Original Article
Efficacy of adjuvant chemotherapy combined with immunotherapy with cytokine-induced killer cells for gastric cancer after d2 gastrectomy

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Abstract: Background and aims: It remains a challenge to prevent the local recurrence or distant metastasis of gastric carcinoma after D2 gastrectomy. Cytokine-induced killer (CIK) cells have shown promising activity against solid tumors *in vitro* and *in vivo*. We investigated the effect of adjuvant chemotherapy combined with autologous CIK therapy after D2 gastrectomy compared with adjuvant chemotherapy alone after D2 gastrectomy in patients with stage II-III gastric cancer. Methods: From January 2009 to December 2011, 226 patients with stage II-III gastric cancer who had had curative D2 gastrectomy were enrolled. Eighty-nine patients (CIK group) received adjuvant chemotherapy combining with autologous CIK therapy and 137 patients (control group) received adjuvant chemotherapy alone. Disease-free survival (DFS) and overall survival (OS) were evaluated. Results: Patients in the CIK group had longer DFS and OS than patients in the control group (DFS 41.0 months vs. 32.0 months, OS 45.0 months vs. 44.0 months, by log-rank test P = 0.006 and P = 0.028, respectively). In subgroup analysis, no significant differences in DFS and OS were observed between the two groups for the patients with stages II disease. Patients with stage III disease in the CIK group had longer median DFS and OS than patients in the control group (P = 0.031 and P = 0.038, respectively). In multivariate analysis, the stage and the interaction of stage and CIK therapy were independent prognostic factors for DFS and OS. Conclusions: The data suggest that adjuvant chemotherapy combined with autologous CIK therapy can improve prognosis for gastric carcinoma patients after D2 gastrectomy, especially for the patients with stage III disease.

Keywords: Gastric cancer, immunotherapy, cytokine-induced killer cells, adjuvant chemotherapy, D2 gastrectomy

Introduction

Gastric cancer is the fourth most common malignant disease worldwide and the second most common cause of death from cancer, with 988,000 new cases (7.8% of all cancers) and 736,000 deaths per year [1-3]. Surgery is the main treatment for operable gastric cancer; however, recurrence rates are as high as 40-80% in advanced cases [3, 4]. In East Asia, D2 gastrectomy is the standard surgical treatment for localized gastric cancer, and chemotherapy alone as adjuvant treatment is now considered the standard of care after adequate D2 gastrectomy [5-7]. However, adjuvant treatment results in only a modest reduction of the risk of cancer-related death by 25-30%, translating into an absolute 5-year survival benefit of only 10-15% [8]. This poor outcome has prompted major efforts to explore different effective adjuvant therapies to prolong the survival of patients with gastric cancer.

In recent years, immune therapy has become the fourth important treatment modality for malignant tumors following surgery, radiotherapy and chemotherapy [9-11]. Adoptive immunotherapy, as an adjuvant or alternative treat-

ment, is anticipated to be an innovative approach to treat most malignancies. The general concept of immunotherapy is to stimulate the patient's immune system *ex vivo* or *in vivo* in order to induce an antitumor immune response and to restore the patient's immune status. Cytokine-induced killer (CIK) cells meet all requirements for application in adoptive immunotherapy [12].

The CIK cells are heterogeneous *ex vivo*-expanded T lymphocytes, with a mixed T-NK phenotype, able to exert a wide MHC-unrestricted antitumor activity against both solid and hematologic malignancies [13, 14]. Among CIK cells it is possible to distinguish two main subsets, positive (CD3+CD56+) and negative (CD3+CD56-) for the membrane expression of CD56. The antitumor activity of CIK cells is mainly due to the CD3+CD56+ fraction, while the CD3+CD56- cells are more similar to conventional T lymphocytes [13, 15, 16]. The high proliferation of CD3+CD56+ cells and cytotoxic activity of T-cell receptor alpha/beta (TCR-α/β) cells in CIK cell cultures leads to the primary effective and substantial lytic activity of CIK cells toward tumor cells by releasing of cytoplasmic cytotoxic granule contents to the extracellular space on stimulation by susceptible target cells [17, 18]. The cytotoxicity of these CD3+CD56+ cells is non-MHC-restricted, perforin-mediated and induced via the natural killer group 2 member D (NKG2D) cell-surface receptors [19, 20]. The NKG2D ligands, for example, MHC class I-related chain (MIC) A/B and UL-16 binding protein 1-4, are over-expressed on both solid and hematologic tumor cells, turning these cells into a favored targets for CIK cells [21, 22].

Our previous studies showed that the high expression of MHC class I chain-related gene A (MICA) is one of the indicators of a poor prognosis for advanced non-small cell lung cancer patients [23], but there is limited data about the CIK in gastric cancer [24-26]. In the present study, we evaluate the clinical outcome of CIK cell immunotherapy as an adjuvant therapy for postoperative patients with gastric cancer.

Methods

Ethics statement

All procedures were conducted in accordance with the Helsinki Declaration, and with approval from the Ethics Committee of Fujian Provincial Cancer Hospital. Written informed consent was obtained from all participants.

Patients

We retrospectively studied all the patients with stage II-III gastric carcinoma diagnosed after surgery who were admitted into the Department of Medical Oncology of Fujian Provincial Cancer Hospital from January 2009 to December 2011. Two hundred and twenty-six consecutive patients were recruited into the study according to the following criteria: 1) All the patients had R0 gastrectomy with D2 lymphadenectomy, 2) All patients were diagnosed and histologically confirmed with stages IIA, IIB, IIIA, IIIB, or IIIC disease according to the American Joint Committee on Cancer (AJCC) TNM Staging Classification for Carcinoma of the Stomach (7th ed., 2010), 3) Eastern Cooperative Oncology Group performance status between 0 and 2 before adjuvant chemotherapy, 4) Complete medical records were available, 5) All patients had received at least four cycles of adjuvant chemotherapy based on 5-fluorouracil (5-FU) or capecitabine doublet regimens, 6) Patients non-randomly received adjuvant immmunotherapy with CIK cells, and patients who received CIK cell treatment were given at least two cycles, 7) Adequate bone marrow function with leukocyte counts 3,000-12,000/mm3, hemoglobin $\geq$ 8.0 g/dl, and platelet counts $\geq$ 100,000/mm^3, 8) Adequate liver function with total serum bilirubin $\leq$ 2.0 mg/dl and serum transaminases $\leq$ 100/UI, 9) Adequate renal function with serum creatinine within the upper limit of normal, 10) An expected survival period of > 3 months.

CIK cells preparation

The CIK cells were isolated and cultured according to a standard protocol as described in our previous studies [23]. Briefly, peripheral blood (50 ml) was drawn from patients using heparin as an anticoagulant. Mononuclear cells were isolated by Ficoll-Conray density gradient centrifugation and their viability assessed by trypan blue exclusion. About 2.0×10^6/ml mononuclear cells were plated onto six-well dishes and cultured with Medium I containing RPMI 1640 plus 1.0×10^6 U/L human interferon gamma (IFN-γ), 5.0×10^5 U/L recombinant human interleukin-2 (IL-2), 10% heat inactivated

Table 1. Patient characteristics

Characteristics		Total 226	Control group 137	CIK group 89	*P*-value
Sex	Male	178	108	70	0.974
	Female	48	29	19	
Age	< 65	155	92	63	0.565
	≥ 65	71	45	26	
Histological grade	G1-G2	105	57	48	0.069
	G3-G4	121	80	41	
Stage	II	72	38	34	0.099
	III	154	99	55	
Adjuvant Chemotherapy	Xelox Folfox4	142	89	53	0.168
	PF	84	48	36	

human serum, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C. After 24 h, 100 µg/L of monoclonal antibody (MAb) against CD3 and 1.0×10^5 U/L IL-1α were added. After another 48 h, the supernatant was aspirated and the cells were cultured in Medium II (Medium I in the absence of INF-γ). The medium was changed every three days. Cell viability was determined using trypan blue staining. The CIK cells were transfused back into the donors following eight days of culture. All CIK cell cultures were tested for contamination (bacteria, fungi, and mycoplasma) throughout the study to assure culture quality and transfusion safety.

Adjuvant chemotherapy

All patients had received at least four cycles of adjuvant chemotherapy based on 5-fluorouracil (5-FU) or capecitabine doublet regimens, including Xelox (Capecitabine, 1,000 mg/m² twice daily on days 1 to 14 of each cycle. Oxaliplatin, 130 mg/m² on day 1 of each cycle), Folfox4 (Oxaliplatin, 85 mg/m² on day 1, 5-florouracil, 2,400 mg/m² 46-hour infusion Leucovorin (400 mg/m² on day 1 of each cycle), PF (Paclitaxel, 135 mg/m² on day 1, 5-florouracil, 2,400 mg/m² 46-hour infusion, and Leucovorin, 400 mg/m² on day 1 of each cycle).

CIK cells treatment

As shown in **Table 1**, 137 patients (control group) received adjuvant chemotherapy alone, and 89 patients (CIK group) received adjuvant chemotherapy combined with autologous CIK cell therapy. The patients in the CIK group received at least three cycles of CIK cell therapy, the first one of which was given within two weeks after surgery, and the others were given once per month starting within six weeks after adjuvant chemotherapy. For each cycle, patients were given an infusion of at least 1.0×10^{10} CIK cells. The patients were eligible for CIK cells therapy until they no longer agreed to continue treatment or until disease recurrence.

With the eventual failure of this adjuvant therapy, the first-line, second-line chemotherapy according to 2010 NCCN Clinical Practice Guidelines in Oncology were recommended to all patients if their performance status was preserved.

Evaluation of toxicity and efficacy

All patients were followed up at the outpatient clinic from the date of surgery to December 31, 2014, or to the time of death. During treatment, patients were evaluated with abdominal computed tomography (CT) scans every 1-2 months according to the Response Evaluation Criteria In Solid Tumors (RECIST) criteria [27]. A complete blood cell count and measurements of liver and renal function were assessed at least once a week during the treatment. Non-hematological toxicities were also verified at least once a week by patient interview and physical examination. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 3.0 [28]. Disease-Free-Survival (DFS) was measured from the day of surgery to the first evidence of recurrence or death. Overall Survival
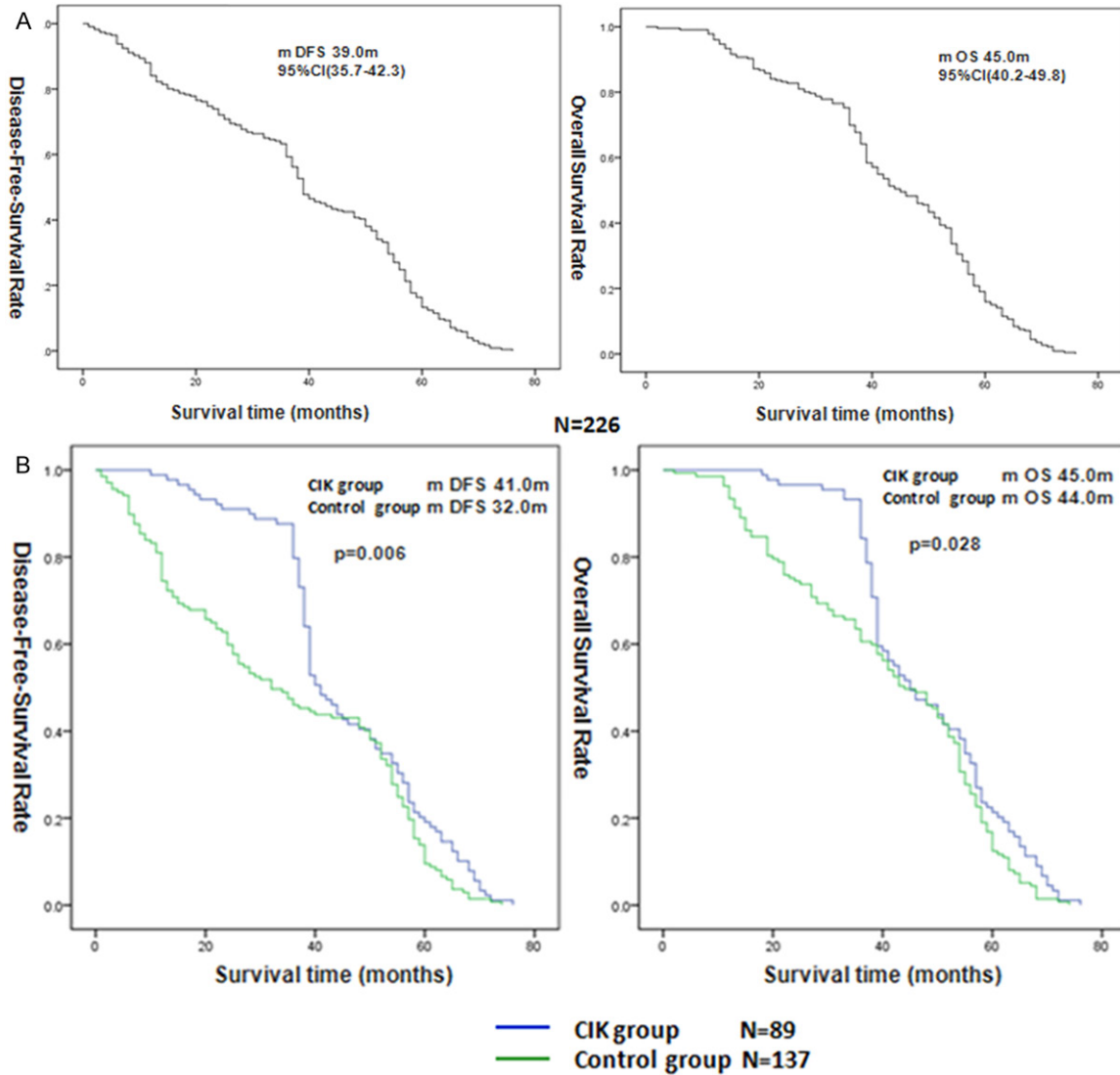
Figure 1. Kaplan-Meier curves for Disease-Free-Survival (DFS) and Overall Survival (OS) in 226 patients (A). DFS and OS in the patients received adjuvant chemotherapy combining with or not CIK therapy (B).

(OS) was defined from the date of surgery to death from any cause.

Statistical methods

Statistical analysis was performed with the SPSS software (Version 18.0, SPSS). For all analyses, the significance level was specified as $P< 0.05$. Comparisons between the immunotherapy and control groups were analyzed using the χ^2 test and the Fisher exact probability test. The OS and DFS variables were estimated by the Kaplan-Meier method and survival curves were plotted. Two-sided log-rank tests were used to compare survival rates between groups. Multivariate analyses using the Cox proportional hazards regression model were performed to assess the impact of the variables on DFS and OS.

Results

From January 2009 to December 2011, 226 patients with stage II-III gastric carcinoma were enrolled in this study and were fully evaluated for DFS and OS. One hundred and thirty-seven patients received adjuvant chemotherapy alone, and 89 patients received adjuvant chemotherapy combining with autologous CIK cell therapy. Patient characteristics are listed in **Table 1**, showing that there were no statistically significant differences in patient characteristics between the two groups.

Table 2. Univariate analysis of factors associated with survival of 226 patients

Variable		n	m DFS	P-value	m OS	P-value
Sex	Male	178	39	0.794	45	0.942
	Female	48	39		45	
Age	< 65	155	39	0.427	46	0.611
	≥ 65	71	38		43	
Histological grade	G1-G2	105	44	0.048	49	0.090
	G3-G4	121	37		42	
Stage	II	72	48	0.007	50	0.013
	III	154	36		42	
CIK therapy Yes		89	41	0.006	45	0.028
	No	137	32		44	
Adjuvant chemotherapy	Xelox Folfox4	142	39	0.883	46	0.721
	PF	84	39		43	

Phenotypic analysis of CIK cells

The phenotype of the cultured cells was determined by flow cytometry (BD FACSCalibur). The cells were labeled with monoclonal antibodies (mAbs) that recognize human CD3, CD4, CD8, CD56, CD19, CD25, and CD127. Phenotypic analysis of cells in the 89 patients after 14 days of culture demonstrated that the percentages of CD8+, CD4+, CD3+, CD3+CD56+, CD19+, and CD4+CD25+CD127- cells were 72.8 ± 4.5%, 8.3 ± 3.2%, 88.4 ± 7.5%, 23.2 ± 6.5%, 0.4 ± 0.2%, and 0.3 ± 0.2% respectively.

DFS and OS

For the 226 patients, the median follow-up period was 44.1 months, 95% CI = 41.9-46.3 months. By the end of follow-up 93 patients (41.2%) had died and 96 patients (42.5%) had been diagnosed with recurrence. The median DFS was 39.0 months, 95% CI = 35.7-42.3 months, and median OS was 45.0 months, 95% CI = 40.2-49.8 months, and the 3-year DFS rate and 3-year OS rate were 59.3% and 69.9%, respectively (**Figure 1A**).

Univariate survival analysis

In the univariate analysis, stage, and CIK therapy were associated with DFS and OS, and histological grade was associated with DFS but not with OS. While the sex, age, and the regimens of adjuvant chemotherapy were not correlated with DFS or OS (**Table 2**). The median DFS and OS of patients in the CIK group were significantly longer than for those in the control group, DFS 41.0 months vs. 32.0 months, OS 45.0 months vs. 44.0 months, by log-rank test P = 0.006 and P = 0.028, respectively (**Figure 1B**).

In the subgroup analysis, it was found that the median DFS and OS of patients with stage III disease in the CIK group were both longer than the median DFS and OS for patients in the control group, P = 0.031 and P = 0.038, respectively (**Figure 2B**). For the patients with stage II disease, the median DFS and OS were not significantly different between the CIK group and control group, P = 0.799 and P = 0.727, respectively (**Figure 2A**).

Multivariate survival analysis

In the multivariate analysis, variables that included sex, age, histological grade, stage, CIK therapy, adjuvant chemotherapy, and the interaction of stage and CIK therapy, were tested to determine their independent effect on DFS and OS. The CIK therapy was an independent prognostic factor for DFS (**Table 3**). The stage and the interaction of stage and CIK therapy were independent prognostic factors for DFS and OS (**Table 3**). This shows that the CIK therapy has a good effect in patients with stage III disease, which was consistent with the results of the univariate subgroup analysis.

Safety of CIK cell treatment

In the CIK group, autologous CIK cell treatment was generally well tolerated. CIK-treatment-related adverse events (Grade 1/2) were observed in 23.6% of patients, with the most common events being flu-like symptoms such as fever and fatigue (13 patients, 14.6%), rash (five patients, 5.6%), and diarrhea (three patients, 3.4%). No patient experienced adverse events consistent with grade 3/4 toxicity or an autoimmune reaction.

Discussion

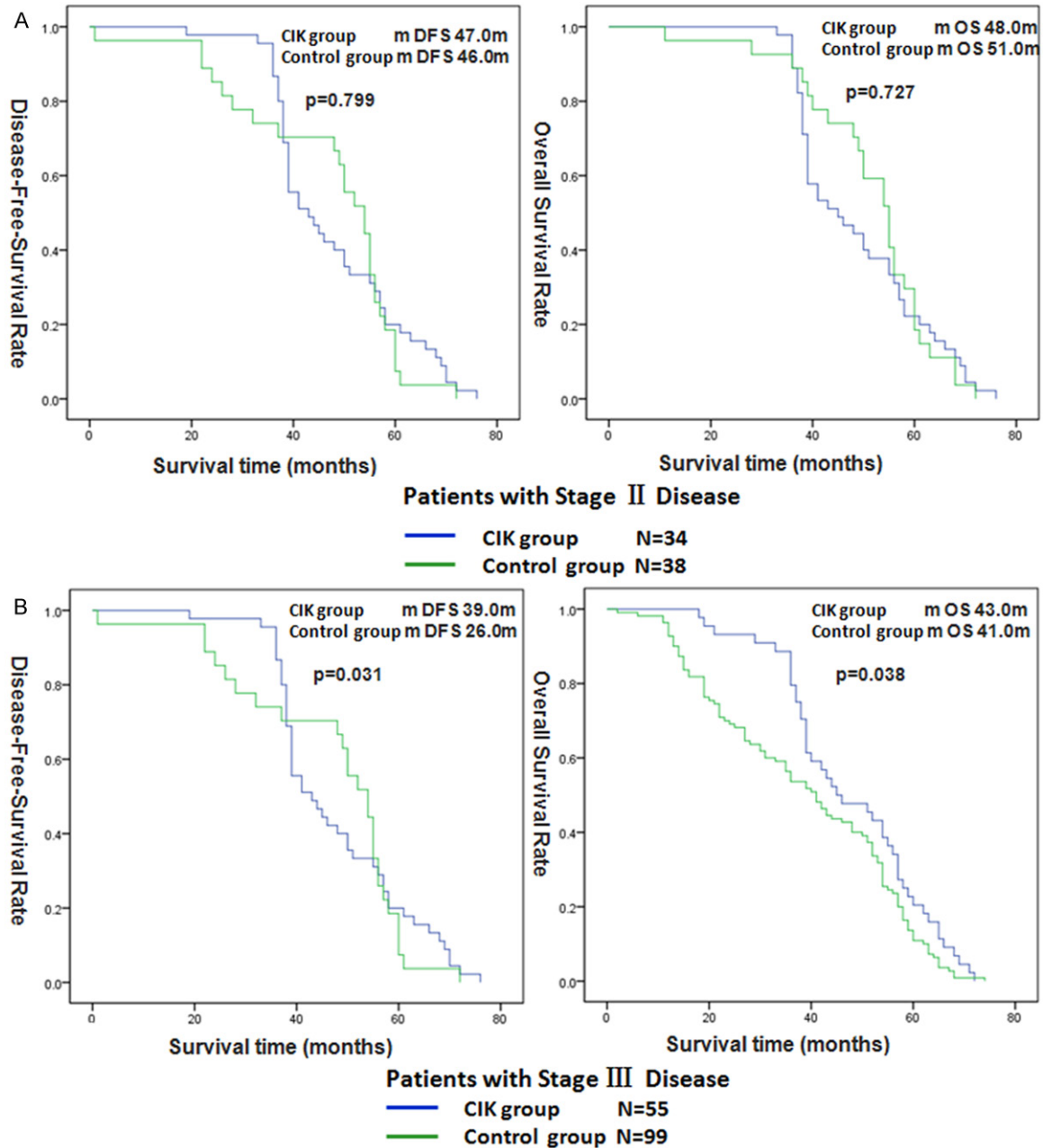In China, gastric cancers are usually diagnosed at a relatively advanced stage. Surgery is the

Figure 2. Disease-Free-Survival (DFS) and Overall Survival (OS) in the patients with stage II by CIK therapy (A). DFS and OS in the patients with stage III by CIK therapy (B).

main treatment for operable gastric cancer, and adjuvant chemotherapy is a standard component of resectable gastric cancer therapy and improves patient outcomes, however, recurrence rates are as high as 40-80% in advanced cases [5, 7, 29, 30]. CIK cells, or NK T-lymphocytes, hold great promise in the quest for new therapeutic approaches in the setting with solid tumors refractory to standard treatments. In the last 10 years efforts have been

made to evaluate the clinical value of CIK cell therapy and to improve cellular effectivity. CIK cell therapy has been combined with different therapies, resulting in promising clinical responses [12, 31].

In this study, we evaluated the potential effects of adjuvant chemotherapy combined with CIK cells in patients with locally advanced gastric carcinoma after gastrectomy. To our knowl-

Table 3. Multivariate analysis (Cox model) of factors associated with disease-free-survival (DFS) and overall survival (OS) of 226 patients

Variable	DFS			OS		
	P value	Hazard ratio	95% CI	P value	Hazard ratio	95% CI
Stage	0.047	1.347	1.004-1.894	0.018	1.408	1.061-1.869
CIK therapy	0.043	1.336	1.009-1.769	-	-	-
Stage\CIK Interaction	0.001	1.146	1.058-1.241	0.003	1.129	1.041-1.224

edge, the present report is the largest prognostic study in gastric cancer treated with adjuvant CIK cells immunotherapy.

Liu et al. combined CIK cell therapy with Folfox4 chemotherapy for the postoperative treatment of 98 patients with gastric carcinoma [32]. Compared with the control group, the 1-, 2- and 3-year recurrence rates were significantly (P < 0.05) reduced in the immunotherapy group while the survival rates were significantly (P < 0.05) improved. In a different study, 151 postoperative gastric carcinoma patients who had received six cycles of adjuvant chemotherapy based on 5-FU were divided into a control group receiving no additional treatment and an immunotherapy group treated with adjuvant CIK cell therapy [33]. Immunotherapy significantly (P = 0.044) prolonged 5-year DFS (28.3% vs. 10.4%) but not 5-year OS (32.4% in the immunotherapy group vs. 23.4% in the control group, P = 0.071).

In order to balance the baseline of our patients, we had enrolled the patients with stage II and III gastric carcinoma who had accepted a D2 radical operation and at least four cycles of postoperative chemotherapy. Our result shows that adjuvant chemotherapy combined with CIK cells could prolong the survival time of patients with stage II-III gastric carcinoma after D2 gastrectomy. However, the single-factor subgroup analysis revealed that the population of beneficiaries was the patients with gastric carcinoma in stage III, which has been substantiated in multi-factor analysis. In the multivariate analysis, the stage and the interaction of stage and CIK therapy were independent prognostic factors for DFS and OS. This means that CIK therapy has a better effect in patients with stage III disease, which is consistent with the results of the univariate subgroup analysis.

We considered that this could be explained by two possibilities. First, the 5-year survival rate of the patients with gastric carcinoma in phase II exceeds 60%. There were almost half of the patients who were not expected to relapse in this time frame among the group under investigation from 2009 to 2011, and the patients in stage II account for a large proportion of the total patient population. So within the period of follow-up visits, it remains impossible to observe a statistically significant effect; perhaps a positive statistical result can be observed over a longer period of follow-up visits. Second, according to other findings, the later the state of malignant cancer, typically the more significantly the patients' immunologic function is inhibited, and relapse tends to occur within three years in patients with stage III gastric carcinoma. CIK can restore the immune function in patients after an operation for a solid tumor. Thus the survival benefit CIK brings to patients with stage III disease is particularly apparent in the survival curve because of restored immune function. But our findings need a larger sample and longer follow-up visit periods in future studies in order to verify this.

We found patients have good tolerance of CIK therapy with only slight untoward side-effects. This result is consistent with other centers' research. With measurable benefits and limited side-effects patients are advised to accept the CIK therapy after the postoperative chemotherapy for gastric carcinoma provided that their condition permits.

Our results are limited by the heterogeneity in the study design and the retrospective nature of the analysis. Therefore, a treatment model for systemic chemotherapy combined with CIK therapy needs further investigation with an expanded sample size in further prospective clinical studies.

In conclusion, these results indicate that CIK cell therapy combined with adjuvant chemotherapy is associated with an improved progno-

sis for gastric carcinoma patients after D2 gastrectomy, especially for the patients with stage III disease. The side effects of this treatment are mild. A prospective randomized trial in our hospital is being carried out to further validate these findings.

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