

## Clinical Activity of Adjuvant Cytokine-Induced Killer Cell Immunotherapy in Patients with Post-Mastectomy Triple-Negative Breast Cancer

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### Abstract

**Purpose:** Triple-negative breast cancer (TNBC) is a high risk form of this disease, even after surgery, due to the absence of targets for hormone treatment and anti-Her-2 therapy. Chemotherapy is the main therapeutic strategy for such patients with breast cancer, although the outcome is often unsatisfactory. Thus, the development of combination adjuvant therapies is essential for improved prognosis in patients with TNBC. In this study, we investigated the efficacy of a sequential combination of cytokine-induced killer cell (CIK) infusion and chemotherapy for patients with post-mastectomy TNBC.

**Experimental Design:** From 2008 to 2012, 90 patients with post-mastectomy TNBC were included in this retrospective study: 45 cases received chemotherapy alone or with sequential radiotherapy; a further 45 cases received chemotherapy with/without radiotherapy and sequential CIK infusion.

**Results:** Survival analysis showed significantly higher disease-free survival (DFS) and overall survival (OS) rates in the CIK treatment group compared with the control group ( $P = 0.0382$ ,  $P = 0.0046$ , respectively; log-rank test). Multivariate survival analysis showed that CIK adjuvant treatment was an independent prognostic factor for OS of patients with TNBC. In subgroup analyses, CIK adjuvant treatment significantly increased the DFS rate of patients with pathologic grade 3, and significantly increased the OS rate of patients in N1, N2, N3, IIB, III TNM (tumor-node-metastasis) stages, and with pathologic grade 3.

**Conclusions:** These data indicate that adjuvant CIK treatment combined with chemotherapy is an effective therapeutic strategy to prevent disease recurrence and prolong survival of patients with TNBC, particularly those with lymph node metastasis, advanced TNM stage, and poor pathologic grade. *Clin Cancer Res*; 20(11); 3003–11. ©2014 AACR.

### Introduction

Breast cancer is the most common female malignancy in both the developing and developed world, and is the leading cause of cancer-related mortality among women globally (1). Today, the incidence rates of breast cancer still continue to increase, especially in developing countries, including China (2). The mortality rate in developed coun-

tries has declined as a result of the introduction of systemic adjuvant treatments (3), such as targeted treatment, which is directed against a nuclear or surface receptor, as exemplified by tamoxifen and trastuzumab, respectively. However, a significant subgroup of patients with breast cancer that is negative for estrogen receptor (ER), progesterone receptor (PR), and HER2, known as "triple-negative breast cancer (TNBC)," gains little benefit from the currently available targeted treatments due to the lack of related receptor expression.

The TNBC category accounts for 10% to 15% of breast cancers, which present aggressive behavior, high risk of early relapse, and poor overall survival (OS) compared with other breast carcinoma subtypes (4, 5). In the absence of a specific therapeutic target, conventional chemotherapy is the mainstay of TNBC treatment according to the majority of national and international guidelines (6). Chemotherapy could certainly improve clinical outcome for some chemosensitive patients with TNBC (7). However, in most patients with TNBC, chemotherapy produces mixed results and has a variable impact on long-term prognosis (8–11). Furthermore, with chemotherapy alone, the residual risk of

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### Translational Relevance

Cytokine-induced killer cell (CIK) infusion is a recently developed technique that has been used successfully in combination with chemotherapy for some solid tumors. In this study, we evaluated the efficacy of sequential CIK infusion and chemotherapy for patients with post-mastectomy triple-negative breast cancer (TNBC). This subset of patients with breast cancer is at high risk of early recurrence and distant metastasis but has limited treatment options due to the absence of currently identified therapeutic targets. Our findings showed that additional CIK treatment significantly enhanced the disease-free survival (DFS) and overall survival (OS) rates in patients with TNBC compared with the rates associated with conventional chemotherapy alone. These data indicate the potential benefits of adjuvant CIK treatment in high-risk patients with TNBC. Furthermore, our study represents the basis of further research into the development of optimized treatment strategies for this unique cohort of patients with breast cancer.

TNBC remains substantially high (30%–40%), and these patients have a high risk of relapse, with a sharp decrease in survival in the first 3 to 5 years after treatment (5, 12–14). Therefore, the identification of more active therapies than are currently provided by chemotherapy for patients with TNBC remains an important clinical challenge.

Previous studies have provided evidence to show that cancer progression and clinical outcomes in patients with TNBC are particularly influenced by tumor immune responses (15–18), indicating that immune-based therapy could be a promising therapeutic approach for patients with TNBC (19). Cytokine-induced killer (CIK) cells, which were developed at Stanford University (Stanford, CA), may be an alternative immunotherapeutic strategy for this type of patient. CIK cells are immune-active host effector cells that can kill cancer cells *in vitro* and *in vivo* (20). Stimulation with a cytokine cocktail, including IFN- $\gamma$ , interleukin (IL)-2, and anti-CD3 antibody results in expansion of CIK cell numbers that mediate MHC-unrestricted cytolytic activity against a broad range of tumor cells (21–24). Notably, a recent series of clinical studies clearly show the potential effectiveness of CIK treatment for clinical applications in patients with cancer, including those with solid and hematologic malignancies (25–35). Several reviews and meta-analysis reports have highlighted the safety and efficacy of CIK therapy in clinics, suggesting that CIK cells represent a realistic new option in the field of cancer immunotherapy (36–40).

Patients with TNBC lack identified overexpressed receptors and antigens; therefore, CIK cells may be a good choice for the treatment of these patients due to the broad-spectrum, antigen nonspecific tumor cytolytic activity of these cells. To date, the therapeutic effects of CIK cells in TNBC, including preclinical and clinical studies, have rarely been

reported; thus, the present study comprised a retrospective analysis of the efficacy of adjuvant CIK cell infusion combined with chemotherapy in patients with post-mastectomy TNBC ( $n = 90$  cases) for the improvement of clinical outcomes in patients with TNBC after tumor resection.

### Materials and Methods

#### Patient population

Between December 1, 2008, and December 31, 2012, the medical records of patients with breast cancer from a computerized database in the Sun Yat-sen University Cancer Center (SYSUCC; Guangzhou, PR China) were reviewed. This database recorded the clinicopathologic information of the patients with breast cancer at accrual and included details about age, menopausal status, tumor characteristics, TNM (tumor-node-metastasis) stage, treatment, and outcome. The definition of TNBC was based on immunohistochemical staining of ER, PR, and HER-2, which is performed routinely in the pathology department of our hospital. The staining was categorized as follows: ER and PR nuclear staining <1%, ER- and PR-negative; HER-2 staining 0 to 2+ by immunohistochemistry or a nonamplified HER-2 by FISH, HER-2-negative. Patients negative for ER, PR, and HER-2 were eligible for this study. All of the female patients with TNBC underwent surgery, including quadrantectomy or mastectomy and axillary lymph node dissection. Subsequently, these patients received 4 to 8 cycles of post-mastectomy anthracyclines and/or taxane-based chemotherapy. After completion of chemotherapy as planned, a subpopulation of the patients received sequential radiotherapy depending on their clinical stage. Following termination of chemotherapy or radiotherapy, another subpopulation of the patients received at least four cycles of CIK immunotherapy. Patients were excluded from the study based on the following criteria: with distant metastasis at diagnosis, a history of other malignancy, treatment with neoadjuvant chemotherapy, the occurrence of serious adverse events during chemotherapy, without receiving post-mastectomy chemotherapy, or receiving CIK treatment after recurrence. After review, 90 patients with TNBC met the described criteria and were included for further analysis. Among them, 45 patients received CIK treatment (CIK group), whereas the other 45 patients without CIK treatment were used as the control group for comparisons. The data of all patients who received immunotherapy with CIK cells were shown in Supplementary Table S1.

#### Chemotherapy and radiation treatment

After surgery, adjuvant chemotherapy (4–8 cycles) was administered to all patients as follows: an anthracycline-based [cyclophosphamide, epirubicin, and fluorouracil (CEF)], a taxane-based [docetaxel, cyclophosphamide (TC)], or an anthracycline- and taxane-based [docetaxel, doxorubicin, and cyclophosphamide (TAC)] regimen. At the completion of post-mastectomy chemotherapy with a 2-week interval, 48 patients ( $n = 20$  for control group;  $n = 28$  in CIK group) underwent definitive radiation treatment. Radiotherapy was delivered mainly to the ipsilateral chest

wall and supraclavicular region on the same side as the tumor. The radiation treatment to the chest wall was administered with 6 to 9 MeV X-ray at a prescribed dose of 50 Gy/25 fractions (5 fractions/week). Regional nodal irradiation was added as clinically indicated, generally for patients with pathologically positive axillary lymph nodes  $>2$ .

### CIK generation

CIK cell-based treatment is observational clinical immunotherapy in our hospital. It was approved by the institutional ethics committee of SYSUCC (A total of 11 votes, 8 votes in favor, 3 abstentions), and the written consent from each patient was obtained. Autologous CIK cells were prepared according to our previous report with some modification (29, 33). In brief, 2 weeks after the patients had completed chemotherapy or radiation treatment and when routine blood examination had returned to normal, samples (50–60 mL) of heparinized peripheral blood were collected. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation and suspended in X-VIVO 15 serum-free medium (Longza). In culture, 1,000 U/mL rhIFN- $\gamma$  (Clone-gamma, Shanghai Clone Company) was added for the first 24 hours, followed by 100 ng/mL mouse anti-human CD3 monoclonal antibody (R&D Systems), 1,000 U/mL rhIL-2 (Beijing Sihuan), and 100 U/mL IL-1  $\alpha$  (Life Technologies) to induce CIK cells. During the culture, fresh medium containing 1,000 U/mL rhIL-2 was added periodically and the cell density was maintained at  $2 \times 10^6$  cells/mL. At 14 days, the CIK cells were harvested. Before transfer to the patients, a fraction of cells was collected for evaluation of number, viability, phenotype, and possible contamination.

### CIK treatment

For transfer to patients, all numbers of harvested autologous CIK cells free of microbial contamination were washed and resuspended with 100 mL normal saline (containing 3–5 mL 20% human serum albumin). Before infusion, 50 to 60 mL heparinized peripheral blood was collected for the next cycle of CIK generation. The autologous CIK cells were administered via intravenous infusion within 30 minutes. During transfusion, vital signs such as pulse, heart rate, breath, blood pressure, and temperature were monitored and recorded. In general, patients will receive at least 4 cycles of CIK cell infusion with 2-week intervals between each cycle. The CIK cell treatment protocol was shown in Supplementary Fig. S1. If disease was stable and patients wanted, more cycles of CIK maintenance treatment will be given using the same protocol as above. Otherwise, the CIK therapy was stopped when the disease is in progression or the patients did not want to continue. An alternative therapy was recommended by physicians.

### Follow-up

After surgery, all patients with TNBC were followed-up regularly at our outpatient department. In general, follow-up was required every 3 months in the first 2 years, every 6 months from year 2 through year 5, and annually thereafter.

Furthermore, telephone consultations were conducted regularly for each patient at our follow-up center. Each follow-up in the outpatient department included a complete examination, basic serum chemistry, chest X-ray, and ultrasound scans of liver and abdomen. Chest computed tomography/MRI was performed when tumor recurrence or metastases were suspected. Disease-free survival (DFS) was defined from the date of definitive surgery to the date of first recurrence (local or distant) or date of last follow-up. Patients who died before experiencing a disease recurrence were considered censored at their date of death. OS was defined from the date of definitive surgery to the date of death from any cause or date of last follow-up. If recurrence or metastases were confirmed during the follow-up, remedial treatments included surgery, chemotherapy beside the anthracycline- and taxane-based regimen or radiation treatment was recommended. The status and correlating treatments of the patients were entered into the medical records after follow-up and were updated accordingly in the database.

### Statistical analysis

Differences in demographic and clinical variables of the two groups were tested using the Pearson  $\chi^2$  test, and the Fisher exact test was used as appropriate. The Kaplan–Meier method was used to analyze the rates of PFS and OS. The log-rank test was used to compare differences in Kaplan–Meier estimates for each group. The Cox proportional hazards regression model was used for univariate and multivariate analyses. SPSS 18.0 (SPSS) was used for the statistical calculations, and a difference with a *P* value of less than 0.05 was considered significant.

## Results

### Patient demographics and clinical characteristics

The present study comprised a retrospective analysis of 90 patients with TNBC divided into two cohorts (CIK group and control group). The demographic data were well matched between the two groups (Table 1). There were no statistically significant differences between the two groups in terms of variables such as age, receipt of radiation or not, T, N, and TNM stages, and pathologic grades (*P* > 0.05).

### Characteristics of final CIK cell cultures

After culturing and expansion, the final number of CIK cells produced was approximately  $8.7 \times 10^9$  to  $1.2 \times 10^{10}$ , with a viability of more than 95%. The percentage of CD3 $^+$ T cells was approximately 80% to 90%; the percentage of CD3 $^+$ CD8 $^+$ T cells was approximately 60% to 80%; and the percentage of CD3 $^+$ CD56 $^+$ T cells was approximately 6% to 22%. All products were free of bacterial and fungal contamination, negative for mycoplasma and contained <5 EU endotoxin. After detection, all fresh autologous CIK cells were infused back to the patients.

### Safety and toxicity of CIK cell immunotherapy

After CIK cell infusion, 12 patients had mild chills and fever, the body temperature was not more than 38°C and

**Table 1.** Demographics and disease characteristics: control patients and CIK immunotherapy treatment patients

	Control group (n = 45)	CIK Group (n = 45)	P
Age			0.833
<50	23	22	
≥50	22	23	
Median (range)	51 (28–73)	50 (26–73)	
Radiotherapy			0.091
Yes	20	28	
No	25	17	
T stage			0.099
T1	11	21	
T2	28	21	
T3	4	3	
T4	2	0	
N stage			0.331
N0	15	21	
N1	16	12	
N2	10	11	
N3	4	1	
TNM stage			0.264
I	6	12	
II	23	21	
III	16	12	
Pathologic grade			0.574
1	0	1	
2	21	22	
3	24	22	

resolve spontaneously within 6 hours. One patient appeared hypersphyxia and recovered to normal after symptomatic treatment. No serious side effects appeared in all patients who receive CIK cell treatment.

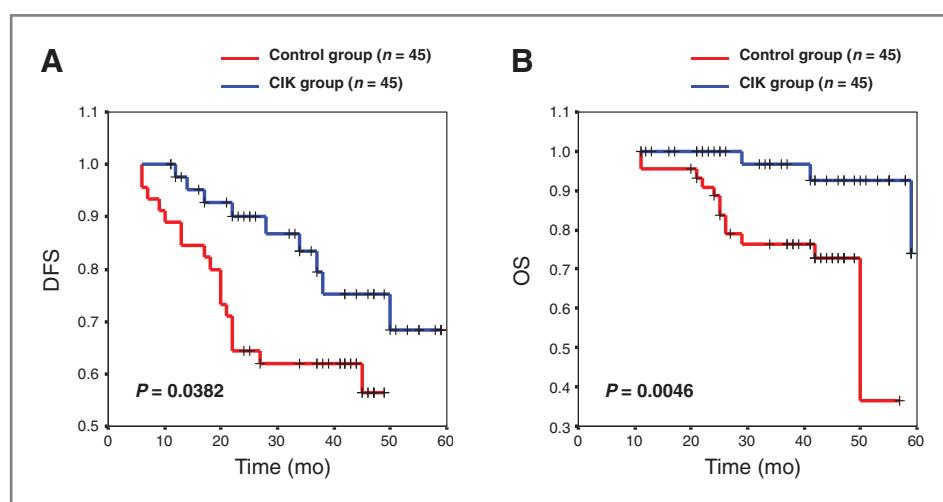
### Survival estimates

The median follow-up time among patients in the CIK group was 41 months (range, 11–59 months); the median follow-up time among patients in the control group was 41 months (range, 11–57 months). The 1-, 2-, 3-, and 4-year DFS rates were 97.7%, 90.1%, 83.4%, and 75.2%, respectively, in the CIK group, and were 88.9%, 64.4%, 62.1%, and 56.4%, respectively, in the control group. Patients in the CIK group had a better DFS compared with the patients in the control group (Fig. 1A). The 1-, 2-, 3-, and 4-year OS rates were 100.0%, 100.0%, 96.7%, and 92.4%, respectively, in CIK group, and were 95.6%, 88.6%, 76.3%, and 72.7%, respectively, in the control group. The patients who received CIK treatment also exhibited a better OS than the control group (Fig. 1B).

The effects of CIK treatment on the prognosis of patients with post-mastectomy TNBC were further evaluated in univariate and multivariate Cox proportional hazards regression analyses. Early stage, low-grade tumors, and CIK treatment showed a significant association with improved DFS and OS in univariate analysis (Tables 2 and 3). Low-grade tumors and CIK treatment remained associated with improved OS in the multivariate analysis, although CIK treatment was not an independent prognostic factor for DFS (Tables 2 and 3).

### Subgroup analysis

Because N and TNM stages, and pathologic grade were associated with prognosis of patients with post-mastectomy TNBC, we subsequently investigated which group of patients with TNBC according to these clinical parameters could benefit most from the CIK cell treatment. In the early-stage group (N0 and I, IIA TNM stages) and the low-grade group (1 and 2 pathologic grades), CIK treatment did not significantly affect the DFS and OS of patients with TNBC (Fig. 2, left). In the advanced-stage group (N1, N2, N3 and IIB, III TNM stages), CIK treatment was not significantly associated with improved DFS of patients with TNBC (Fig. 2A and B, right), but the OS of patients with TNBC was



**Figure 1.** Survival analysis in patients with TNBC. A, DFS curves. B, OS curves. The Kaplan–Meier method was used to compare the survival rates between the sequential CIK cell adjuvant therapy group (CIK group, n = 45) and chemotherapy with/without the radiation treatment alone group (control group, n = 45).

**Table 2.** Univariate and multivariate analysis of DFS in patients with TNBC

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
Age (<50 vs. ≥50)	0.552 (0.252–1.206)	0.136		
Radiotherapy (no vs. yes)	0.623 (0.284–1.366)	0.238		
T stage (1 vs. 2, 3, 4)	0.407 (0.164–1.011)	0.053		
N stage (0 vs. 1, 2, 3)	0.205 (0.070–0.598)	0.004 <sup>a</sup>	0.500 (0.127–1.972)	0.322
TNM stage (I, IIA vs. IIB, III)	0.191 (0.072–0.511)	0.001 <sup>a</sup>	0.378 (0.107–1.322)	0.130
Pathologic grades (1, 2 vs. 3)	0.353 (0.154–0.809)	0.014 <sup>a</sup>	0.427 (0.184–0.993)	0.048 <sup>a</sup>
Treatment (CIK vs. control)	0.427 (0.186–0.984)	0.046 <sup>a</sup>	0.551 (0.235–1.292)	0.170

Abbreviation: CI, confidence interval.

<sup>a</sup>P < 0.05.

enhanced significantly (Fig. 2D and E, right). In the high-grade group (3 pathologic grade), CIK treatment significantly improved the DFS and OS of patients with TNBC compared with the control group (Fig. 2C and F, right).

## Discussion

During more than a decade of clinical studies, CIK treatment has been confirmed to provide positive clinical efficacy in several types of patients with cancer (25–35). However, to date, there are few reports on the therapeutic effects of CIK treatment in patients with TNBC. Thus, in the present study, we evaluated the efficacy of sequential CIK infusion combined with chemotherapy in patients with post-mastectomy TNBC through a retrospective analysis.

Compared with the control group that received only postoperative chemotherapy (with or without radiation treatment), patients with TNBC who received additional sequential CIK infusion displayed improved DFS and OS rates. Furthermore, multivariate survival analysis showed that the CIK cell treatment was an independent prognostic factor for OS, indicating that CIK treatment is an effective intervention that prevents disease recurrence and prolongs the survival of patients with post-mastectomy TNBC. Our

results are in accordance with previous studies in patients with lung cancer (41), nasopharyngeal carcinoma (33), gastric cancer (32), and colorectal cancer (42) who received CIK infusion combined with chemotherapy. These clinical analyses provide strong evidence in support of the efficacy of CIK cell-based adjuvant treatment combined with chemotherapy for improved outcomes of patients with cancer.

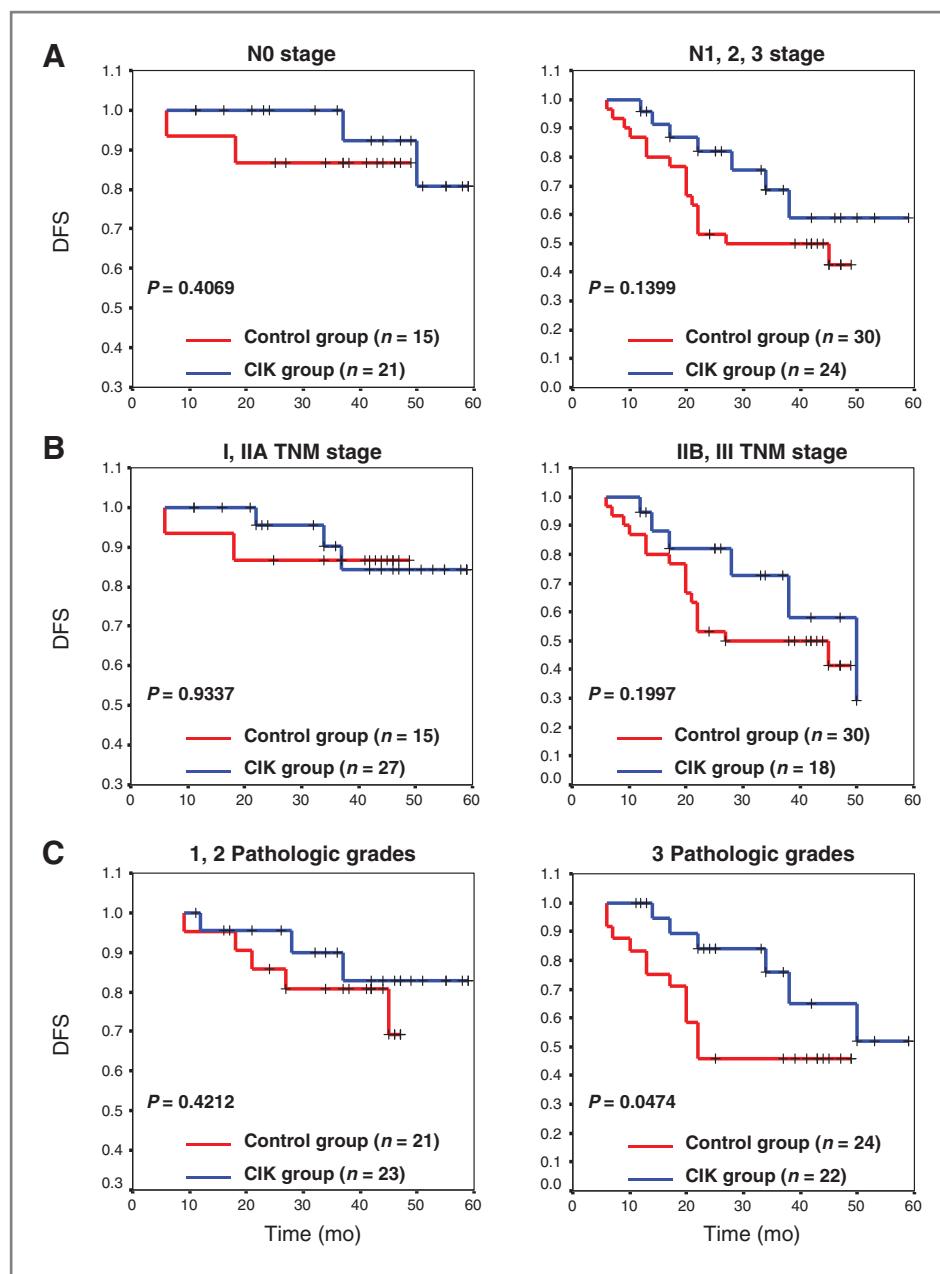
It can be speculated that the mechanism by which CIK immunotherapy enhances the efficacy of chemotherapy in patients with cancer is based on their synergistic effects. First, CIK cells can additionally eliminate potential or residual tumor cells after chemotherapy, including even drug-resistant tumor cells (43, 44). Furthermore, recent two reports showed that CIK cells also had intense tumor killing activity *in vitro* and *in vivo* against putative cancer stem cells, which were resistant to chemotherapy, supporting the clinical activity of CIK cells to reduce recurrence and metastasis risk (45, 46). Second, CIK cells produce large amount of inflammatory cytokines such as IL-2, IFN-γ, and TNF-α (47), which can alleviate immune damage caused by cytotoxic drug and enhance the immunosurveillance capabilities of patients with cancer. On the other hand, chemotherapy can remove immune suppressor factors such as regulatory T cells and myeloid-derived suppressor cells, which

**Table 3.** Univariate and multivariate analysis of OS in patients with TNBC

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
Age (<50 vs. ≥50)	0.553 (0.189–1.621)	0.280		
Radiotherapy (no vs. yes)	0.433 (0.137–1.374)	0.156		
T stage (1 vs. 2, 3, 4)	0.348 (0.098–1.236)	0.103		
N stage (0 vs. 1, 2, 3)	0.089 (0.012–0.682)	0.020 <sup>a</sup>	0.212 (0.016–2.775)	0.237
TNM stage (I, IIA vs. IIB, III)	0.125 (0.028–0.567)	0.007 <sup>a</sup>	0.580 (0.085–3.953)	0.578
Pathologic grades (1, 2 vs. 3)	0.143 (0.032–0.634)	0.011 <sup>a</sup>	0.188 (0.042–0.845)	0.029 <sup>a</sup>
Treatment (CIK vs. control)	0.139 (0.029–0.662)	0.013 <sup>a</sup>	0.186 (0.039–0.896)	0.036 <sup>a</sup>

Abbreviation: CI, confidence interval.

<sup>a</sup>P < 0.05.

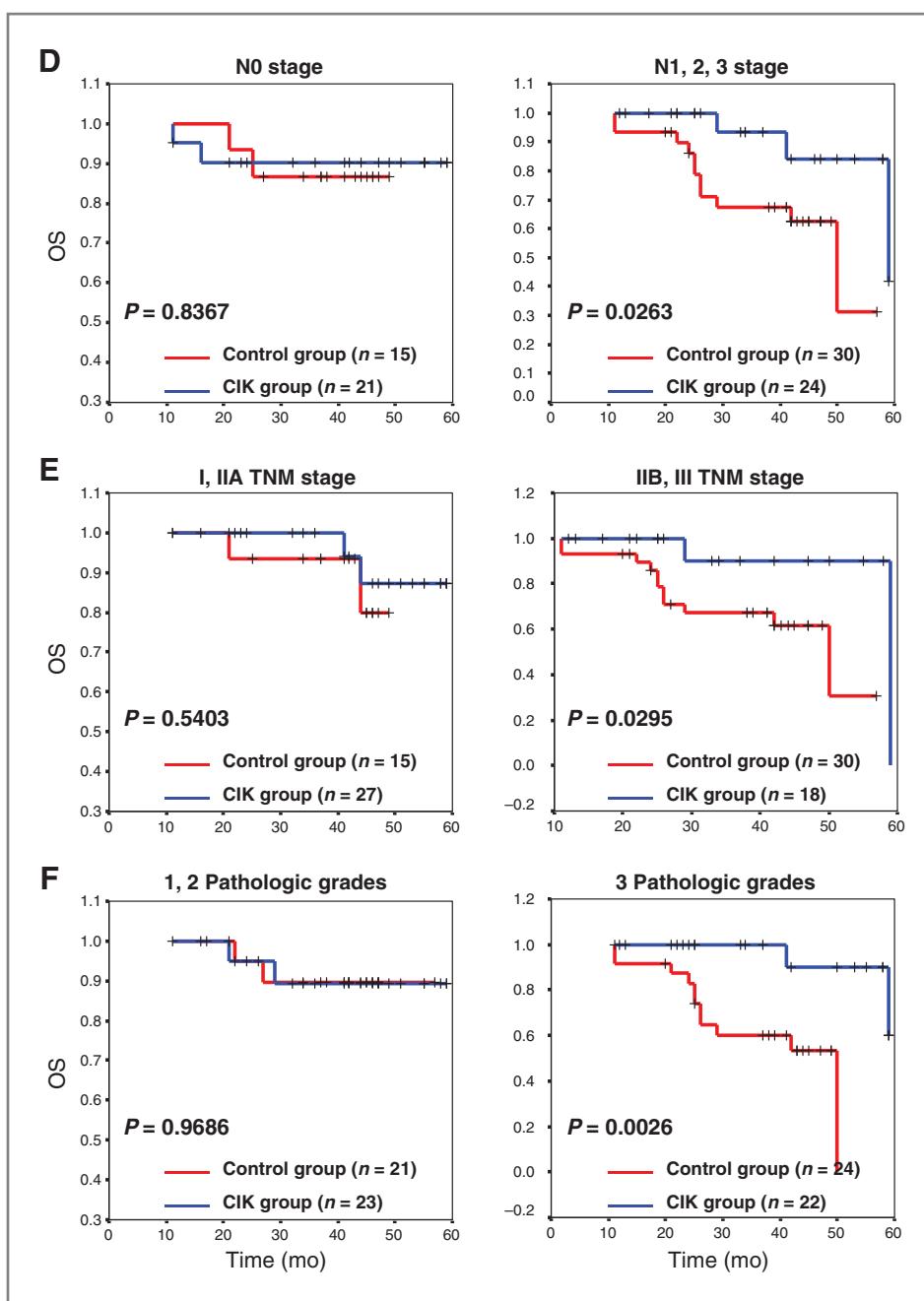


**Figure 2.** Subgroup analysis to estimate the benefits of additional CIK treatment according to N, TNM stages, and pathologic grades. A, DFS curves. Left, N0 stage; right, N1, N2, N3 stages. B, DFS curves. Left, I and IIA TNM stages; right, IIB and III TNM stages. C, DFS curves. Left, 1 and 2 pathologic grades; right, 3 pathologic grade. (Continued on the following page.)

would favor the antitumor functions of immune effector cells (48). Furthermore, a number of chemotherapeutic agents, including anthracycline-based drugs, can not only directly kill tumor cells but also increase the susceptibility of tumor cells to immune effector cells (49). Thus, these observations, together with clinical findings indicate that conventional chemotherapy in combination with CIK immunotherapy represents an optimization strategy to gain improved therapeutic efficacy in the patients with cancer, including patients with TNBC.

Although TNBC is characterized by an especially poor prognosis, an excellent survival rate has been reported following chemotherapy in a subset of patients with TNBC

with early-stage disease (50). Consistent with previous observations, our study also found that patients with TNBC who were lymph node negative, early TNM stage, and low pathologic grade had better DFS and OS rates. In subgroup analyses, adjuvant CIK treatment did not significantly improve the prognosis of this subset of patients with TNBC. However, adjuvant CIK treatment was significantly associated with an improved OS rate in patients with TNBC who were lymph node positive, advanced TNM stage, and high pathologic grade. Adjuvant CIK treatment was also associated with an improved DFS rate in this cohort of patients even though there was no statistical difference for lymph node-positive and advanced TNM stage patients. Thus, our



**Figure 2. (Continued.)** D, OS curves. Left, N0 stage; right, N1, N2, N3 stages. E, OS curves. Left, I and IIA TNM stages; right, IIB and III TNM stages. F, OS curves. Left, 1 and 2 pathologic grades; right, 3 pathologic grade.

findings provide evidence to support the recommendation of additional CIK treatment for patients with high-risk TNBC after surgery and chemotherapy.

In conclusion, in this single-institution study, we provide the first clinical evidence linking the use of sequential CIK infusion with chemotherapy with improved DFS and OS outcomes in patients with single TNBC. Our data show that addition of CIK treatment significantly improves the prognosis of patients with TNBC, especially for those with high-risk disease. In a population with limited targeted options and high risk of early relapse, the efficacy of this intervention should be studied further. Prospective randomized

studies are warranted to confirm the present findings and to further define optimal combinational treatment approaches for this unique subtype of breast cancer.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** K. Pan, J.-J. Li, D.-S. Weng  
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