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*Original Article*

# MHC I-related chain a expression in gastric carcinoma and the efficacy of immunotherapy with cytokine-induced killer cells

Yu Chen[1], Jing Lin[5], Zeng-Qing Guo[3,5], Wan-Song Lin[3,4], Zhi-Feng Zhou[3,4], Chuang-Zhong Huang[3,4], Qiang Chen[1,3], Yun-Bin Ye[2,3]

*[1]The Union Clinical Medical College of Fujian Medical University, Fuzhou, Fujian Province, People's Republic of China; [2]The School of Basic Medical Sciences Fujian Medical University, Fuzhou, Fujian Province, People's Republic of China; [3]Fujian Key Laboratory of Translational Cancer Medicine, Fuzhou, Fujian Province, People's Republic of China; [4]Laboratory of Immuno-Oncology, Fujian Provincial Cancer Hospital, Fujian Medical University Teaching Hospital, Fuzhou, Fujian Province, People's Republic of China; [5]Department of Medical Oncology, Fujian Provincial Cancer Hospital, Fujian Medical University Teaching Hospital, Fuzhou, Fujian Province, People's Republic of China*

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**Abstract:** Cytokine-induced killer (CIK) cells have shown promising activity against gastric cancer in vitro and in vivo. Previous studies showed that cell signaling through MHC I-related Chain A (MICA)-Natural killer group 2, member D (NKG2D) results in CIK cell activation leading to cytolytic activities against tumor cells. In this study, we investigate the MICA status in patients with gastric carcinoma, and determine the potential relationship between MICA and clinical outcome of a CIK containing therapy. Two hundred and forty-three patients with gastric cancer who had received curative D2 gastrectomy were enrolled. The MICA expression of their tumors was determined by immunohistochemistry (IHC). Disease-free survival (DFS) and overall survival (OS) were evaluated. One hundred and forty-eight patients received adjuvant chemotherapy alone, and 95 patients received adjuvant chemotherapy combined with autologous CIK cell therapy. Patients who received adjuvant chemotherapy plus CIK had significantly longer DFS, 42.0 months vs. 32.0 months (P = 0.012), and OS, 45.0 months vs. 42.0 months (P = 0.039), by log-rank test. MICA high-expression, IHC scores of 5-7, was found in tumors from 89 of 243 patients (36.6%). The MICA expression was significantly correlated with the stage (P = 0.007) and there was a borderline association with histological grade (P = 0.054). In the adjuvant chemotherapy plus CIK group (n = 95), patients with high MICA expression had longer DFS, 46.0 months vs. 41.0 months (P = 0.027), and OS, 48.0 months vs. 42.0 months (P = 0.031). In the adjuvant chemotherapy alone group (n = 148), the median DFS and OS had no significant correlation with the MICA status. In a multivariate analysis stage, CIK therapy, and the interaction of MICA status and CIK therapy were independent prognostic factors for DFS and OS. Our study indicated that adjuvant chemotherapy plus CIK immunotherapy is a promising modality for treating gastric cancer patients after D2 gastrectomy. MICA status was associated with the outcome measures in CIK therapy, validation in prospective clinical trials is required to assess the value of this biomarker in the clinical decision-making process.

**Keywords:** Gastric cancer, cytokine-induced killer cells, MICA, NKG2D, adjuvant chemotherapy

## Introduction

Gastric cancer is the fourth most commonly diagnosed cancer worldwide, and 70-85% of patients die within 5 years of diagnosis, making it the third most lethal cancer worldwide [1]. The high mortality associated with gastric cancer (nearly 800,000 deaths per year) is mainly a result of late diagnosis, and limited therapeutic options [1-3]. In East Asia, D2 gastrectomy followed by adjuvant treatment is considered the standard therapy [4-6]. However, adjuvant treatment only results in 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% [7]. This poor outcome has prompted major efforts to explore different effective adjuvant therapies to prolong the survival of patients with gastric cancer.

An innate immune response is thought to be the first line of defense at mucosal surfaces.

**Table 1.** Baseline characteristics of 243 patients

| Characteristics | | Total 243 | Chemotherapy 148 | Chemotherapy plus CIK 95 | *P*-value |
|---|---|---|---|---|---|
| Age | <65 | 163 | 96 | 67 | 0.359 |
| | ≥65 | 80 | 52 | 28 | |
| Sex | Male | 183 | 117 | 66 | 0.091 |
| | Female | 60 | 31 | 29 | |
| Histological grade | G1-G2 | 106 | 58 | 48 | 0.082 |
| | G3-G4 | 137 | 90 | 47 | |
| Stage | II | 83 | 55 | 28 | 0.217 |
| | III | 160 | 93 | 67 | |
| Regimen of Chemotherapy | Xelox, Folfox4 | 150 | 93 | 57 | 0.168 |
| | PF | 93 | 55 | 38 | |

Natural killer (NK) cells are the most important effector cells of the innate immune system, and each NK cell expresses several different activating receptors and a few different inhibitory receptors [8, 9]. Natural killer group 2, member D (NKG2D) is expressed on NK cells and subsets of T cells, such as CD8+ cytotoxic T cells and γδ-T cells, and acts as an activating receptor after ligand binding, supporting the cytotoxic activity of NK cells and T cells against tumor cells [10, 11].

Human NKG2D ligands (NKG2DLs) consist of two members of the MHC class I-related chain (MIC) family (MICA and MICB) and six members of the UL16 binding protein or retinoic acid early transcript (ULBP/RAET) family (ULBP1, ULBP2, ULBP3, RAET1E, RAET1G, and RAET1L) [9, 12, 13]. NKG2DLs expression is highly restricted in healthy tissues, but can be stimulated by multiple stimuli, including infection and heat shock, and by cellular transformation. It is also broadly expressed in a variety of tumors, including hematologic and epithelial tumors [14, 15]. Tumor cells stably transfected to express high levels of MICA and the murine versions of the NKG2D ligands RAE-1 or H60, are removed by CD8+ T cells and NK cells. This indicates that tumor cells over-expressing MICA or other NKG2DLs become more sensitive to immune cell-mediated cytolysis [16-18].

Cytokine-induced killer (CIK) cells are a unique population of cytotoxic T lymphocytes with a characteristic CD3+/CD56+ phenotype, and they can be generated from cytokine cocktail-induced peripheral blood mononuclear cells (PBMC) [19]. Our previous studies showed that CIK cells expressed high-level NKG2D and their cytotoxicity could be induced via the NKG2D receptor. High expression of MICA is one of the indicators of a poor prognosis for advanced non-small cell lung cancer patients and might be one of the predictive factors for successful CIK therapy [20]. But until now, there is limited data about MICA expression in gastric carcinoma and whether high MICA expression can be used as a predictive factor of CIK therapy for patients with gastric cancer still remains to be determined. In the present study, we explored MICA expression in gastric carcinoma to evaluate their prognostic significance and association with other clinicopathological factors, and to determine the effect of CIK therapy.

## Methods

### *Study design and patients*

We collected retrospective tumor samples from 243 patients with gastric cancer from the Department of Medical Oncology of Fujian Provincial Cancer Hospital from January 2009 to March 2012. Eligibility criteria were; 1) R0 gastrectomy with D2 lymphadenectomy, 2) histologically confirmed stages II or III disease, 3) received at least four cycles of adjuvant chemotherapy based on 5-fluorouracil (5-FU) or capecitabine doublet regimens, 4) patients who received CIK cell treatment were given at least two cycles, 5) availability of adequate formalin-fixed paraffin-embedded (FFPE) tumor tissue for biomarkers evaluation. 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.

### *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/m$^2$ twice daily on days 1 to 14 of each cycle. Oxaliplatin, 130 mg/m$^2$ on day 1 of each cycle), Folfox4 (Oxaliplatin, 85 mg/m$^2$ on day 1, 5-flo-

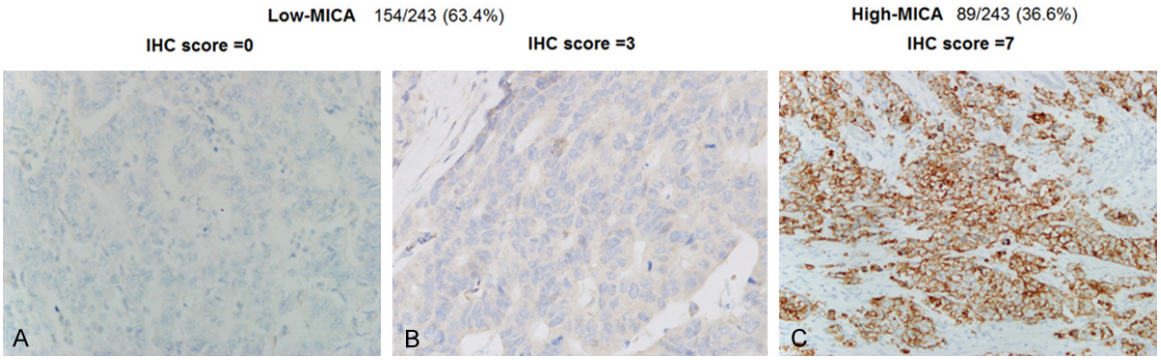


**Figure 1.** MICA Staining by Immunohistochemistry in 243 patients with gastric cancer, showing the Scoring Patterns. A: a score of 0. B: a score of 3. C: a score of 7.

rouracil, 2,400 mg/m$^2$ 46-hour infusion) Leucovorin (400 mg/m$^2$ on day 1 of each cycle), PF (Paclitaxel, 135 mg/m$^2$ on day 1, 5-florouracil, 2,400 mg/m$^2$ 46-hour infusion, and Leucovorin, 400 mg/m$^2$ on day 1 of each cycle).

*CIK cells preparation and treatment*

CIK cells were prepared according to a standard protocol as described in our previous studies [20]. Patients received adjuvant chemotherapy combined with at least two cycles of autologous 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.

*Analysis the phenotype of CIK cells*

The phenotype of the CIK cells was detected by flow cytometry (BD FACSCalibur). The cells were labeled with monoclonal antibodies (m Abs) that recognize human CD3, CD4, CD8, CD56, and NKG2D.

*Immunohistochemistry (IHC) for MICA protein*

Immunohistochemical detection of MICA was performed as described in our previous study [20]. Briefly, FFPE tissue sections of 4um thickness were stained for MICA with the rabbit polyclonal anti-MICA antibody (ab62540) on an automated staining platform (Benchmark; Ventana). Sections were counter-stained with haematoxylin. The omission of the primary antibody and its replacement with PBS was used as the negative control, and tumor infiltrating leukocytes were used as internal positive controls for MICA staining. A section of colorectal tissue which had been shown to be positive for MICA was also used as a positive control.

MICA expression was evaluated on tumor cells, and typically showed membrane and cytoplasmic staining. The sections were scored semi-quantitatively under light microscopy by two pathologists. The intensity of the immunostaining was classified into four categories: 0 = no staining or only a nonspecific background color, 1 = light yellow, 2 = yellow or deep yellow, and 3 = brown or tan. The percentage of positive cells was assessed semi-quantitatively and classified into five groups: 0 = ≤5% positive cells, 1 = 6%-25% positive cells, 2 = 26-50% positive cells, 3 = 51-75% positive cells, and 4 = 76%-100% positive cells. The IHC score of immunoreactivity was obtained by adding the intensity and percentage scores. We used a cutoff of the IHC scores of ≤4 and >4 to define MICA low-expression and high-expression; the cutoff was based on preliminary findings from previous studies.

*Outcomes*

All patients were followed up at the outpatient clinic from the date of surgery until March 31, 2015, or until the time of death. Patients were assessed by clinicians from Medical Oncology and Radiology using the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. Disease-Free-Survival (DFS) was measured from the day of surgery to the first evidence of recurrence or death. Overall Survival (OS) was defined from the date of surgery to death from any cause. Patients who died without a reported previous progression were considered to have progressed on the date of their death. Patients

**Table 2.** Patient and tumor characteristics according to MICA status assessed by IHC

| Characteristics | | Total 243 | MICA high (n) | MICA low (n) | *P*-value |
|---|---|---|---|---|---|
| Age | <65 | 163 | 54 | 109 | 0.106 |
| | ≥65 | 80 | 35 | 45 | |
| Sex | Male | 183 | 67 | 116 | 0.994 |
| | Female | 60 | 22 | 38 | |
| Histological grade | G1-G2 | 106 | 46 | 60 | 0.054 |
| | G3-G4 | 137 | 43 | 94 | |
| Stage | II | 83 | 40 | 43 | 0.007 |
| | III | 160 | 49 | 111 | |
| Adjuvant Chemotherapy plus CIK | Yes | 95 | 38 | 57 | 0.382 |
| | No | 148 | 51 | 97 | |

who neither progressed nor died were censored on the date of March 31, 2015.

*Statistical analysis*

Statistical analysis was performed with SPSS software (Version 21.0, SPSS). The $\chi^2$ test and the Fisher exact probability test were used for binary variable comparisons. The DFS and OS were estimated using Kaplan-Meier curves, and tests were performed using the log-rank test. The relationship between survival and potential prognostic factors were assessed using the log-rank test in univariable analyses. Multivariable models were performed to assess the impact of the variables on DFS and OS. A *P*-value ≤0.05 was considered statistically significant.

## Results

A total of 243 patients with stage II-III gastric cancer were recruited for this study from January 2009 to March 2012. One hundred and forty-eight patients received adjuvant chemotherapy alone, and 95 patients received adjuvant chemotherapy combined with autologous CIK cell therapy. Patient characteristics are summarized in **Table 1**.

*Immunohistochemical analysis of MICA*

The expression of MICA was evaluated within the tumor, and was mainly detected at the cell membrane and in the cytoplasm (**Figure 1**). The high-expression MICA status, IHC scores of 5-7, was found in 89 of 243 patients (36.6%). The MICA expression was significantly correlated with the stage, P = 0.007 and there was a borderline association with histological grade P = 0.054. Various patient and tumor characteristics according to the MICA IHC scores are shown in **Table 2**.

*Analysis of CIK cells' phenotype*

Phenotypic analysis of cells in the 95 patients who were receiving CIK therapy after 12 days of culture demonstrated that the percentages of CD3+/CD56+, CD3-/CD56+, CD3+/CD8+, and CD4+ cells were 25.5±5.8%, 61.2±6.7%, 15.6±4.4%, and 9.5 ±4.3%, respectively. Further analysis of NKG2D on in vitro expanded CIK cells showed that the percentage of NKG2D+ in CD3+/CD56+, CD3+, CD8+, and CD4+ cell populations were 97.2±1.4%, 87.7±2.4%, 95.6± 2.1%, and 0.5±0.2%, respectively.

*Survival analysis*

For the 243 patients, the median follow-up period was 46.3 months, 95% CI = 42.21-48.32 months. The median DFS was 41.0 months, 95% CI = 38.39-43.61 months, and median OS was 44.0 months, 95% CI = 40.19-45.81 months, and the 3-year DFS rate and 3-year OS rate were 56.0% and 69.1%, respectively (**Figure 2A**). In the univariate analysis, histological grade, stage, and CIK therapy were associated with DFS and OS, while the sex, age, the regimens of adjuvant chemotherapy, and MICA status were not correlated with DFS or OS (**Figure 3A**). Patients who received adjuvant chemotherapy plus CIK were associated with significantly longer DFS and OS, DFS 42.0 months vs. 32.0 months and OS 45.0 months vs. 42.0 months, by log-rank test P = 0.012 and P = 0.039, respectively (**Figure 2B**, **Table 3**).

To determine the relation between MICA status and CIK therapy, we conducted a subgroup analysis. In the adjuvant chemotherapy plus CIK group (n = 95), patients with high MICA expression had longer DFS and OS than those with low MICA expression, DFS 46.0 months vs. 41.0 months and OS 48.0 months vs. 42.0 months, by log-rank test P = 0.027 and P = 0.031, respectively (**Figure 3B**). In the adjuvant chemotherapy alone group (n = 148), the median DFS and OS showed no significant correla-

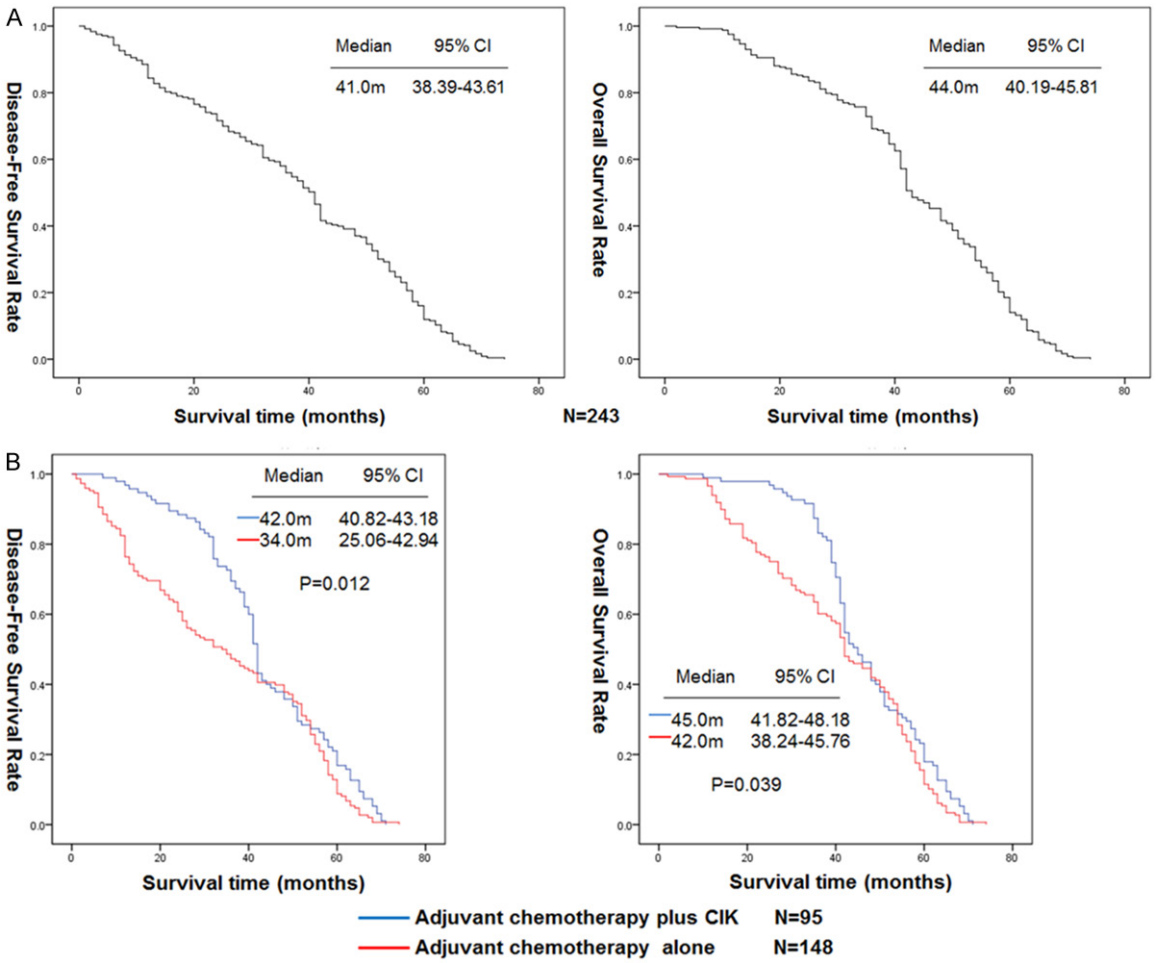


**Figure 2.** A: Kaplan-Meier estimates of Disease-Free-Survival (DFS) and Overall Survival (OS) in 243 patients. B: Kaplan-Meier estimates of DFS and OS for patients according to treatment group.

tion according to MICA status, P = 0.890 and P = 0.747, respectively (**Figure 3C**).

We performed a multivariate analysis of DFS and OS to determine the roles of MICA in determining the treatment outcome for patients after gastric resection who received adjuvant chemotherapy plus CIK therapy. The multivariate analysis included adjustments for variables that included sex, age, histological grade, stage, CIK therapy, MICA status, the regimen of adjuvant chemotherapy, and the interaction of the MICA status and CIK therapy. As a result, we found that the stage, CIK therapy, and the interaction of the MICA status and CIK therapy were joint significant predictors for DFS and OS even after adjustment for all other covariates (**Table 4**).

## Discussion

The data from this study show that adjuvant chemotherapy plus CIK cell therapy improved the prognosis for gastric cancer patients after D2 gastrectomy, and the data support the hypothesis that gastric tumors with high MICA expression are more responsive to CIK therapy than are tumors with low MICA expression.

The class I-like molecule MICA, unlike the classical class I molecules, encodes a membrane-bound protein which acts as a ligand to stimulate an activating receptor, NKG2D [13, 15]. Generally, MICA is constitutively expressed in low levels on epithelial cells in the thymus and gut, endothelial cells, fibroblasts and monocytes, and is upregulated under stressed conditions, such as during bacterial and viral infections, DNA damage, heat shock, oncogenic transformation, and in autoimmune conditions. The upregulation of MICA in distressed cells may alert the immune system that the cells are undergoing pathological changes and enhance the innate immune function [12, 18, 21-23].

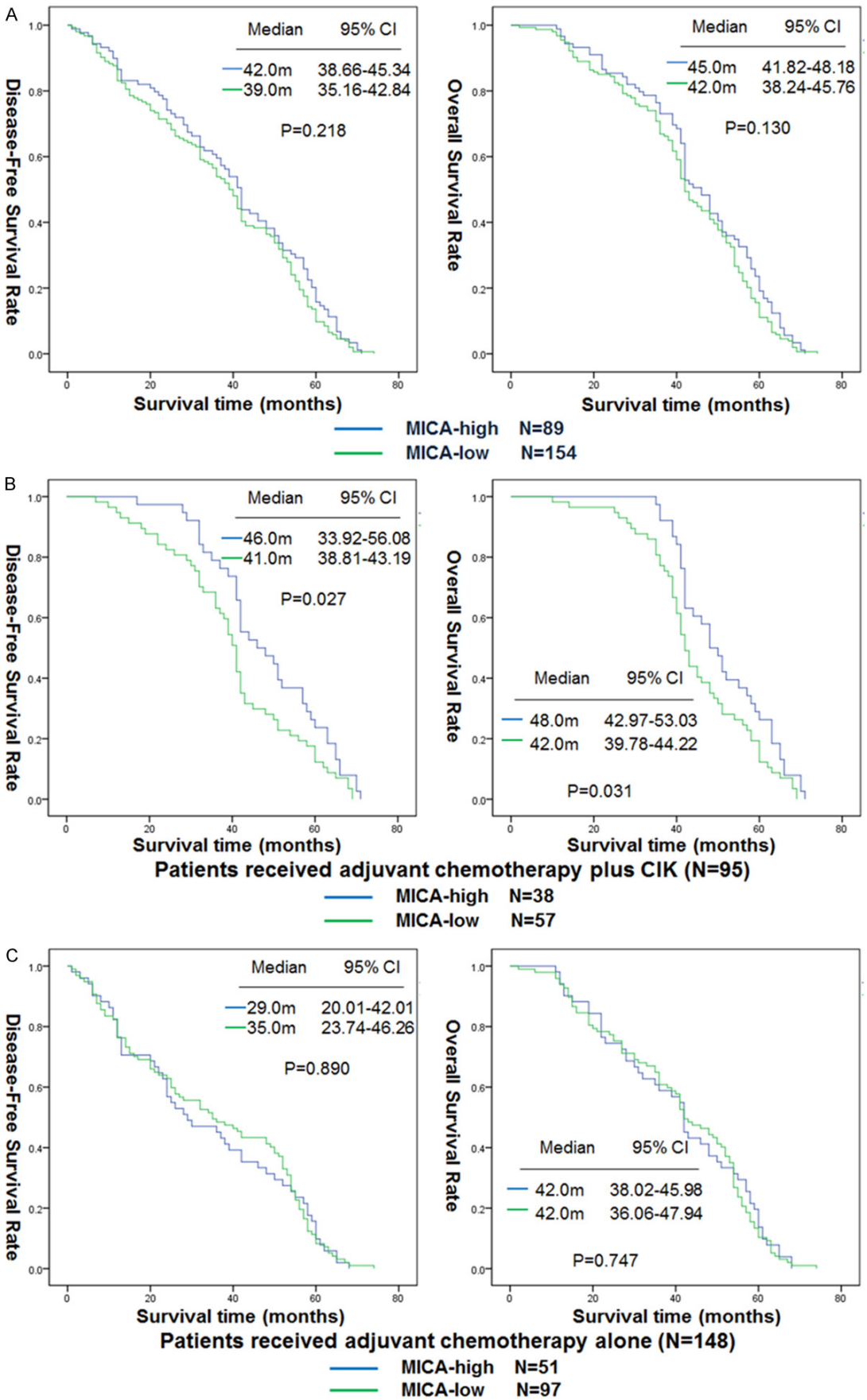
A
Median 95% CI
42.0m 38.66-45.34
39.0m 35.16-42.84
P=0.218
Disease-Free Survival Rate
Survival time (months)
Median 95% CI
45.0m 41.82-48.18
42.0m 38.24-45.76
P=0.130
Overall Survival Rate
Survival time (months)
MICA-high N=89
MICA-low N=154
B
Median 95% CI
46.0m 33.92-56.08
41.0m 38.81-43.19
P=0.027
Disease-Free Survival Rate
Survival time (months)
Median 95% CI
48.0m 42.97-53.03
42.0m 39.78-44.22
P=0.031
Overall Survival Rate
Survival time (months)
Patients received adjuvant chemotherapy plus CIK (N=95)
MICA-high N=38
MICA-low N=57
C
Median 95% CI
29.0m 20.01-42.01
35.0m 23.74-46.26
P=0.890
Disease-Free Survival Rate
Survival time (months)
Median 95% CI
42.0m 38.02-45.98
42.0m 36.06-47.94
P=0.747
Overall Survival Rate
Survival time (months)
Patients received adjuvant chemotherapy alone (N=148)
MICA-high N=51
MICA-low N=97

**Figure 3.** A: Kaplan-Meier estimates of DFS and OS for patients according to MICA status in 243 patients. B: DFS and OS for patients according to MICA status in patients received adjuvant chemotherapy plus CIK therapy group. C: DFS and OS for patients according to MICA status in patients received adjuvant chemotherapy alone group.

**Table 3.** Univariate analysis with Kaplan-Meier estimates of survival

| Variable | | n | m DFS | *P*-value | m OS | *P*-value |
|---|---|---|---|---|---|---|
| Age | <65 | 163 | 40.0 | 0.485 | 43.0 | 0.409 |
| | ≥65 | 80 | 41.0 | | 42.0 | |
| Sex | Male | 183 | 40.0 | 0.652 | 43.0 | 0.646 |
| | Female | 60 | 41.0 | | 44.0 | |
| Histological grade | G1-G2 | 106 | 44.0 | 0.033 | 48.0 | 0.046 |
| | G3-G4 | 137 | 36.0 | | 42.0 | |
| Stage | II | 83 | 48.0 | 0.010 | 50.0 | 0.017 |
| | III | 160 | 33.0 | | 41.0 | |
| Adjuvant Chemotherapy plus CIK | Yes | 95 | 42.0 | 0.012 | 45.0 | 0.039 |
| | No | 148 | 34.0 | | 42.0 | |
| Adjuvant Chemotherapy | Xelox, Folfox4 | 150 | 40.0 | 0.877 | 44.0 | 0.911 |
| | PF | 93 | 41.0 | | 42.0 | |
| MICA status | High | 89 | 42.0 | 0.218 | 46.0 | 0.130 |
| | Low | 154 | 39.0 | | 42.0 | |

MICA is broadly expressed in a variety of malignant tumors such as melanoma, breast, colorectal, lung, pancreatic, ovarian, and hepatocellular cancers. Watson et al. shown that on 449 colorectal carcinomas, MICA expression was an independent marker of good prognosis [24]. Liang et al. also showed that the MICA expression level was significantly and negatively associated with the tumor-node metastasis (TNM) stages, and a low level of MICA expression showed a trend towards a shorter survival time [25], and in cervical cancer it was found that high expression of MICA/B is an indicator of a good prognosis [26]. These results indicated that MICA, as a stress-induced molecule, that has been associated with immune surveillance, provided a marker of "altered self" to the immune system. Paradoxically, our previous study showed that high-expression MICA is one of the indicators of a poor prognosis for advanced non-small cell lung cancer patients [20]. Some other studies also confirmed that induced expression of MICA may be an indicator of a poor prognosis in breast cancer, oral squamous carcinoma, and pancreatic cancer [27-30]. One potential mechanism was that may explain why high expression of MICA leads to poor prognoses is the shedding of MICA molecules. MICA in the membrane can be cleaved by proteolytic activity and released into the bloodstream or tissue culture medium as sMICA, which may leads to impairment of T and/or NK cell immune-surveillance function [23, 31]. In our present study, MICA expression was significantly correlated with stage III, and there was a borderline association with histological grades G3-G4, which is consistent with breast cancer, pancreatic cancer, and other types of tumors [27, 28]. It means that MICA high-expression may contribute to the invasion and metastasis of gastric cancer.

Current evidence indicates that cell signaling through MICA-NKG2D results in CIK cell activation leading to degranulation and cytotoxicity [32-34]. Our study also demonstrates that NKG2D was highly expressed in the subset of expanded CIK cells, CD8+, CD3+/CD56+, and CD3+ cells, which seem to play an important role in the clinical outcome [19]. MICA, the most important NKG2D ligand, was found to be highly expressed (IHC scores of 5-7) in 89 of 243 patients (36.6%). In subgroup analysis, it was shown that in the adjuvant chemotherapy plus CIK group, patients with high MICA expression had longer DFS and OS, P = 0.027 and P = 0.031, respectively, while in the adjuvant chemotherapy alone group, the median DFS and OS were not significantly correlated with the MICA status, P = 0.890 and P = 0.747, respectively. This result was confirmed by multivariate analysis. It means that MICA high-expression was a key factor in the CIK therapy. The tumoricidal effects of CIK cells are exerted through recognition of inducible molecules on tumor cells, such as MICA, by the NKG2D receptor.

Based on our findings, we considered that adjuvant chemotherapy combined with CIK immunotherapy improved prognosis for gastric cancer patients after D2 gastrectomy. High MICA expression according to an IHC score of 5-7 seems to be the effective pretreatment bio-

**Table 4.** Multivariable analysis of 243 patients' clinicopathologic characteristics and survival

| Variable | DFS | | | OS | | |
|---|---|---|---|---|---|---|
| | *P* value | Hazard ratio | 95% CI | *P* value | Hazard ratio | 95% CI |
| Stage | 0.014 | 1.400 | 1.072-1.828 | 0.022 | 1.365 | 1.045-1.781 |
| CIK therapy | 0.019 | 1.326 | 1.052-1.777 | 0.037 | 1.186 | 1.011-1.356 |
| MICA\CIK Interaction | 0.039 | 1.118 | 1.005-1.242 | 0.044 | 1.115 | 1.003-1.241 |

marker identified for CIK therapy. Immunohistochemistry as a technique has been a well-established and widely used approach in routine clinical practice, and the reproducibility of the classification of tumors into high and low MICA expression levels according to an IHC score has been widely-used in malignant tumors such as breast, colorectal, lung, pancreatic, ovarian, and hepatocellular cancers [20, 24, 28-30, 35]. And so, a standard immunohistochemistry test for MICA needs to be established, which may provide a new individualized treatment for future immunotherapy.

In conclusion, our study indicated that adjuvant chemotherapy plus CIK immunotherapy is a promising modality for treating gastric cancer patients after D2 gastrectomy. In view of clinically meaningful improvement in both DFS and OS in patients with high MICA expression who received CIK therapy, we consider that high MICA expression might now be applied clinically as a predictive biomarker to identify patients with gastric cancer patients after D2 gastrectomy that will benefit from the addition of CIK therapy to adjuvant chemotherapy. However, these were the results of a retrospective study, and validation in prospective trials is required to assess the value of this bio-marker in the clinical decision-making process.

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Yun-Bin Ye, The School of Basic Medical Sciences Fujian Medical University, Fujian Key Laboratory of Translational Cancer Medicine, Fuzhou, Fujian Province, People's Republic of China. Tel: 008613635225232; E-mail: zjyunbin@189.cn; Dr. Qiang Chen, The Union Clinical Medical College of Fujian Medical University, Fujian Key Laboratory of Translational Cancer Medicine, Fuzhou, Fujian Province, People's Republic of China. Tel: 008613805019896; E-mail: cqiang8@189.cn

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