclitaxel on day 2. Patients with lymph node metastasis or distant metastasis received sequential intensity-modulated radiation therapy before finishing their chemotherapy regimen, with irradiation of 50 Gy/25f applied outside the axillary +/- lymphatic drainage area. The tumor bed received 10-16 Gy.

Generation of CIK cells and DC from peripheral blood

Percoll density gradient centrifugation was performed to isolate peripheral blood mononuclear cells (PBMCs) from 50 mL heparinized whole blood samples. The lymphocytes were collect-

Table 1. Characteristics of the cohort examined

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>DC-CIK (n=25)</th>
<th>non-DC-CIK (n=42)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient age</td>
<td></td>
<td></td>
<td>0.798</td>
</tr>
<tr>
<td>&lt;50 y</td>
<td>18</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>≥50 y</td>
<td>7</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Median age, y (range)</td>
<td>45 (31-75)</td>
<td>46 (32-60)</td>
<td>0.527</td>
</tr>
<tr>
<td>KPS (mean ± SD)</td>
<td>87.60±5.97</td>
<td>86.67±5.70</td>
<td></td>
</tr>
<tr>
<td>TNM</td>
<td></td>
<td></td>
<td>0.353*</td>
</tr>
<tr>
<td>I-IIA</td>
<td>11</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>IIB-IV</td>
<td>14</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
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</tr>
<tr>
<td>No</td>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>25</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
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<td>0.525*</td>
</tr>
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<td>2</td>
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<td>Yes</td>
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<td>40</td>
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</tr>
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<td></td>
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</tr>
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</tr>
<tr>
<td>Yes</td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

*Fisher’s Exact Test.
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ed, plated at a concentration of 2×10^6 into 75 cm^2 culture flasks (BD, USA), and incubated under 5% CO_2 at 37°C. After 1-2 h, complete GT-T551 medium supplemented with 50 ng/mL interleukin-4 (IL-4) (Amoytop) and 100 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Amoytop, China) was added to each culture flask. The medium was replenished every 3 days. On day 5, the medium was supplemented with 500 U/mL tumor necrosis factor-α (TNF-α; Peprotech, USA) and 100 µg/mL WT1 antigen (Miltenyi Biotec, Germany). Mature tumor antigen-pulsed DCs were harvested at day 7.

The suspended cells that were previously collected were transferred to a 75 cm^2 culture flask that was pre-coated with 50 µg (5 µg/mL) CD3 monoclonal antibody (Takara, Japan). On day 1, complete GT-T551 medium (Takara) supplemented with interferon (IFN)-1000 U/mL (Novoprotein, Xiamen, China) and 1000 U/mL IL-2 (Sihuanpharm, Beijing, China) was added to adjust the cell density to 5×10^6 cells/mL. Every 3 days, the cells received fresh medium containing IL-2 (500 U/mL). On day 7, the CIK cells were mixed with tumor antigen-pulsed DCs at a ratio of 1:10. The mixed DC-CIK cells were maintained in complete GT-T551 medium supplemented with IL-2 (500 U/mL).

**DC-CIK treatment schedule**

Bacterial contamination and endotoxin tests were conducted prior to performing transfusions. Results <0.06 EU were needed within 48 h of testing to proceed. Briefly, DC-CIK cells were harvested on day 12 and added to a 0.9% normal salt solution (Hai Wang, Fuzhou, China). The DC-CIK cells were then administered at 2-5×10^9 cells a day for 4 d via intravenous infusion during the intervals of chemotherapy. One cycle was defined as four transfusions, and it included 1.2×10^10 cells. The interval of each cycle extended over at least 14 d.

**Follow-up**

All patients completed follow-up examinations that were required every three months for the first two years, every six months for the next three years, and then annually thereafter until the 28th of February 2017. Both clinical and laboratory examinations or telephone consultations were conducted. Physical examinations and blood tests were also performed at each follow-up visit. A colonoscopy, chest/abdominal computed tomography (CT) scan, and ultrasound scans of the liver and abdomen were performed every 6-12 months. Positron emission tomography-computed tomography (PET-CT) was used to monitor suspected metastasis or tumor recurrence. Short-term efficacy was evaluated based on assigned Karnofsky performance scale (KPS) scores.

**Statistical analysis**

Statistical analyses were performed with SPSS 16.0. Data are presented as the mean ± standard deviation (SD) and were compared with the Kaplan-Meier method. The Cox proportional hazard regression model was used for multivariate analysis. A p-value <0.05 was statistically significant.

**Results**

**Baseline patient characteristics**

Sixty-seven TNBC patients were histologically confirmed and agreed to participate in this study. Twenty-five patients were assigned to the DC-CIK group and 42 patients were assigned to the non-DC-CIK group. The clinical and demographic characteristics of the two groups did not significantly differ (P>0.05) (Table 1).

**Adverse effects of DC-CIK cell transfusions**

Among the patients who underwent DC-CIK cell transfusions, one patient (4%) experienced a mild fever and chills, while another patient experienced an allergic reaction, anaphylactic shock, hypotension, and chest tightness. Both patients recovered with symptomatic treatment. None of the patients in the cohort exhibited problems with hepatic or renal function.

**Assessment of KPS scores**

The baseline KPS scores for the DC-CIK and non-DC-CIK groups were 87.6±5.97 and 86.07±5.70, respectively (P=0.53). After treatment, the KPS scores for the two groups were 83.02±4.32 and 72.67±5.01, respectively (P<0.05).

**OS and DFS estimates**

Kaplan-Meier estimates of 3-year DFS rates for the DC-CIK and non-DC-CIK groups were signifi-
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Significantly improved ($P=0.021$ Figure 1), and the 5-year DFS rates for the DC-CIK and non-DC-CIK groups were improved from 20.08% to 39.32%, although it only approached statistical significance ($P=0.052$; Figure 2). Kaplan-Meier estimates of 3-year and 5-year OS rates for the two groups had no statistical significance ($P=0.106$, $P=0.137$, respectively; Figures 3, 4).

**Subgroup analysis**

A subgroup analysis was performed to investigate whether TNM stage affects the prognosis of postoperative TNBC patients with or without DC-CIK treatment. TNBC patients in the early stage group (which included TNM stages I and IIA) did not appear to benefit from DC-CIK treatment based on their 3-year and 5-year DFS rates (Figures 5, 6, $P=0.755$, $P=0.934$, respectively) as well as 3-year and 5-year OS rates (Figures 7, 8, $P=0.784$, $P=0.945$, respectively).

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**Figure 1.** Kaplan-Meier estimates of 3-year DFS for the DC-CIK and non-DC-CIK groups.

**Figure 2.** Kaplan-Meier estimates of 5-year DFS for the DC-CIK and non-DC-CIK groups.

**Figure 3.** Kaplan-Meier estimates of 3-year OS for the DC-CIK and non-DC-CIK groups.

**Figure 4.** Kaplan-Meier estimates of 5-year OS for the DC-CIK and non-DC-CIK groups.

**Figure 5.** Kaplan-Meier estimates of 3-year DFS for patients with TNM stage I or IIA TNBC.
In contrast, the 3-year DFS rate and 5-year DFS rate for the advanced-stage group (which included TNM stages IIb, III, and IV), was significantly affected by DC-CIK treatment (Figures 9, 10, \( P=0.026, P=0.042 \) respectively). The 3-year OS rate was also significantly improved for the advanced-stage group (Figure 11, \( P=0.025 \)). Although compared with non-DC-CIK group, the improvement of 5-year OS rate with DC-CIK treatment in the advanced-stage subgroup had only borderline statistical significance (Figure 12, \( P=0.051 \)), the 5-year OS rate was still improved from 22.72% to 41.56%.

**Discussion**

TNBC exhibits biological behaviors, including an invasive phenotype, which is distinct from other breast cancers. Currently, anthracycline-based chemotherapy and radiotherapy are the primary treatments for TNBC, while endocrine
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and targeted therapeutic targets have not been identified. For early stage TNBC, surgical treatment combined with radiotherapy improves local control and is currently the best treatment available. However, for patients with advanced stage TNBC, radiotherapy has not significantly affected prognosis [20]. Moreover, neopathy of radiotherapy remains a cause of increased mortality [21]. The potential side effects of chemotherapy should also be considered, and these include immunosuppression and toxicity to kidneys, liver, lung, and heart. Unfortunately, these side effects remain difficult to overcome. Consequently, immunotherapy represents an attractive option for cancer treatment due to its low toxicity and high specificity [22]. Moreover, it has been demonstrated that cell immunotherapy combined with conventional treatment can improve treatment outcomes [23]. The mechanism responsible for this improvement may derive from the synergistic effects of these two approaches. For example, chemotherapy not only kills and/or slows the growth of cancer cells, but it can also increase the sensitivity of tumor cells' response to immune effector cells. Meanwhile, immunotherapy can restore an immune system which is compromised by chemotherapy and can also stimulate antitumor immunity [24]. Currently, DC-CIK cell treatment is widely used for the treatment of cancer patients. Antigen-presenting DCs are able to activate naïve CD4+ T helper cells and they have overcome immune tolerance and immunosuppression in many cancer patients [25]. CIK cells mediate cytotoxicity that can directly eradicate tumor cells, while secretion of Th1 cytokines, such as IFN-, IL-2, and IL-12, by CIK cells can modulate the immune system to reduce tumor recurrence and metastasis [26]. Correspondingly, the incubation of DCs with CIK cells has led to an increase in Th1 cytokine secretion and stronger cytotoxicity [27]. Furthermore, many studies have also reported the successful application of DC-CIK immunotherapy as an adjuvant treatment to chemotherapy and radiotherapy to colon cancer [28], hepatocellular cancer [29], and gastric cancer [30].

In this retrospective study, adjuvant DC-CIK therapy was associated with fewer adverse effects and an improved 5-year DFS rate from 20.08% to 39.32% in TNBC patients, although it only approached statistical significance (P=0.052). However, the 5-year OS rates did not significantly improve in the DC-CIK group compared with the non-DC-CIK group, and this result is inconsistent with the results reported by Pan et al. [19]. Therefore, a larger sample size is needed to further investigate these findings since the sample size in our study and in the Pan et al. study are both small. In the subgroup analysis that was conducted based on TNM stage, the advanced-stage subgroup exhibited a significant improvement in their 3-year and 5-year DFS rate with DC-CIK therapy. The 3-year OS rate was significantly affected by DC-CIK treatment for this group, and the 5-year OS was also improved albeit without statistical significance. The relatively small sample size of the present study may have contributed to the absence of a statistical result. Moreover, the early-stage subgroup did not exhibit signifi-
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Significant improvements in their 5-year DFS and OS rates. Thus, the present results indicate that patients with advanced stage TNBC (e.g., IIb, III, and IV) may greatly benefit from adjuvant DC-CIK treatment, and this observation is consistent with previously reported results for advanced cancers [28-31].

Due to the limitations associated with a retrospective study, a randomized, double-blind, parallel-group, multicenter prospective trial needs to be conducted to further examine and confirm the present results. Additional studies are also needed to examine the effects of using a combination of several alternative therapy modalities to achieve a more comprehensive tumor treatment. These modalities could include conventional therapies, cell immunotherapies, and antibody-based immunotherapies, including those targeting programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) immune proteins. For example, pembrolizumab and ipilimumab are monoclonal antibodies which have been developed to target PD-1 and CTLA-4, respectively. Despite having different targets, and distinct cellular mechanisms, both induce an expansion of CD8$^+$ T cells to mobilize the body's immune system to kill tumors [32]. Nanda et al. recently showed consistent benefits of pembrolizumab treatment for metastatic TNBC patients, with a total remission rate of 18.5% achieved [33]. Thus, PD-1 may represent a new target in treatments of TNBC. However, only 20-30% of TNBC cases have been found to be positive for programmed death-ligand 1 expression [34]. Consequently, additional combinational treatment options are being considered and developed for TNBC. Of particular interest is the capacity for cell immunotherapies to improve immune function. Furthermore, cell immunotherapies in combination with antibody-based immunotherapies could provide more effective treatment.

Conclusions

Among the 67 TNBC patients examined, 25 underwent DC-CIK therapy combined with chemotherapy with or without radiotherapy, while the remaining patients did not undergo DC-CIK therapy yet still received chemotherapy with or without radiotherapy and served as the control group. There were several findings made based on these two groups. First, the KPS score for the DC-CIK group was significantly higher than the KPS score for the non-DC-CIK group. Second, patients in the DC-CIK group had a lower risk of cancer recurrence despite no significant improvement in their 3-year and 5-year OS rate. Third, a subgroup analysis showed that the advanced stage TNBC patients who received chemotherapy with or without radiotherapy after surgery benefited from DC-CIK therapy. For example, the 3-year and 5-year DFS rate for these patients significantly improved compared with the non-DC-CIK group. The 3-year OS rate was also significantly improved for these advanced stage patients, and the 5-year OS rate also improved although the improvement approached statistical significance. Thus, adjuvant DC-CIK cell therapy may represent a useful strategy for the treatment of cases involving advanced stage cancer.

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This study was approved by the ethics committee of Teaching Hospital of Fujian Medical University, Fujian Provincial Tumor Hospital and was carried out in accordance with the Declaration of Helsinki of the World Medical Association. All enrolled patients provided written informed consent.

Disclosure of conflict of interest

None.

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