



Cell-based Immunotherapy for Colorectal Cancer with Cytokine-induced Killer Cells

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Colorectal cancer is the third leading cancer worldwide. Although incidence and mortality of colorectal cancer are gradually decreasing in the US, patients with metastatic colorectal cancer have poor prognosis with an estimated 5-year survival rate of less than 10%. Over the past decade, advances in combination chemotherapy regimens for colorectal cancer have led to significant improvement in progression-free and overall survival. However, patients with metastatic disease gain little clinical benefit from conventional therapy, which is associated with grade 3 ~ 4 toxicity with negative effects on quality of life. In previous clinical studies, cell-based immunotherapy using dendritic cell vaccines and sentinel lymph node T cell therapy showed promising therapeutic results for metastatic colorectal cancer. In our preclinical and previous clinical studies, cytokine-induced killer (CIK) cells treatment for colorectal cancer showed favorable responses without toxicities. Here, we review current treatment options for colorectal cancer and summarize available clinical studies utilizing cell-based immunotherapy. Based on these studies, we recommend the use of CIK cell therapy as a promising therapeutic strategy for patients with metastatic colorectal cancer.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent cancers, along with lung and breast cancers. In 2012, approximately 1.4 million new cases and 700,000 CRC-related deaths were estimated worldwide (1). Over the last decade, CRC incidence and mortality in the United States have been on a steady decline as a result of advances in its prevention, screening, and treatment. Nevertheless, it is still the second leading cause of cancer deaths (2). Moreover, there has been a significant rise in CRC incidence in newly developed or economically transitioning countries, such as South America, Eastern European countries, and most parts of Asia (3). Although CRC is highly curable at an early stage, approximately 50% of patients with CRCs eventually develop metastatic disease (4). Five-year survival rate is approximately 10% in patients with a stage IV disease (5).

There are many known risk factors for CRC including age, sex, smoking, excessive alcohol consumption, high intake of red and processed meat, obesity, diabetes, inflammatory bowel disease, inherited genetic disorder, and

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Abbreviations: CIK cells, cytokine-induced killer cells; CRC, colorectal cancer; LOH, loss of heterozygosity; CIN, chromosomal instability; MSI, microsatellite instability; MMR, mismatch repair; DFS, disease-free survival; OS, overall survival; 5-FU, 5-fluorouracil; LV, leucovorin; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; DCs, dendritic cells; CEA, carcinoembryonic antigen; MAGE, melanoma-associated antigen; ACT, adoptive cell therapy; TILs, tumor-infiltrating lymphocytes; SLN, sentinel lymph node

family history of CRC (6-12). Among them, the most significant risk factor is age. Incidence significantly increases in adults 50 years and older (13). Furthermore, inflammatory bowel disease—ulcerative colitis and Crohn's disease—increase the overall risk of colitis-associated cancer (14). A link between inflammation and cancer is well-established. Approximately 20% of patients with inflammatory bowel disease develop colitis-associated cancer within 30 years of disease onset (15). Inherited CRC, including Lynch syndrome and familial adenomatous polyposis, represent less than 5% of CRC cases (16).

In this review, we briefly review currently available drugs for the treatment of colorectal cancer and summarize clinical studies that use cell-based cancer immunotherapy as promising therapeutic strategy. We also suggest the use of cytokine-induced killer (CIK) cells as an additional therapeutic approach for metastatic colorectal cancer.

THE MOLECULAR PATHOGENESIS OF COLORECTAL CANCER

Colorectal cancer is a heterogeneous disease. Most CRCs occur without a known genetic risk factor and significant family history. Only a small proportion (5%) of inherited CRCs, which is also known as familial CRC, have been thoroughly characterized (17), and additional 20~25% are yet to be completely understood (17). CRC develops over the course of more than 10 years, and entails multistep genetic events for tumor progression. A Multistep genetic model of colorectal carcinogenesis was proposed by Fearon and Vogelstein. Colorectal cancers occur as a result of oncogenes activation, along with the inactivation of tumor suppressor genes by mutations or loss of heterozygosity (LOH) (18). Mutations in at least four to five genes are required for the formation of a malignant tumor (18).

The molecular pathogenesis of colorectal cancer is commonly classified into two main pathways of genomic instability: chromosomal instability (CIN) and microsatellite instability (MSI) pathways. CIN leads to an imbalance in chromosome number (aneuploidy) such as chromosome gains or losses (including LOH events at specific tumor suppressor gene loci), and is observed in 80~85% of sporadic CRCs (18-21). A mutation coupled with LOH of the adenomatous polyposis coli (APC) tumor suppressor gene, followed by activating mutations of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), lead to the

initial appearance of adenoma from normal colonic epithelium (18). APC mutations are typically observed in earliest stages of tumors, including in adenomas as small as 0.5 cm (22). This indicates that a functional loss of APC, which is part of the Wnt signaling pathway, plays a role in early tumorigenesis. In contrast, MSI, which is observed in 15~20% of sporadic CRCs (21), is a hypermutable phenotype caused by impaired DNA mismatch repair (MMR) system to correct errors such as single-base mismatches and insertion-deletion loops that spontaneously occur during DNA replication (23,24). MSI arises from impaired DNA MMR genes, such as MutL homolog 1 (MLH) 1, MutS homolog (MSH) 2, and to a lesser degree MSH6 (25,26). Faulty DNA fidelity caused by defects in DNA MMR causes frameshift and point mutations, mainly in repetitive sequences (microsatellites) (26). Lynch syndrome is a well-established hereditary predisposition to colorectal cancer caused by a germline mutation in a DNA MMR gene. The syndrome accounts for approximately 3% of all CRCs (16). However, 12% of MSI exhibit sporadic CRC with CIN (27). Because pathways are not mutually exclusive, CRC can display features of multiple pathways. The significance of these features is not fully understood (28).

CURRENT TREATMENT OF COLORECTAL CANCER

Surgery is the cornerstone treatment for colorectal cancer. In stage I colon cancer, surgery is the definitive treatment without the use of adjuvant chemotherapy. Benefit of adjuvant chemotherapy for patients with a stage II disease is highly debatable due to minimal gains in overall response. However, adjuvant chemotherapy is generally acceptable for the use patients with node-positive cancer following surgery (high-risk stage II disease) (29). Adjuvant chemotherapy is required for all patients with a stage III colon cancer after surgical resection, which have a high risk of recurrence of 15~50% (30). Conventional chemotherapeutic agents include 5-fluorouracil (5-FU) with leucovorin (LV), capecitabine (an orally administered prodrug of 5-FU), oxaliplatin, and irinotecan. Adjuvant chemotherapy using the 5-FU/LV regimens following surgery provides significant reduction in mortality by 22% and improves event-free survival by 35% (31). To improve disease-free survival (DFS) and overall survival (OS), currently, FOLFOX (5-FU/LV and oxaliplatin) or FOLFIRI

(5-FU/LV and irinotecan) is widely used for standard first- or second-line treatment in patients with node-positive colon cancer following surgical resection (32,33). However, FOLFOX and FOLFIRI demonstrated greater toxicities, including grade 3 or higher acute toxicity, compared to 5-FU/LV alone (34).

In 2004, targeted therapeutic agents for metastatic colorectal cancer, such as anti-epidermal growth factor receptor (EGFR) antibodies (cetuximab and panitumumab), vascular endothelial growth factor (VEGF) inhibitors (bevacizumab, ziv-aflibercept, and ramucirumab), and a multikinase inhibitor (regorafenib), were approved by the US Food and Drug Administration (FDA) for combinational use with standard chemotherapy treatment (35). Currently, there are various regimens of combinational therapies using targeted therapeutic agents and conventional chemotherapy drugs (36). Treatment strategy is often dependent on tumor-specific factors, such as KRAS and/or v-Raf murine sarcoma viral oncogene homolog B (BRAF) mutation, and condition of patients and their disease progression following first-line treatment (33,36). Since RAS mutations lead to continuous activation of signaling pathways downstream of EGFR, the use of anti-EGFR antibodies is effective in patients with wild-type KRAS exon 2, but not in patients with KRAS and/or BRAF mutations. Thus, approximately 30~40% of CRCs are associated with KRAS gene mutation and do not respond to anti-EGFR antibodies (37). Over the past decade, multidisciplinary advances in the treatment of metastatic colorectal cancer with targeted therapy have statistically improved progression-free and overall survivals. Despite these advances, only a relatively small effect on survival outcomes was observed, especially in patients with distant metastatic disease. Therefore, development of new therapeutic strategy against metastatic colorectal cancer is urgently needed.

DENDRITIC CELL THERAPY FOR COLORECTAL CANCER

Dendritic cell (DC) therapy is one of several strategies in therapeutic cancer vaccines. Sipuleucel-T, a DC-based cancer vaccine, has been approved by the US FDA for advanced prostate cancer in 2011. The goal of DC vaccination is to elicit anti-tumor response by inducing tumor-specific effector T cells. DCs are generated *ex vivo* by culturing hematopoietic progenitor cells or monocytes

with a combination of cytokines and then pulsing them with antigens *ex vivo* (38). Tumor antigen choice for DCs loading is crucial to achieve optimal clinical outcomes. There are various type of cancer antigens for DCs loading, including mutated and non-mutated antigens (39).

Now, we can find 9 clinical trials for the treatment of patients with CRC from 2004 to 2015 (Table 1). Antigens that are most commonly used for colorectal cancer include carcinoembryonic antigen (CEA) peptides (40,41), melanoma-associated antigen (MAGE) from allogeneic melanoma cell lysates (42,43), and autologous cell lysates from biopsy material (44). While CEA expression in normal colon epithelial cells is relatively low, it is over expressed in most colorectal carcinomas as well as in many cancers (45). Therefore, CEA has been the major immunological target of DC-based cancer vaccines for colorectal cancer. Clinical trials of CEA-pulsed DCs demonstrated its immune-stimulatory capacity and it was well tolerated in patients without any observable toxicities. However, the overall clinical response was rather unimpressive (40,41,46), which may reflect severely impaired immune functions in patients with excessive tumor burdens and tumor immunoediting mechanisms. Nevertheless, several clinical studies of MAGE-pulsed DCs showed 24~40% clinical benefit rate with durable responses and tumor regression (42,43). Moreover, regulatory T cell levels declined upon DC vaccination (42). Recently, Hunyadi J. and colleagues demonstrated that autologous tumor cell lysates-loaded DCs led to an increase in 6-years survival rate in colorectal cancer patients and more efficient induction of T-lymphocytes proliferation *in vitro* when compared to CEA-pulsed DCs (44). However, because of limited number of patients, additional evaluations in large-scale clinical trials are needed. Although significant advances have been made over the past decade, further studies are required to fully determine the potential antitumor effects of DC vaccination for colorectal cancer.

T CELL THERAPY OF COLORECTAL CANCER

Adoptive cell therapy (ACT) for metastatic melanoma was first described in 1988 (47). In ACT, tumor-infiltrating lymphocytes (TILs) are collected from solid tumor specimen, and are activated and expanded *ex vivo*. Subsequently, TILs are administered intravenously to the autologous patient. In 2002, Dudley ME et al. demonstrated that host im-

Table I. Summary of clinical studies of dendritic cell-based immunotherapy for CRC (a search of the PubMed from 2004 to 2015)

Author	Cell types	Number of patients	Clinical outcome	Adverse events
Hunyadi et al. (44)	DC+autologous tumor cell lysates DC+CEA peptide	6 patients	Survival for 6-years (5 of 6)	No adverse effects
Morse et al. (65)	DC+Poxvectors encoding CEA and MUC1 (PANVAC)	37 patients	Survival for 6-years (3 of 3) 2 of 37 death	No grade 3/4 toxicity except grade 3 urticaria on the DC+PANVAC
Sakakibara et al. (41)	PANVAC+GM-CSF DC+CEA peptide	37 patients 10 patients	5 of 37 death PD (7), SD (1), withdrawal (2)	No adverse effects except grade 1 fever Mild grade 1 or 2 toxicity
Toh et al. (42)	DC+MAGE	20 patients	PD (11), PR (1), SD (7), withdrawal (1)	No adverse effects
Burgdorf et al. (43)	DC+MAGE	20 patients	24% of SD (4)	No adverse effects
Kavanagh et al. (46)	DC+CEA peptide	21 patients	11 of all patients evaluated, PD (11)	No significant toxicity
Morse et al. (66)	DC+Fowlpox vector encoding CEA and costimulatory molecules	11 patients, 3 patients (NSCLC)	PD (8), SD/MR (6)	No grade 3/4 toxicity
Liu et al. (40)	DC+CEA peptide	10 patients	PD (8), SD (2)	No grade 2/3 toxicity
Matsuda et al. (67)	DC+CEA peptide	7 patients	PD (5), SD (2)	No adverse effects

DC, dendritic cell; CEA, carcinoembryonic antigen; GM-CSF, granulocyte-macrophage colony-stimulating factor; PD, progressive disease; SD, stable disease; PR, partial response; MR, minor response; MAGE, melanoma-associated antigen; CRC, colorectal cancer; NSCLC, non-small cell lung cancer.

munodepletion using cyclophosphamide and fludarabine prior to TIL infusion resulted in dramatic improvement in clinical outcomes for patients with metastatic melanoma (48). Although ACT has been evaluated in various cancers, only a small number of ACT trials for CRC have been performed. In a phase I clinical trial, 14 patients with colorectal liver metastases received ACT with TILs in combination with high-dose IL-2 (49). Unfortunately, no significant difference in DFS rates was observed between TILs therapy and conventional chemotherapy. A novel ACT approach for CRC was conducted in two separate studies; sentinel lymph node (SLN) T lymphocytes were used in a pilot study (seven with stage II-III and nine with stage IV CRC) (50) and in a phase I/II study (46 with stage I-III and nine with stage IV CRC) (51), respectively. The pilot study proposed feasible clinical outcomes in patients with CRC (50). All patients with stage IV disease responded to the treatment with the following outcomes: four stable disease, one partial response, and four complete response with no detectable cancer cells remaining. The overall survival of ACT-treated patients was also significantly improved to 2.6 years compared to 0.8 years in conventionally-treated control patients. The latest phase I/II study also showed favorable clinical outcomes (51). The median overall survival of SLN-T lymphocytes-treated and control groups in stage IV patients were 28 and 14 months, respectively. In the two studies, no significant toxicity was observed following SLN-T lymphocytes treatment. These studies demonstrated that SLN-T lymphocyte immunotherapy is indeed feasible and safe for patients with metastatic CRC. However, surgeon's proficiency may be required for the intraoperative sentinel node detection. In a phase I study of autologous genetically engineered T cells, all patients developed severe colitis despite showing a decrease in serum CEA levels by CEA-specific T cells (52).

CYTOKINE-INDUCED KILL CELL THERAPY FOR COLORECTAL CANCER

Cytokine-induced killer (CIK) cells are *ex vivo*-expanded lymphocytes used for cancer immunotherapy. CIK cells are generated by culturing peripheral blood mononuclear cells (PBMCs) with a combination of IL-2 and anti-CD3 monoclonal antibodies for 14 days (53). Subsequently, CIK cells are harvested and administered intravenously to the autologous patient. CIK cells consist of heterogeneous

cell population, mainly CD3⁺CD56⁻, CD3⁺CD56⁺, and a minor population of CD3⁻CD56⁺ cells. Importantly, CD3⁺CD56⁺ cells are the distinguishing cell population among the CIK cells and have the most potent cytotoxic function (53). CD3⁺CD56⁺ cells originated from CD3⁺CD8⁺CD56⁻ cells but not from CD3⁺CD56⁺ cells during the *ex vivo* expansion (54). CIK cell cytotoxicity is mediated by perforin release and dependent on several activating receptors such as NKG2D, NKp30, and DNAM-1 (55,56). CIK cells also exhibit non-specific and non-MHC-restricted cytotoxicity (56).

Over the past decade, CIK cell therapy has been evaluated in numerous clinical studies in patients with various types of cancer, such as hepatocellular carcinoma, non-small cell lung cancer, renal cell carcinoma, and gastric cancer (57). CIK cell therapy can be used as a post-operative adjuvant treatment as well as a palliative treatment following standard therapies. CIK cell therapy was evaluated in a limited number of clinical studies in patients with colorectal cancer. In a retrospective study, 21 of 96 colorectal cancer patients who underwent surgery as well as adjuvant chemotherapy received one to three cycles of CIK cell transfusion for immunotherapy (58). Patients in the CIK-treated group had significant improvement in their 2-year DFS rates than those in the control group (59.65±24.80% vs 29.35±6.39%). CIK cell transfusions were well tolerated without any observable toxicity. Other studies used CIK in a combination therapy with DCs, specifically tumor lysate-pulsed DCs. In 2014, two clinical studies of DC vaccine and CIK cell combinational therapy for colorectal cancer patients were published. One study demonstrated that overall survival rates were significantly improved in the DC-CIK therapy compared to the control group (p=0.04) (59). Moreover, most patients that received DC-CIK therapy displayed improvement in quality of life, including physical strength, appetite, sleep, and body weight. The adverse effects were mild and self-resolving. In the other study, Gao, D. and colleagues evaluated the clinical benefits of DC-CIK therapy in 54 gastric and colorectal patients following surgery with or without chemo-radiotherapy (60). The study demonstrated that DFS and 5-year survival of colorectal cancer patients were significantly prolonged in DC-CIK treatment groups (DFS rate: 66% and 5-year survival rates: 75%) when compared to patients in control groups (DFS rate: 8% and 5-year survival rates: 15%; p<0.01). Serum levels of cytokines were

also evaluated. IFN- γ and IL-12 levels were significantly increased in patients that received the DC-CIK treatment. Besides fever, no adverse events were observed in patients that received the DC-CIK therapy. Since they did not compare the efficacy of combination therapy with each alone, we could not clarify whether combination therapy is better than each therapy. However, when we considered the efficacy of the single therapy of DCs and CIK cells described above, we could presume that The combination of CIK and DC might exert better antitumor activity than each alone.

Here, we provide additional evidence that CIK cell therapy can effectively prevent growth of colorectal cancers in a xenograft mouse model. CIK cells were generated from PBMCs of healthy volunteers. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation, washed three times with PBS, and cultured in the presence of immobilized anti-CD3 antibody (5 μ g/ml) and recombinant human IL-2 (700 U/ml) for five days. The cell suspension

was further incubated in complete medium containing IL-2 only (170 U/ml) for nine days. Thereafter, medium containing IL-2 was replenished every 2 or 3 days. On day 14, cell phenotypes were examined by flow cytometry. CIK cells comprised of 94% CD3⁺, 3% CD3⁻CD56⁺, 16% CD3⁺CD56⁺, 14% CD4⁺, and 81% CD8⁺ cells (Fig. 1A). To examine the cytotoxicity of CIK cells, two cell lines, SW620 and K-562 were used. SW620 cells are metastatic human colon cancer cell lines derived from lymph node (61). K-562 cell lines are human leukemic cells commonly used as reference target cells of NK cells due to the very low level of MHC class I antigens on their cell membrane (62). A 4-h ⁵¹Cr release assay revealed that CIK cells were able to kill 14%, 22%, and 36% of SW620 cells at effector:target ratios of 10:1, 30:1, and 100:1, respectively (Fig. 1B). Moreover, CIK cells showed strong cytotoxicity against K562 cells, a known NK-sensitive target.

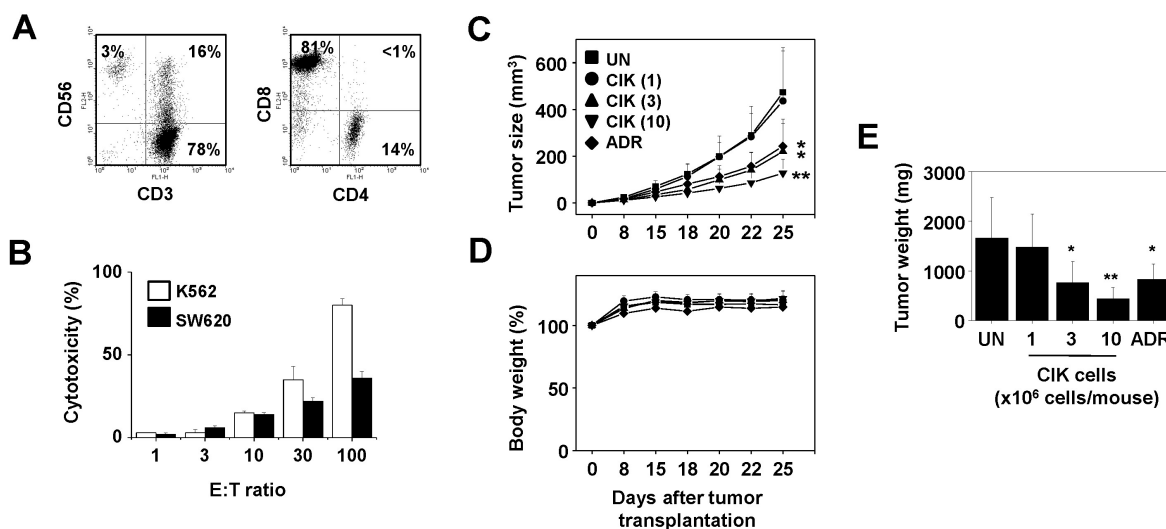


Figure 1. Antitumor efficacy of cytokine-induced killer (CIK) cells for CRC in a mouse xenograft model. CIK cells were generated by culturing human peripheral blood mononuclear cells (PBMCs) with a combination of IL-2 and anti-CD3 antibody for 14 days. CIK cells were stained with human monoclonal antibodies against CD3, CD56, CD4, and CD8 (A). *In vitro* cytotoxicity of CIK cells was evaluated by a 4-h ⁵¹Cr-release assay that used SW620 and K562 as target cells (B). Effector and target cells (1×10^4 cells/ 100μ l/well) were mixed at different ratios (1:1 to 100:1). The percentage of cytotoxicity was calculated as following: cytotoxicity = [(sample-spontaneous)/(maximum-spontaneous)] \times 100. Spontaneous release was measured from target cells incubated in medium alone, whereas maximum release was measured after cells were treated with 2% Nonidet P-40. Nude mice (n=7) were implanted subcutaneously with 6×10^6 SW620 cells. CIK cells (1 to 10×10^6 cells/mouse) were injected intravenously once a week. Adriamycin (ADR) was injected intravenously at 2 mg/kg. Tumor size was estimated as length (mm) \times width (mm) \times height (mm)/2 (C). Body weight was measured to estimate toxicity (D). Mice were sacrificed on day 25 and tumors were weighed (E). Statistical significance was determined by Student's *t*-test versus the PBS-treated control group (**p* < 0.05, ***p* < 0.01). All experimental procedures were approved by the Animal Experimentation Ethics Committee and by the Institutional Ethics Committee of Chungbuk National University. Informed consents have been obtained from volunteers.

A nude mouse xenograft assay was used to examine the *in vivo* antitumor activity of CIK cells. SW620 cells (6×10^6) in 300 μ l of PBS were injected subcutaneously on day 0, followed by intravenous once-a-week injection of CIK cells and adriamycin (ADR). On day 25, mice were sacrificed and the tumor mass and body weight (to determine toxicity) were measured. In control mice, SW620 cells grew to a tumor volume of $473 \pm 190 \text{ mm}^3$ ($n=7$) (Fig. 1C). A strong dose-dependent anti-tumor effect of CIK cells was observed. CIK cells injected at doses of 1×10^6 , 3×10^6 , and 10×10^6 cells per mouse were able to inhibit tumor growth by 7%, 53%, and 73%, respectively. ADR also strongly inhibited the growth of SW620 cells (Fig. 1C). In control mice, weight of SW620 cells reached $1,676 \pm 530 \text{ mg}$ at 25 days post-implantation. CIK cells did not affect body weight gain of nude mice (Fig. 1D). CIK cells injected at doses of 1×10^6 , 3×10^6 and 10×10^6 cells per mouse reduced tumor weight by 10%, 54% and 73%, respectively (Fig. 1E). Thus, our preclinical efficacy data showed that CIK cells were able to kill SW620 cells *in vitro* and *in vivo*, suggesting that CIK cells might be a good immunotherapy candidate for colorectal cancer.

CONCLUSION

Patients with metastatic colorectal cancer have significant risk of recurrence following surgery and conventional adjuvant therapy. Although advances in therapeutic agents for CRC have reduced the risk of recurrence and increased overall survival, patients with metastatic disease have a poor prognosis and a particularly low 5-year survival rates of less than 10%. Additionally, current combination therapy is occasionally discontinued due to a grade 3~4 toxicity with negative effects on quality of life (63). Overall quality of life should be considered for patients with stage IV disease for palliative therapy. Also, elderly patients with stage III disease often avoid adjuvant chemotherapy due to anxiety of old age, comorbidities, side effects, and perceived minimal benefit (64).

Cell-based cancer immunotherapy is a promising therapeutic strategy. Clinical and preclinical studies of CRC treatment with DCs, T cells, and CIK cells showed promising outcomes, although only limited information have been available till recently. Among the immune effector cells, CIK cells have certain advantages in a clinical application: they are relatively easy to generate and expanded in

large-scale from PBMCs, their non-MHC-restricted cytotoxic activity could eliminate MHC class I-negative tumor cells, and importantly, CIK cell therapy causes mild, transient, and easy-to-manage side effects. Over the last decades, CIK cell therapy has been evaluated in numerous clinical studies in patients with various cancers. In most patients, combination therapy using conventional agents and CIK cells showed superior clinical outcomes than standard therapy alone. Clinical studies previously reported by others and our preclinical data suggest that immunotherapy of CRC with CIK cells can be a promising strategy to limit the growth and metastasis of CRC.

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CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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