Original Article

FOXP3+ Tregs exhibit different infiltrating status and predict a distinct prognosis in primary lesions and hepatic metastases in stage III&IV advanced gastric cancer

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Abstract: Advanced gastric cancer (AGC) patients with hepatic metastasis have a somber prognosis. Furthermore, understanding the molecular mechanisms and immune cells infiltrating status in the hepatic metastases event in gastric cancer become quite imperative and pressing. In this study, CD3+ T lymphocytes, CD8+ T lymphocytes and PD-L1 were favorable prognostic indicators. The positive expression of PD-L1 indicates better prognosis, and FOXP3highPD-L1neg could be regarded as a poor prognostic factor in the multivariate analysis in primary lesions. The infiltration of FOXP3+ Treg is significantly higher in primary tumor lesions than paired hepatic metastatic lesions (P<0.0001). In AGC patients with hepatic metastasis, low infiltration of FOXP3+ Tregs both on primary lesions and metastatic lesions indicate better prognosis. Besides, compared with this in hepatic metastases, the proportion of PD-1+CD8+ T lymphocytes in CD8+ T lymphocytes was elevated in the primary lesions. Moreover, compared with Tregs which were infiltrated in primary lesions, they exhibit higher immunosuppressive effects on hepatic metastases despite the decrease in number. Thus, FOXP3+ Tregs exhibit different infiltrating status and predict a distinct prognosis in primary lesions and hepatic metastases, implying the immunological heterogeneity of primary and metastatic lesions in AGC. These conclusions would provide further theoretical basis and a potential target for immunotherapy of AGC.

Keywords: Gastric carcinoma, FOXP3+ regulatory T cell, PD-L1, hepatic metastasis, prognosis

Introduction

Gastric cancer (GC), still ranking the fifth most frequently diagnosed cancer and the third leading cause of cancer death, is a high-mortality disease with limited effective therapeutic strategies [1, 2]. GC is most often diagnosed at an advanced stage, and patients with advanced disease have a somber prognosis [3].

At the time of diagnosis, 35% of gastric cancer patients have evidence of distant metastases, 31% with peritoneal disease, 14% with hepatic metastases, and 16% with lung metastases. Furthermore, distant metastasis have commonly been considered invariably fatal situations of gastric cancer [4]. Actually, clinical approach to gastric cancer patients with hepatic metastasis is still debated, only a few patients are candidates for hepatic resection because these are often multiple, scattered, bilobar metastases, and recurrence usually occurs with a combination of various patterns, such as peritoneal dissemination, lymph node metastases, and distant metastases [5, 6]. However, the researches about the mechanisms of remodelling the immune microenvironment of gastric cancer hepatic metastases were quite rare [7]. Thus, understanding the molecular mechanisms and immune cells infiltrating status which may drive the hepatic metastases event in gastric cancer become quite imperative and pressing.

In recent years, immunotherapy with immune checkpoint inhibitors has revolutionised the
oncology landscape by targeting the host immune system in advanced cancer and exhibited quite promising perspective [8-10]. Early-phase clinical trials demonstrating the potential applications of Programmed death-1 (PD-1) therapy in gastric cancer were performed in patients with positive immunohistochemical expression of Programmed death-ligand 1 (PD-L1), a ligand for PD-1 [11], however, the therapeutic effect is still not ideal [12]. As for the expression of PD-L1 in tumor cells be an independent factor which could indicate either better or worse prognosis in gastric cancer had been widely reported [13]. Thus, a study of tumor PD-L1 expression in hepatic metastases from gastric cancer is needed.

Tumor infiltrative forkhead box P3-positive (FOXP3+) regulatory T cells (Tregs) can suppress anticancer immunity, thereby hindering protective immunosurveillance of neoplasia and hampering effective antitumour immune responses in tumor-bearing hosts, thus promoting tumor development and progression in human cancers including gastric cancer [14-17]. FOXP3 is a transcription factor that is specifically expressed by natural Tregs. Its expression and stability are crucial for Tregs to regulate effector T cells’ function [18, 19], in addition, CD4+ CD25+CD127low/- T cell population had typical characteristics of Treg cells and FOXP3 expression was significantly higher than other groups and correlated positively with the classic regulatory T cells [20]. However, predicting the prognosis of patients with the infiltration of Tregs in the microenvironment of GC is still controversial [21, 22]. Moreover, the studies about the infiltrating status between primary tumors and hepatic metastases, and the correlation between the infiltration of Tregs in hepatic metastases and the GC patients’ prognosis are still quite rare.

In this study, a tissue microarray (TMA) including 266 advanced gastric cancer (AGC) primary lesion specimens (including 68 paired hepatic metastases lesions) was used to investigate the expression of PD-L1, quantified tumor infiltrating CD3+, CD8+ T lymphocytes, FOXP3+ Tregs density to determine their relationships with clinicopathological features and patients’ prognosis in advanced gastric cancer patients (Figure 1A-D). The immune microenvironment of hepatic metastasis was also assessed and compared with that of the primary tumor from the same case.

Materials and methods

Patients and samples

To evaluate the immune indices in the advanced gastric cancer samples, we retrospectively assessed 266 advanced stage III-IV gastric cancer samples (using TMA, including 68 cases with hepatic metastasis) from patients who underwent primary or metastatic tumor resection between December 2010 to June 2016 at Department of Gastrointestinal Surgery, RenJi Hospital, School of Medicine, Shanghai Jiao Tong University. All the samples were definitely diagnosed as gastric cancer by Department of Pathology.

In this study, we excluded the following types of patients: (1). Patients without complete clinical information, follow-up data, etc. (2). Patients with non-neoplastic resection such as palliative gastrointestinal bypass surgery and non-adenocarcinoma patients. (3). Patients receiving other neoadjuvant treatment or previous radiotherapy. (4). Patients with perioperative death from various surgical complications. (5). Broken tissue samples were unavailable for TMA. Overall Survival time was defined as the interval between the gastrectomy and patient death or survival, and the final follow-up date was January 8, 2018, for all cases examined [23, 24]. All patients received the standard treatments such as D2 radical resection for primary lesions and hepatic metastases resection for the hepatic metastases lesions. Tumor TNM stage was assigned based on pathological tumor, node, and metastasis staging by the American Joint Committee on Cancer (AJCC 8th edition) staging system. For each case, the diagnosis was confirmed by a senior pathologist.

Every patient’s tumor formalin-fixed, paraffin-embedded (FFPE) tissues on the TMA was consecutive, and the TMA was constructed using a tissue arrayer with 5 μm thickness. According to the manufacturer’s instructions, the immunohistochemical staining was performed with the manual of Dako REAL EnVision Detection System (K5007, Dako). The following primary antibodies were used: Anti-CD3 (1:100, ab-16669, Abcam); Anti-CD8 (1:100, ab4055,
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Figure 1. FOXP3+ Tregs, CD3+ T lymphocytes, CD8+ T lymphocytes and PD-L1 expression in primary lesions or hepatic metastases by immunohistochemistry (×400). The representative images of FOXP3+ Tregs (A); CD3+ T lymphocytes (B); CD8+ T lymphocytes (C); the representative positive or negative expression of PD-L1 in tumor tissues (D); the correlations between FOXP3+ Tregs and CD3+ T lymphocytes in primary lesions or hepatic metastases (E); the correlations between FOXP3+ Tregs and CD8+ T lymphocytes in primary lesions or hepatic metastases (F).
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Abcam); Anti-FOXP3 (1:100, ab20034, Abcam); Anti-PD-L1 (1:100, 22C3, Dako). 6 pairs of TNM stage IV Fresh AGC samples with hepatic metastasis were collected from treatment-naive adults undergoing surgery for AGC between October 2017 to September 2019 after informed consent and approval from the Shanghai Jiaotong University School of Medicine, Renji Hospital Ethics Committee. This retrospective study was performed according to the criteria of the Ethical Committee of the Shanghai Jiao Tong University School of Medicine. Renji Hospital consent from the patient was obtained.

Quantitative immunohistochemical analysis

In brief, each primary antibody-probed section was incubated with 0.3% hydrogen peroxide for 30 minutes, and then blocked with 10% BSA (Sangon, Shanghai, China) at 4°C overnight, and followed by the HRP second antibody (Thermo Scientific, US) at room temperature for 1 h. After that, positive staining was visualized with DAB substrate liquid (Gene Tech, Shanghai) and counterstained with hematoxylin. Digital images of the sections were obtained at 40× and 200× magnifications by ZEISS Axio Vert. A1 microscope system. 5 visual areas showing highest infiltrating densities at 40× magnification were chosen firstly and then counted the cell numbers at 200× magnification. After counting the numbers, cell destiny were calculated in mm² for further statistics. In a two-category immunoscore analysis, patients with cell numbers greater than the median were defined as ‘high’ and those were smaller cell numbers were defined as ‘low’ (The median count of hepatic metastatic lesions was comparable to that of the primary lesion). And PD-L1 positivity was defined as staining in 1% or more of tumor cells [25, 26]. PD-L1 expression on tumor cells, instead of stroma, was immunohistochemically analyzed by an experienced pathologist [27].

Flow cytometry

For analysis of surface markers, cells were stained in PBS containing 2% fetal bovine serum (FBS) with antibodies as indicated. Antibodies staining was performed according to the manufacturer’s instructions (eBioscience). Cells were stained with fixable viability dye eFluorTM 780 and antibodies as indicated. All samples were processed on a LSRS FortessaTM X-20 flow cytometer (BD Biosciences) and data were analyzed by FlowJo software (Tree-Star). The following flow cytometry antibodies were purchased from Biolegend: CD3 (OKT3), CD8 (RPA-T8), PD-1 (EH12.2H7), CD127 (A01-9D5); FOXP3 (PCH101), CD25 (BC96), CTLA-4 (14D3) were from eBiosciences.

In vitro suppression assay

All tissue samples were cut into small pieces and incubated in 300 U/mL type IV collagenase (SIGMA) for 40 min at 37°C. After passing through a 300 mesh filter, cells were washed twice with PBS. Tumor infiltrating lymphocyte was isolated by density gradient centrifugation with Ficoll-Paque (GE Healthcare). Tregs isolated from gastric cancer tumor tissues by FACS on a BD FACS ARIA II sorter (BD Biosciences). Responder T cells were sorted from human peripheral blood and labelled with CellTrace Violet following the protocol from CellTrace Violet Cell Proliferation Kit (Invitrogen). Labeled responder T cells were then either cultured alone or mixed with different ratios of Tregs from primary tumor or hepatic metastasis (1:0; 2:1; 4:1; 8:1). Cell mixtures were stimulated under anti-CD3/anti-CD28 antibody for 48 h. Proliferative cells were further detected by flow cytometry.

Statistical analysis

SPSS 23.0 and GraphPad Prism 6.0 were used for statistics in this study. Box and whiskers plot diagrams represent the median, the interquartile range, minimum (Min) and maximum (Max) of positive cell counting numbers per mm² (Including CD3, CD8, FOXP3). Chi-square tests were performed to compare CD3, CD8, FOXP3 or PD-L1 expression with clinical features. Overall survival analysis was performed using the Kaplan-Meier method and the long-rank test (Time unit: month). The hazard ratio (HR) of mortality was assessed by the Cox regression model. Two-sided, P-values <0.05 were considered to be significant.

Results

Clinicopathologic characteristics

A retrospective study of 266 AGC patients, including 193 TNM stage III cases, and 73 TNM stage IV cases, was conducted (68 cases with paired primary lesions and hepatic meta-
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The median age of the AGC patients was 63 (33-89) years, and the median OS time was 32 (0-99) months. A total of 201 (75.56%) patients died during the follow-up period. The detailed clinicopathological characteristics of the patients are presented in Table 1.

**Correlation of CD3+ lymphocytes, CD8+ lymphocytes and PD-L1 with AGC patients’ clinicopathological parameters**

In this study, CD3+ T lymphocytes could be a favorable prognostic factor (HR: 0.694, 95% CI: 0.524-0.918, P=0.010), high CD3 expression indicates better prognosis (5-year OS: 17.5 ± 0.034 vs 32.5 ± 0.042, P=0.010), at the same time, CD8+ T lymphocytes could also be a favorable prognostic factor (HR: 0.645, 95% CI: 0.487-0.854, P=0.002), high CD8 expression indicates benign prognosis (5-year OS: 15.3 ± 0.032 vs 35.0 ± 0.043, P=0.002). Besides, 53 out of 266 cases (19.9%) showed positive PD-L1 expression in primary tumor cells, whereas 213 patients out of 266 cases (80.1%) showed negative PD-L1 expression. Nevertheless, 6 out of 68 cases (8.8%) showed positive PD-L1 expression in hepatic metastatic tumor cells, whereas 62 patients out of 68 cases (91.2%) showed negative PD-L1 expression (Figure 2B). The positive expression of PD-L1 in primary tumor indicates better prognosis (HR: 0.623, 95% CI: 0.430-0.901, P=0.012; 5-year OS: 22.0 ± 0.029 vs 36.1 ± 0.069, P=0.012). However, in hepatic metastases, all of these indicators have no statistically significant indication for AGC patients’ prognosis (Tables 1, 2; Figure 3B-D).

**PD-L1 expression and FOXP3+ Treg Infiltration are associated with AGC patients’ overall survival**

In this research, high FOXP3 expression indicates poor prognosis in primary tumors, while the correlation between FOXP3+ Tregs and patients’ prognosis features did not show significant differences in hepatic metastases (Figure 3A). The ratio of Tregs to CD3+ T cells or CD8+ T cells was dichotomous, in primary tumors, both high ratio of FOXP3/CD3 and FOXP3/CD8 exhibit worse prognosis (5-year OS: 29.5 ± 0.041 vs 20.2 ± 0.036, P=0.013; 30.4 ± 0.042 vs 19.3 ± 0.035, P=0.026, respectively). Then, when FOXP3 was combined with PD-L1, FOXP3negPD-L1neg could be regarded as a poor prognostic factor in the multivariate analysis (HR: 1.514, 95% CI: 1.119-2.049, P=0.007; 5-year OS: 30.6 ± 0.040 vs 18.3 ± 0.036, P=0.007) (Table 2; Figure 4B, 4C).

**Different immune cells infiltrating status between primary tumor lesions and hepatic metastatic lesions indicates diverse prognosis**

68 pairs of primary tumor lesions and hepatic metastatic lesions were analysed about the infiltrating status of FOXP3+ Tregs, CD3+ T cells, CD8+ T cells. The infiltration ratio of FOXP3+ Treg is significantly higher in primary tumor lesions than paired hepatic metastatic lesions, (P<0.0001= nevertheless, the infiltration of CD3+, CD8+ lymphocytes did not show significant difference between primary lesions and metastatic lesions) (Figure 2A). This phenomenon is also confirmed in 6 pairs of fresh advanced gastric cancer samples by Flow Cytometry, the proportion of CD4+CD25+CD127low Tregs is significantly upregulated in primary tumors than metastases. However, the proportion of CD3+ and CD8+ T lymphocytes didn’t show difference (Figure 5A-C). Also, the patients without metastases have a significantly lower Treg infiltration than the ones with metastases in primary lesions, adversely, the patients without metastases have a significantly higher CD3+ T cells infiltration than the ones with metastases in primary lesions (Figure 2C). In addition, CD8+ T lymphocytes and FOXP3+ Tregs show a slight positive correlation in primary tumors without hepatic metastasis, however, CD3+ T lymphocytes and FOXP3+ Treg did not show any correlation (Figure 1E, 1F). Moreover, in AGC patients with hepatic metastases, low infiltration of FOXP3+ Tregs both on primary lesions and metastatic lesions show better prognosis. It would also be an independent favorable prognostic factor (HR: 0.244, 95% CI: 0.075-0.792, P=0.019) (Figure 4D).

**Tregs exhibit higher immunosuppressive effects on hepatic metastases than primary tumors despite the decrease in number**

To further investigate the differences of Treg’s functional markers and the expression of PD-1 on CD8+ T lymphocytes between the primary tumors and the hepatic metastases, we examined them using fresh AGC samples by Flow Cytometry. Compared with this in the hepatic metastases, the proportion of PD-1+CD8+ T lymphocytes...
Treg exhibit different infiltrating status in hepatic metastases in AGC

Table 1. Clinicopathologic characteristics of the total 266 gastric cancer patients

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Treg exhibit different infiltrating status in hepatic metastases in AGC

Figure 2. Cell counts comparison between primary tumor and paired hepatic metastasis. A: Cell counts comparison between primary tumor and paired hepatic metastasis about FOXP3+ Tregs, CD3+ T lymphocytes and CD8+ T lymphocytes; B: The proportion of PD-L1 positive patients between primary tumor and paired hepatic metastasis; C: Cell counts comparison between primary tumor (with or without hepatic metastasis) and hepatic metastasis about FOXP3+ Tregs, CD3+ T lymphocytes, CD8+ T lymphocytes (PT: primary tumor; stage III: without hepatic metastasis; stage IV: with hepatic metastasis).
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In this study, CD8+ T lymphocytes, CD8+ T lymphocytes also generally be favorable prognostic indicators, and the positive expression of PD-L1 indicates better prognosis, which is consistent with the trend in some previous reports [30, 31]. Besides, accumulating studies have demonstrated that a large number of Tregs infiltrate into various types of tumors in humans. And high frequency of tumor-infiltrating FOXP3+ Tregs was often significantly negatively correlated with patients’ survival [32, 33]. Our results also exhibit that high infiltrating of the FOXP3+ Tregs indicate poor prognosis in stage III-IV AGC patients. A relative study reported that high densities of PD-L1 in patients with high CD8/FOXP3 and low CD8/PD-L1 ratios correlated with increased survival in GC [34]. In our study, it showed that the combination of FOXP3 and CD3 or CD8 can predict the prognosis in the primary lesions of AGC patients. Combining two indicators to predict prognosis would be a good idea, suggesting that high expression of FOXP3 in AGC indicates poor prognosis, while higher infiltrating of CD3+ T cells and CD8+ T cells suggests a favorable prognosis. In addition, the combination of FOXP3+ Tregs infiltration and the expression of PD-L1 in tumor cells can be considered as an independent predictor of multivariate analysis.

In order to elucidate the difference of the expression of immune indices between primary lesions and hepatic metastatic lesions, we used the paired primary focus and metastatic...
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A. Primary tumor vs. Metastasis

- FOXP3+ Tregs
  - Overall survival (%)
  - Time (months)
  - P = 0.0019

B. Primary tumor vs. Metastasis

- CD3+ T cells
  - Overall survival (%)
  - Time (months)
  - P = 0.013

C. Primary tumor vs. Metastasis

- CD8+ T cells
  - Overall survival (%)
  - Time (months)
  - P = 0.0026

D. Primary tumor vs. Metastasis

- PD-L1
  - Overall survival (%)
  - Time (months)
  - P = 0.0118

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Figure 3. Correlation of immune indicators expressed on primary lesions or hepatic metastases with AGC patients’ overall survival. Correlation of FOXP3+ Tregs (A); CD3+ T lymphocytes (B); CD8+ T lymphocytes (C) and PD-L1 (D) expressed on primary lesions or hepatic metastases with AGC patients’ overall survival.
Treg exhibit different infiltrating status in hepatic metastases in AGC

lesions in AGC patients with hepatic metastasis. Compared with the primary lesion, although the infiltration of Tregs is significantly higher than the metastatic lesions, it exhibited that the expression of the Treg functional marker CTLA-4 in Tregs in metastatic lesions was elevated than matched primary lesions in fresh clinical AGC samples, which suggest a more robust inhibition. Moreover, Tregs also show higher immunosuppressive effects on hepatic metastases than primary tumors by in vitro suppression assay. At present, there are a number of studies dedicated to discovering the heterogeneity of tumor-infiltrating Tregs, the subsets with strong inhibiting capacity [35, 36], and the Tregs infiltrated in hepatic metastases just exhibit a strong inhibitory function.

At the same time, the expression of PD-1 in the infiltrating CD8+ T lymphocytes in the hepatic metastases was lower than that in the matched primary lesions. Moreover, the expression of PD-L1 in hepatic metastases was also lower than that of primary lesions. Up to now, the predictive role of PD-L1 expression is still controversial in the clinic, the tumor stratification based on the presence of T lymphocytes and PD-L1 might be a promising predictive tool.
Treg exhibit different infiltrating status in hepatic metastases in AGC

Figure 5. FOXP3^+ Tregs, CD3^+ T lymphocytes, CD8^+ T lymphocytes and PD-L1 expression in primary lesions or hepatic metastases of AGC by FACS. Proportions of CD4^+CD25^+CD127^low Tregs (A); CD3^+ T lymphocytes (B); CD8^+ T lymphocytes (C) between primary lesions and paired hepatic metastases. Proportions of PD-1^+CD8^+ T lymphocytes between primary lesions and paired hepatic metastases (D). *, P<0.05; **, P<0.01; ns: no significance.
Treg exhibit different infiltrating status in hepatic metastases in AGC

Figure 6. Tregs exhibit higher immunosuppressive effects on hepatic metastases. MFI of various Treg functional marker expression by Treg cells within primary lesions and hepatic metastases as indicated (A) (n=6). *, P<0.05; quantitative analysis of the MFI of Treg functional markers between primary lesions and paired hepatic metastases; (B) *, P<0.05. In vitro suppression assay was performed in Tregs from primary tumor or hepatic metastasis. Labeled responder T cells were either cultured alone or mixed with different ratios of Tregs from primary tumor or hepatic metastasis (1:0; 2:1; 4:1; 8:1) (C); percentage of proliferated responder T cells was assessed (D). All data represent mean ± S.D. *, P<0.05; **, P<0.01.
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to define optimal therapy for patients with advanced cancer. In this research, the above immune indicators suggest the immunological heterogeneity of primary and metastatic lesions, which may indicate that anti-PD-L1/PD-1 treatment is more difficult to achieve the desired effect on hepatic metastases, while targeting the certain subset of Tregs, which exhibit strong inhibition in the tumor immune microenvironment, may become a promising therapeutic strategy. However, the number of samples of matched primary and metastatic lesions is still quite rare, more samples are needed to enhance persuasiveness. Due to the limitations of single-center retrospective studies, more multicenter studies should be conducted to validate all of these results, moreover, the specific pathways or related mechanisms may require further in-depth exploration in the future.

Conclusions

In this study, large scale advanced gastric cancer samples (including AGC with hepatic metastasis) were used to elucidate both CD3+, CD8+ T lymphocytes and PD-L1 in tumor cells with positive prognostic effects, the FOXP3+ Tregs, with poor prognostic implications. At the same time, the differences of immune molecules between primary and metastatic lesions were also been compared. In addition, the immunological heterogeneity of primary and metastatic lesions was also exhibited. It would provide further theoretical basis and potential target for immunotherapy of advanced gastric cancer.

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Disclosure of conflict of interest

None.

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