Regulatory T-cell density and cytotoxic T lymphocyte density are associated with complete response to neoadjuvant paclitaxel and carboplatin chemoradiotherapy in gastric cancer

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Abstract. Regulatory T-cell density and cytotoxic T lymphocyte density are crucial in regulating antitumor immune responses. Tumor infiltration has marked therapeutic effects in gastric carcinoma, and there is evidence that chemoradiotherapy (CRT) exhibits an immune-mediated component. In the present study, the density of $CD4^+$ and $CD8^+$ cells were evaluated in post-CRT surgical samples from 68 patients with gastric cancer using immunohistochemistry. The associations between T-cell density, cytotoxic T lymphocyte density and clinical survival rate were also analyzed. Cytotoxic T lymphocyte density was associated with gastric carcinoma regression grade and regulatory T-cell density was also associated with gastric carcinoma regression grade. Of the patients who had a pathologic complete response, 84 and 76% were found to have a high $CD3^+$ and $CD4^+$ cell density, which was significantly different to patients who had a low $CD3^+$ and $CD4^+$ cell density. High cytotoxic T lymphocyte density was also associated with improved survival rates of patients with gastric cancer. In conclusion, these outcomes indicated that regulatory T-cell density and cytotoxic T lymphocyte density in the tumor microenvironment were associated with the response to neoadjuvant CRT and may represent a therapeutic target for gastric cancer.

Introduction

Gastric cancer is one of the most common human malignancies and is the second most common cause of cancer-associated mortality in the world (1-3). A high risk of metastasis of gastric cancer cells and the resistance of cancer cells to apoptosis in patients with gastric cancer has been reported in the previous reports (4-6). Patients with gastric cancer present with higher morbidity and mortality rates, compared with other types of carcinoma of the digestive system (7-9). Therefore, investigating efficient antitumor treatments are required in the field of cancer research and treatments.

Currently, neoadjuvant chemoradiotherapy (CRT) is widely used for the treatment of locally advanced gastric cancer to inhibit tumor growth, making it more amenable to resection, and to decrease the risk of tumor cell metastasis (10-13). Paclitaxel chemotherapy is an effective drug for advanced gastric cancer with acceptable toxic side-effects, and has been considered as an effective anticancer agent due to the smooth transition to the subsequent regimen (14). Carboplatin radiotherapy in combination with 2-(8-hydroxy-6-methoxy-1-oxo-1H-2-benzopyran-3-yl) propionic acid can inhibit gastric tumor growth, which may be an effective drug in the treatment of gastric cancer (15). However, the combined therapeutic effects of paclitaxel and carboplatin CRT in the CRT response in gastric cancer remain to be elucidated.

The presence of tumor-infiltrating lymphocytes (TILs) is associated with improved clinical outcome in gastric cancer, and TILs can suppress further invasion and/or metastasis of gastric carcinoma (16). A previous study indicated that the density of tumor-infiltrating T cells is a notable prognostic indicator for gastric cancer following treatment with CRT (17). TILs in gastric adenocarcinoma also present with marked compartmentalization with high numbers of lymphocytes in the stroma and low intraepithelial lymphocyte counts, which underline the importance of inflammation for tumor therapy (18). Another study showed that immunoscoring involves the assessment of T-cell density in the central tumor and invasive margin based on expression of pairs of T-cell markers (CD3 and CD8, and CD3 and CD45), which can be used to evaluate the prognostic and histopathologic tumor-node-metastasis stage for patients with cancer (19). Therefore, evaluating the association between therapeutic effects of antitumor agents and the pathologic complete response (pCR) to neoadjuvant CRT is crucial for the treatment of patients with gastric cancer.

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In the present study, the T regulatory-Treg-lymphocyte density and cytotoxic T lymphocyte density were analyzed in surgically resected samples from 68 patients with locally advanced gastric cancer treated with CRT (paclitaxel and carboplatin). The results showed that regulatory T-cell density and cytotoxic T lymphocyte density were associated with pCR to neoadjuvant CRT (paclitaxel and carboplatin) in the gastric cancer response to CRT.

Materials and methods

Patients, treatment and follow-up. A total of 68 patients with gastric cancer were recruited at The First Affiliated Hospital of Hebei North University (Hebei, China) between February 2010 and May 2015. Clinical gastric tumor stage was determined by computed tomography and magnetic resonance imaging prior to treatment with CRT. Neoadjuvant radiotherapy, comprising paclitaxel and carboplatin, was administered for 4 weeks. Surgical resection (20), involving curative (R0) resection and D2 lymphadenectomy, of the gastric tumor was performed following the completion of neoadjuvant treatment. The follow-up period in the present study was a total of 60 months, every 5 months. The survival rate of patients with gastric cancer was recorded and cancer-specific survival (CSS) was analyzed as the time between the date of surgery and the date the patient succumbed to mortality from gastric cancer. Recurrence-free survival (RFS) was evaluated as the time between the date of surgery and the date of first gastric cancer recurrence.

CRT administration. The patients received paclitaxel (80 mg/m^2 per day intravenously on days 1, 8, and 15; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in combination with carboplatin (75 mg/m^2 per day intravenously on days 1, 8, and 15, Sigma-Aldrich; Merck KGaA) in 4-week treatment cycles.

Histopathologic assessment. Tumor regression grading was analyzed as part of routine pathological review using the Dworak system (21) and defined as grade 4 corresponding to pCR and grade 0 corresponding to no regression.

Distinguishing regulatory T cells and cytotoxic T lymphocytes. Regulatory T cells were distinguished using typical markers $CD4^+CD25^+Foxp3^+$, as described previously (22). Cytotoxic T lymphocytes were distinguished using typical markers $CD3^+CD4^-CD8^+$, as described previously (23).

Immunohistochemistry. The fresh tissue specimens were deparaffinized in xylene and dehydrated through a graduated alcohol series. The paraffin-embedded tissue sections (4-μm) were prepared and epitope retrieval was performed using Tris-EDTA buffer solution (pH 9.0, Sigma-Aldrich; Merck KGaA; cat. no. SRE0063) for 60 min at 65˚C. The paraffin sections were subjected with hydrogen peroxide (3%) for 15 min at 37˚C and then blocked with 5% BSA (Sigma-Aldrich; Merck KGaA) for 2 h at 37˚C. The sections were incubated with anti-CD3 (1:1,000, cat. no. ab16669; Abcam, Cambridge, UK), anti-CD4 (1:1,000, cat. no. ab133616; Abcam), and anti-CD8 (1:1,000, cat. no. ab4055; Abcam), respectively, for 12 h at 4˚C. All sections were washed three times with PBS for 15 min at 37˚C and then incubated with goat anti-rabbit IgG H&L secondary antibodies (Alexa Fluor® 488, 1:1,000, cat. no. ab150077; Abcam) for 2 h at 37˚C. The tumor sections were observed in three-random views under a fluorescence microscope (YS100; Nikon Corporation, Tokyo, Japan). Densitometric quantification of the immunohistochemistry data was performed by using Quantity-One 1.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Assessment of T-cell density. Images of the gastric tumor sections (4-μm) were captured using a high-resolution digital scanner (Aperio Scanscope XT; Leica Biosystems, North Ryde, NSW, Australia) at x40 magnification. The T-cell density (cells per mm^2 tissue) was analyzed using software (Aperio Imagescope version 11; Leica Biosystems).

Statistical analysis. Data are presented as the mean and standard error of the mean and statistical analyses were performed using SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA). Differences in T-cell density in each group were analyzed using general linear models. Associations of clinical variables and pCR were analyzed using logistic regression. The Kaplan-Meier method with and the log-rank test was used to analyze time-to-event outcomes (CSS and RFS). The prognostic value of $CD3^+$, $CD4^+$ and $CD8^+$ for cancer-specific survival rate was analyzed using the Kaplan-Meier method and multivariate Cox proportional hazard model analysis.

Table I. Characteristics of patients with gastric cancer.

Characteristic	n
Patients	68
Male	34
Female	34
Age in years, mean (SD)	63 (12.3)[a]
Weeks between end of CRT and surgery, median (IQR)	6 (5,7)[b]
Pretreatment clinical T stage, n (%)	
T2	12 (17.6)
T3	48 (70.6)
T4	4 (5.9)

CRD, chemoradiotherapy.

Table II. Clinical and pathological response of patients with gastric cancer to neoadjuvant chemoradiotherapy paclitaxel and carboplatin.

Stage	n (%)
T2	12 (17.6)
T3	48 (70.6)
T4	4 (5.9)
Metastatic	4 (5.9)

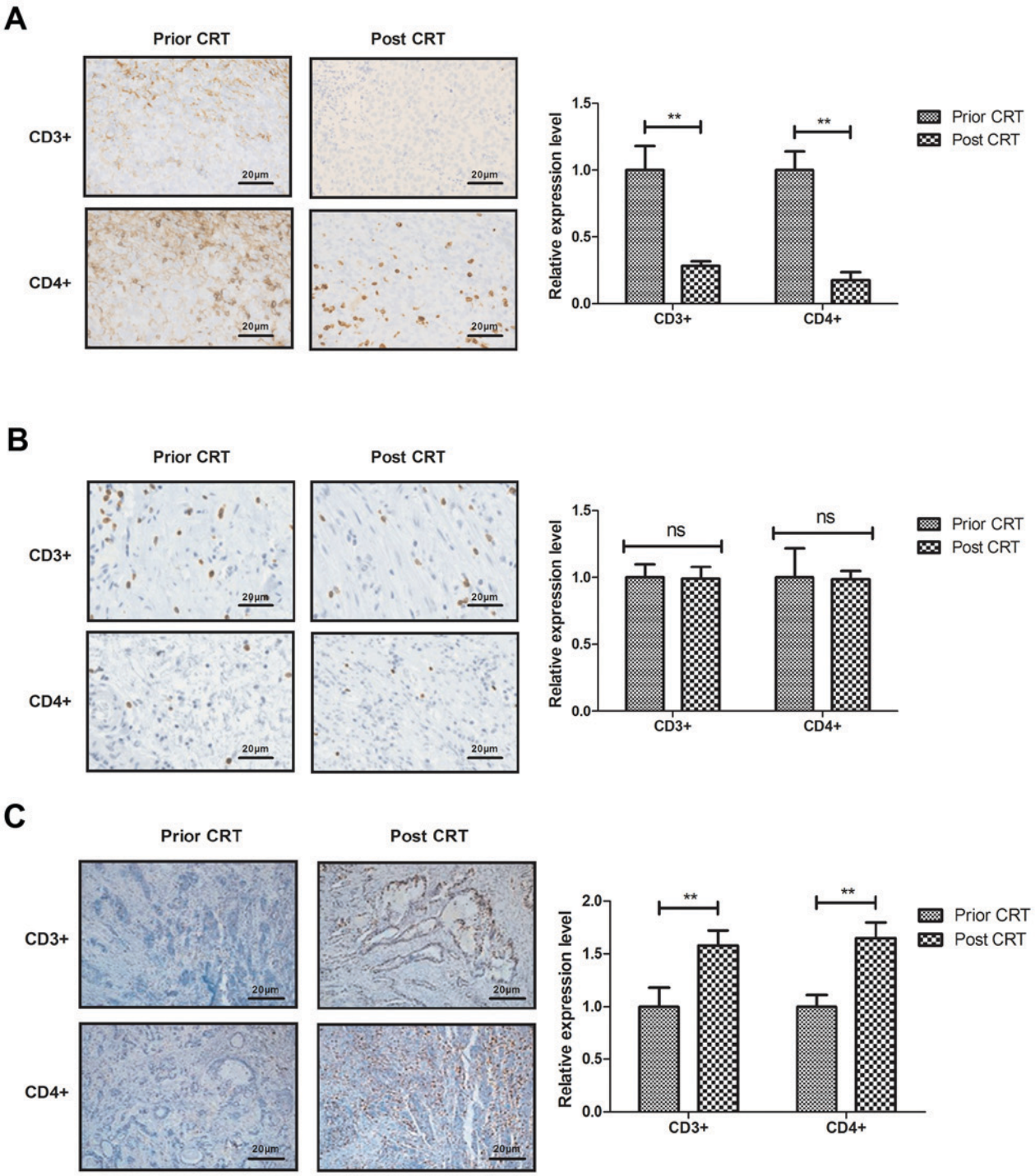

Figure 1. Analysis of regulatory T-cell density in the gastric tumor microenvironment following CRT. The densities of $CD3^+$ and $CD4^+$ T cells in (A) T4, (B) T3 and (C) T2 stage gastric tumors were determined by immunohistochemistry. $^{**}P<0.01$, vs. prior CRT. CRT, chemoradiotherapy.

P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics and treatment response. A total of 68 patients with gastric cancer were identified and tumor samples from these 68 patients were included in the tissue microarrays. The clinical characteristics of the 68 patients with gastric cancer are shown in Table I. There were 48 (70.6%) patients with clinical T3 stage, 12 (17.6%) patients with clinical T2 stage 2, four (5.9%) patients with clinical T4 stage and four (5.9%) patients with metastatic disease (Table II).

Regulatory T-cell density in the gastric tumor microenvironment following CRT. The present study analyzed the regulatory T-cell density in the gastric tumor microenvironment following CRT. The density of $CD3^+$ and $CD4^+$ T cells were decreased in the T4 stage gastric tumor tissue (Fig. 1A). The T3 stage gastric tumor tissue showed a similar

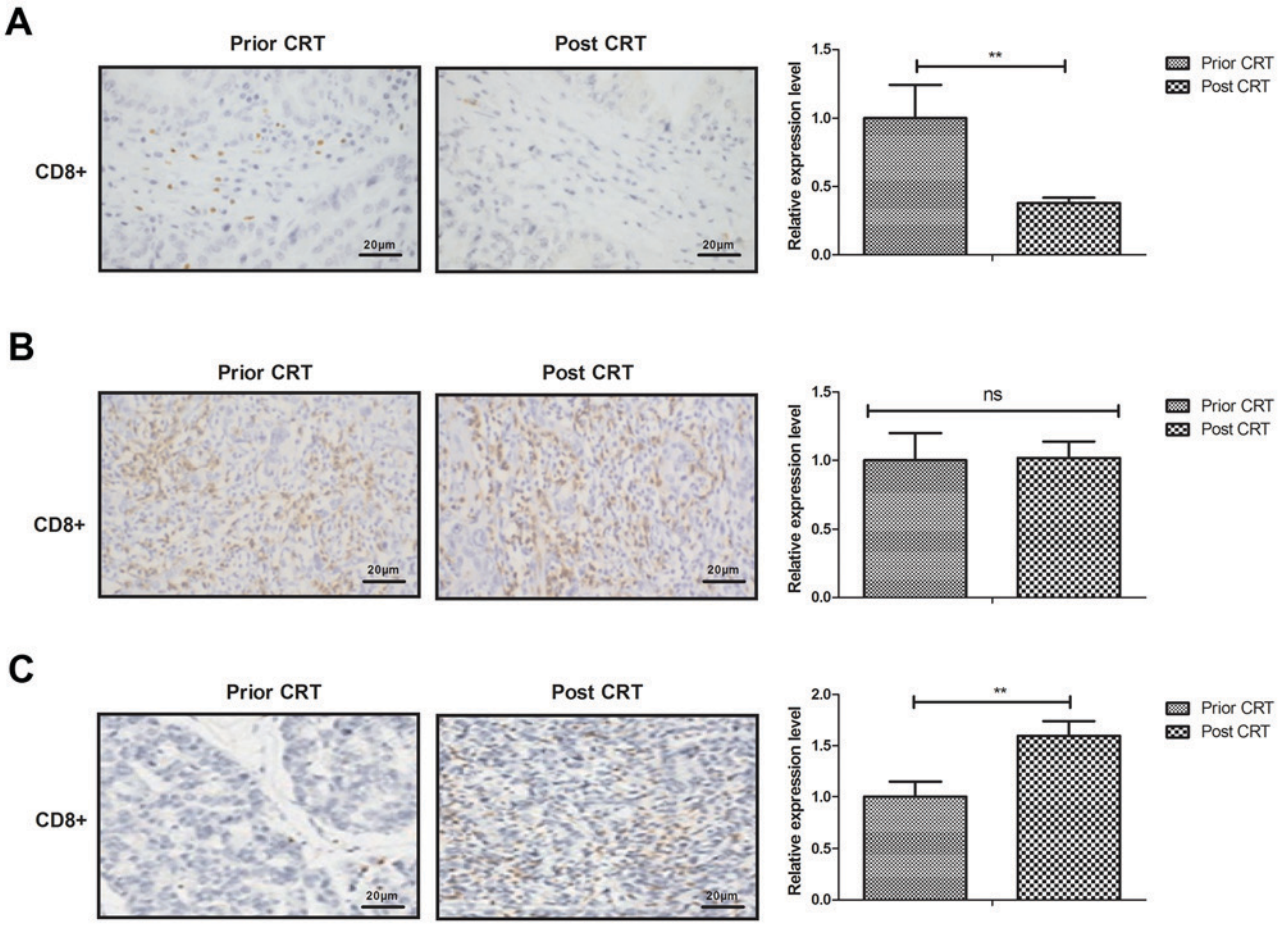

Figure 2. Analysis of cytotoxic T lymphocytes in the gastric tumor microenvironment following CRT. The densities of $CD8^+$ T cells in (A) T4, (B) T3 and (C) T2 stage gastric tumors were determined by immunohistochemistry. $^{**}P<0.01$, vs. prior CRT. CRT, chemoradiotherapy; ns, not significant.

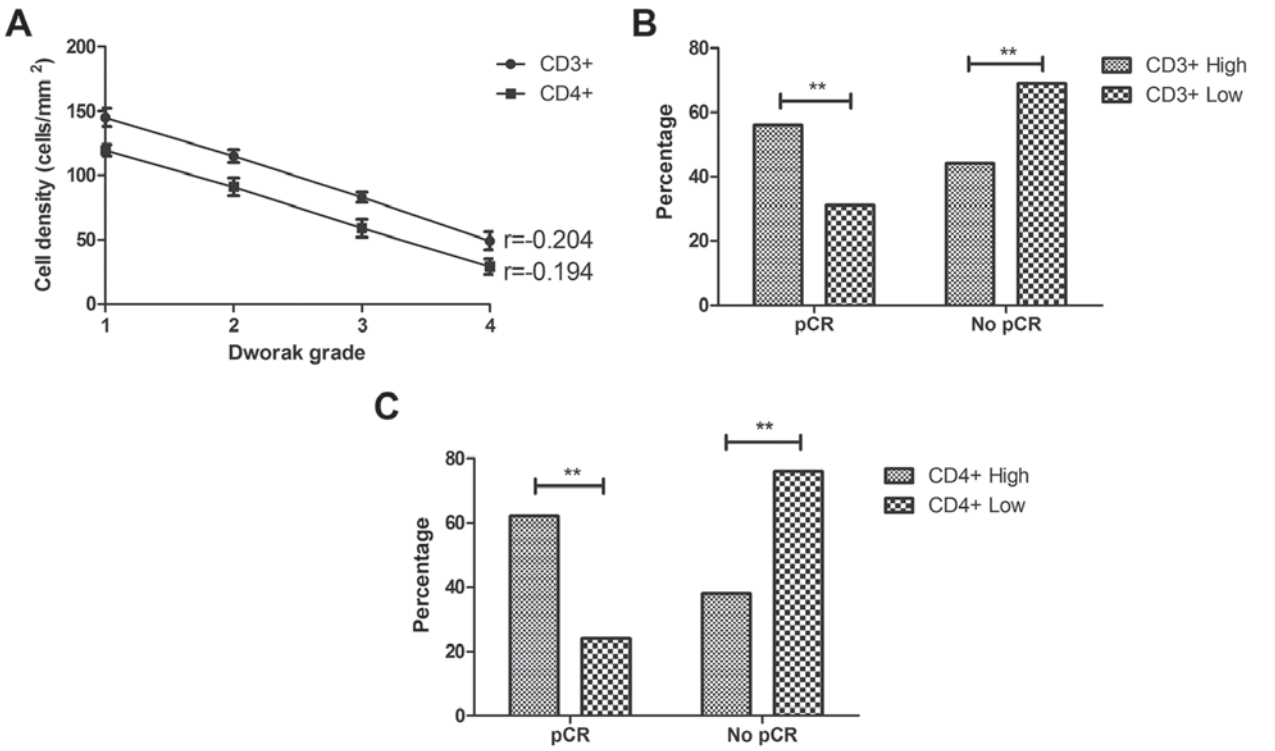

Figure 3. Regulatory T-cell density is associated with response to chemoradiotherapy. (A) Association between regulatory T-cell density ($CD3^+$ and $CD4^+$) and gastric cancer regression. The association between (B) $CD3^+$ and (C) $CD4^+$ regulatory T-cell density and pCR in patients with gastric cancer were examined. $^{**}P<0.01$, vs. $CD3^+$ low. pCR, pathologic complete response.

Table III. Clinical outcomes of patients with gastric cancer following neoadjuvant chemoradiotherapy with paclitaxel and carboplatin.

Outcome	n (%)
Mortality	12 (21)
Survival without cancer recurrence	28 (50)
Local recurrence	12 (21)
Colon metastases	4 (8)

distribution of $CD3^+$ and $CD4^+$ T cells (Fig. 1B). However, the density of $CD3^+$ and $CD4^+$ T cells were increased in the T2 gastric tumor tissue (Fig. 1C).

Cytotoxic T lymphocyte in the gastric tumor microenvironment following CRT. The present study analyzed the cytotoxic T lymphocyte density in the gastric tumor microenvironment following CRT. The T4 stage gastric tumor tissue exhibited a lower density of $CD8^+$ cells compared with prior to CRT (Fig. 2A). The density of $CD8^+$ cells in T3 stage gastric tumor tissue did not differ significantly between pre-CRT and post CRT (Fig. 2B). The density of $CD8^+$ cells were increased in T2 stage gastric tumor tissue (Fig. 2C).

Regulatory T-cell density is associated with the response to CRT. Analysis of the regulatory T-cell density revealed an inverse correlation between $CD3^+$, $CD4^+$ cell density in gastric cancer regression (r=-0.204 and -0.194, respectively, Fig. 3A). Among the patients who had pCR, 31% had a low density of $CD3^+$ cells, which was lower than 56% of those with pCR with a high $CD3^+$ cell density (Fig. 3B). The results also demonstrated that, of patients with a high $CD4^+$ cell density, 62% had a pCR, which was significantly higher than that in patients with a low $CD4^+$ cell density (Fig. 3C).

Cytotoxic T lymphocyte density is associated with the response to CRT. The association between cytotoxic T lymphocytes and pCR was also analyzed in the present study. As shown in Fig. 3A, cytotoxic T lymphocyte $CD8^+$ cells had an inverse correlation with gastric cancer regression (r=-0.312, Fig. 4A). It was observed that 65% patients with a high $CD8^+$ cell density showed pCR, whereas 32% patients with a low $CD8^+$ cell density showed pCR (Fig. 4B).

Regulatory T-cell and cytotoxic T lymphocyte density as a prognostic marker in neoadjuvantly treated gastric cancer. A total of 56 patients (82%) survived the 60-month follow-up from the date of surgery, 12 (21%) patients succumbed to mortality during the follow-up period, 28 patients (50%) survived without cancer recurrence, 12 patients (21%) showed local recurrence, and four patients (8%) showed colon metastases (Table III). It was shown that $CD3^+$ and $CD4^+$ cell density were associated with the survival rate of patients with gastric cancer (Fig. 5A-D). The probabilities of 5-year CSS and RFS were 79, vs. 64% and 72, vs. 56% in the $CD3^+$ high, vs. low groups, respectively (Fig. 5A and B). The probabilities of 5-year CSS and RFS were 84, vs. 64% and 82, vs. 62% in the $CD4^+$ high, vs. low groups, respectively (Fig. 5C and D). It was also shown that $CD8^+$ cell density was associated with the survival rates of patients with gastric cancer (Fig. 5E and F). The probabilities of 5-year CSS and RFS were 75%, vs. 60% and 81, vs. 62% in the $CD8^+$ high, vs. low groups, respectively (Fig. 5E and F). The densities of $CD3^+$, $CD4^+$ and $CD8^+$ cells were associated with CSS of patients with gastric cancer (Table IV). In addition, tumor size and concomitant diseases markedly affected the CSS of patients with gastric cancer.

Discussion

Although previous reports have shown that CRT treatment can inhibit gastric cancer growth in the process of cancer progression (24-26), the associations between regulatory T-cell density, cytotoxic T lymphocyte density and pCR to paclitaxel and carboplatin have not reported in gastric cancer. In the present study, the densities of regulatory T-cell density and cytotoxic T lymphocytes were analyzed in gastric cancer tissues from a total of 68 patients. An association was found between regulatory T-cell density and cytotoxic T lymphocyte density in the local tumor microenvironment following neoadjuvant CRT of paclitaxel and carboplatin in locally gastric cancer. Owing to the evidence of an immune-mediated component to chemoradiotherapy, it was

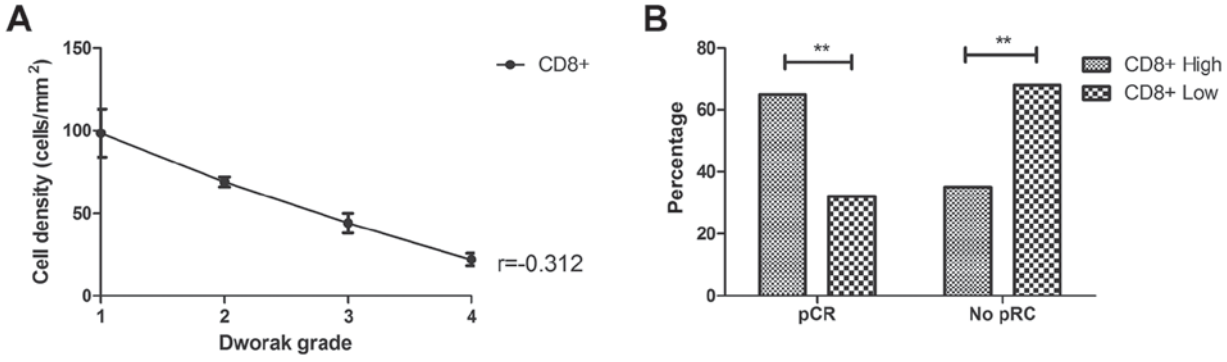

Figure 4. Cytotoxic T lymphocyte is associated with response to chemoradiotherapy. (A) Cytotoxic T lymphocyte $CD8^+$ was inversely correlated with gastric cancer regression. (B) Association between regulatory T-cell $CD8^+$ density with pCR in patients with gastric cancer. $^{**}P<0.01$, vs. $CD8^+$ low. pCR, pathologic complete response.

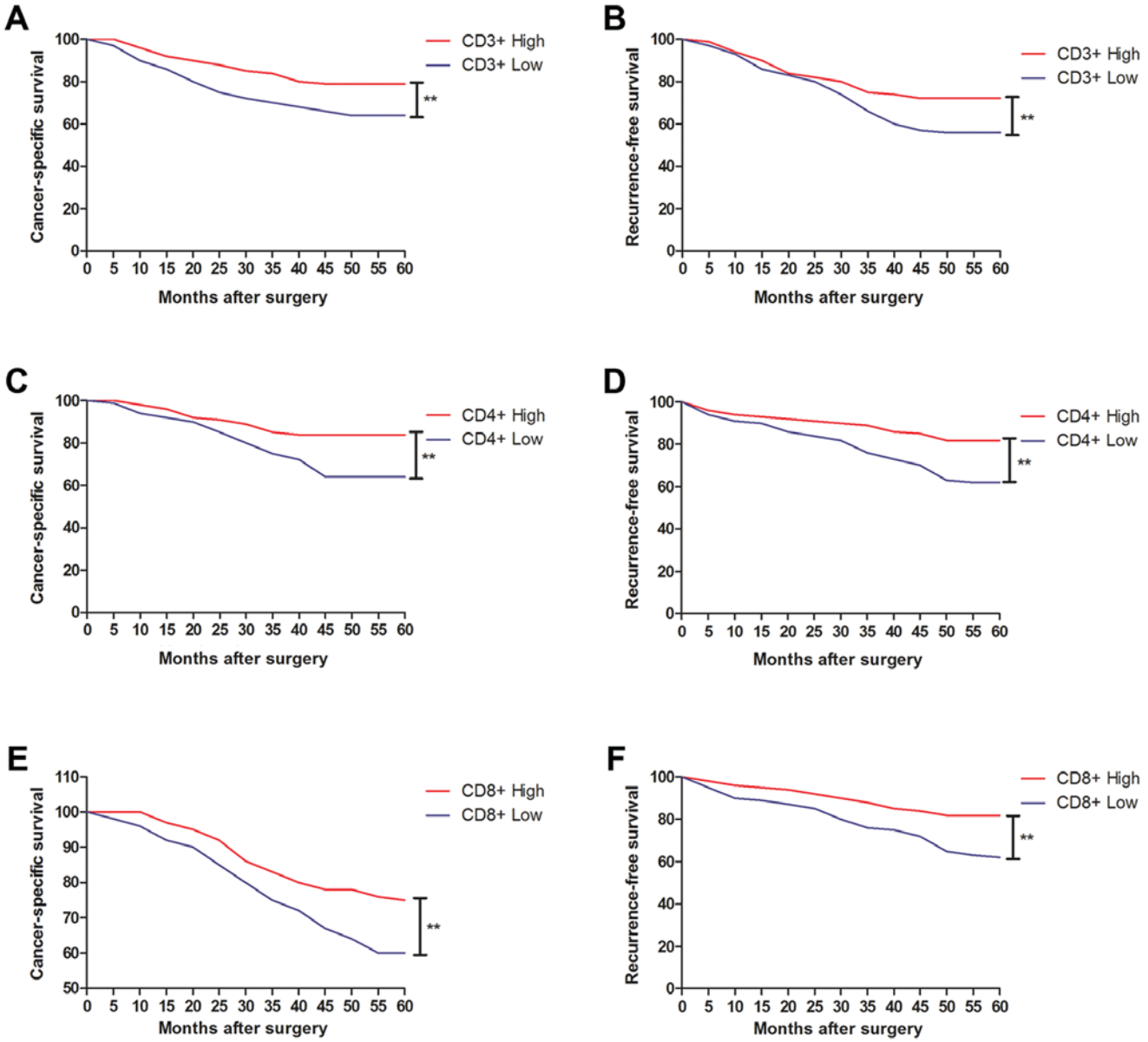

Figure 5. Survival rates and regulatory T-cell and cytotoxic T lymphocyte density in patients with gastric cancer. Analysis of (A) CSS and (B) RFS by $CD3^+$, cell density. Analysis of (C) CSS and (D) RFS by $CD4^+$, cell density $CD4^+$. Association between $CD8^+$ cell density and (E) CSS and (F) RFS. $^{**}P<0.01$ vs. $CD8^+$ low. CSS, cancer-specific survival; RFS, recurrence-free survival.

hypothesized that regulatory T-cells inhibited the response of gastric carcinoma to neoadjuvant CRT and cytotoxic T lymphocytes promoted the response of gastric carcinoma to neoadjuvant CRT.

Currently, paclitaxel is one of the most widely used clinical anticancer drugs in chemotherapy (27). The combined administration of paclitaxel has been reported to markedly enhance tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in resistant cancer cells *in vitro* and *in vivo* (28), and Zhu *et al* indicated that $CD4^+Foxp3^+$ regulatory T-cell impairment by paclitaxel is independent of Toll-like receptor 4 (29). In the present study, the results revealed that the regulatory T-cell density of $CD3^+$ and $CD4^+$ T cells were decreased in T4 stage gastric tumor and increased in T2 gastric tumor following CRT. However, T3 stage gastric tumor showed similar distribution of $CD3^+$ and $CD4^+$ T cells between pre-CRT and post-CRT gastric cancer tissues. The findings also indicated that regulatory T-cell $CD3^+$, $CD4^+$ cell density was inversely correlated with gastric cancer regression. However, further analysis is required in larger gastric cancer populations.

Carboplatin is active in advanced gastric and esophageal cancer (30). A report found that carboplatin and paclitaxel added to radiation and fluoropyrimidine analogs is a well-tolerated regimen in the adjuvant setting, which is a safe and feasible regimen in this relatively high-risk group of patients with gastric cancer and is of interest for future development (31). The density of tumor-infiltrating T cells ($CD8^+$ T cells), is an indicator of improved survival rate in patients with gastric cancer, and the CD8 T-cell density predicts the likelihood of tumor regression following CRT (32). In the present study, it was found that cytotoxic T lymphocyte $CD38^+$ $CD8^+$ density was decreased in T4 stage gastric tumor tissue and increased in T2 stage gastric tumor tissue. Cytotoxic T lymphocyte

Table IV. Univariate and multivariable analyses of prognostic factors for CSS following curative gastric cancer resection.

	CSS				
	Univariate			Multivariate	
Prognostic factor	Log-rank	P-value	HR	95% CI	P-value
$CD3^+$ cell density					
Interaction					
No interaction	1.193	0.026[a]	1.704	1.104-2.824	0.038[a]
$CD4^+$ cell density					
Interaction					
No interaction	1.826	0.058	0.886	0.914-2.320	0.026[a]
$CD8^+$ cell density					
Interaction					
No interaction	2.520	0.037[a]	2.158	1.104-2.824	0.025[a]
Concomitant disease					
Interaction					
No interaction	4.726	0.016[a]	1.862	1.426-3.026	0.018[a]
Tumor size (mm)					
<50	3.746	0.014[a]	1.638	1.242-2.858	0.0163[a]
≥50					

[a]P<0.05 was considered to indicate a statistically significant difference. CSS, cancer-specific survival; HR, hazard ratio; CI, confidence interval.

$CD3^+$ $CD8^+$ density was also inversely correlated with gastric cancer regression.

In conclusion, the results of the present study indicated that regulatory T-cell and cytotoxic T lymphocyte density may be considered as prognostic markers in neoadjuvantly treated gastric cancer. Notably, regulatory T-cell and cytotoxic T lymphocyte density were associated with pCR and the improved long-term outcome of patients with gastric cancer. These results suggest that regulatory T-cells in the local microenvironment may inhibit the response of gastric cancer to neoadjuvant CRT and cytotoxic T lymphocytes may promote the response of gastric cancer to CRT.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DH performed the experiments. YY, SZ, ZS, TP, XW and YZ prepared and analysed experimental data. SL designed the experiments.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the First Affiliated Hospital of Hebei North University. All patients provided written informed consent prior to undergoing any protocol-specific screening procedures or treatments.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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