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Intratumoural FOXP3-positive regulatory T cells are associated with adverse prognosis in radically resected gastric cancer

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ABSTRACT

We investigated the clinical significance of tumour-infiltrating FOXP3-positive regulatory T cells (Tregs) in radically resected (R0) gastric cancer. From a single-institution database, tumors of 110 patients who underwent R0 resection for stage II–III disease were studied for FOXP3-positive Tregs by immunohistochemistry. The observed median number of FOXP3-positive Tregs was used as the cut-point in analyses (<6 versus ⩾6 count). Tregs were significantly higher in gastric carcinomas than in normal tissue ($P = 0.0001$). Tregs count ⩾6 was significantly associated with vascular/lymphatic/perineural invasion (VELIPI) in the tumour ($P = 0.03$). Multivariate analysis showed association between adverse relapse-free survival and grading 3, stage III, VELIPI and Tregs count ⩾6 ($P = 0.02$). Adverse overall survival was associated with grading 3, stage III, VELIPI and Tregs count ⩾6 ($P = 0.006$). FOXP3-positive Tregs may be a novel marker for identifying high-risk gastric cancer patients. Present findings deserve additional investigation as Tregs may also represent an innovative therapeutic target.

1. Introduction

Tumours often contain infiltrates of immune cells. These infiltrates represent the result of an interplay between the host immune system and tumours during their development and growth.[1] Intratumoural T cells with an effector (memory) phenotype showed favourable influence on the prognosis of patients with colorectal[2,3] and epithelial ovarian cancer.[4] On

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the other hand, tumours have elaborated methods to circumvent such a response. T regulatory (Treg) cells are functionally defined as T cells that inhibit an immune response by influencing the activity of another cell type.[5] Treg cells fall into two main categories. One is naturally occurring thymus-derived CD4+ T cells; the other is peripherally antigen-induced CD4+ T cells deriving from CD4+CD25- non-Treg T cells. Treg cells, which were originally defined by CD4 and $CD25^{hi}$ positivity, have been identified in peripheral blood, primary tumours and draining lymph nodes of cancer patients.[5] The body of evidence suggests that Treg cells within the tumour microenvironment might play a significant role in the suppression of local antitumour immune responses.[5] This may also explain why the presence of high numbers of intratumoural FOXP3-positive T cells has been associated with a higher risk of recurrence and poor overall survival of patients with some solid neoplasms.[6–12] Thus, Tregs are under investigation as a potential prognostic factor and they may also represent a novel therapeutic target in several cancers. In this perspective, a major issue concerns their precise identification and the possibility of selectively targeting these cells for therapeutic purposes. In fact, CD25 is expressed by all activated effector T cells. Other markers (e.g. CTLA-4, GITR, FOXP3) have been proposed to identify Tregs, but their expression is not exclusively restricted to Tregs. Nonetheless, FOXP3 has been widely accepted as the best marker for Treg identification in humans thus far.[13,14]

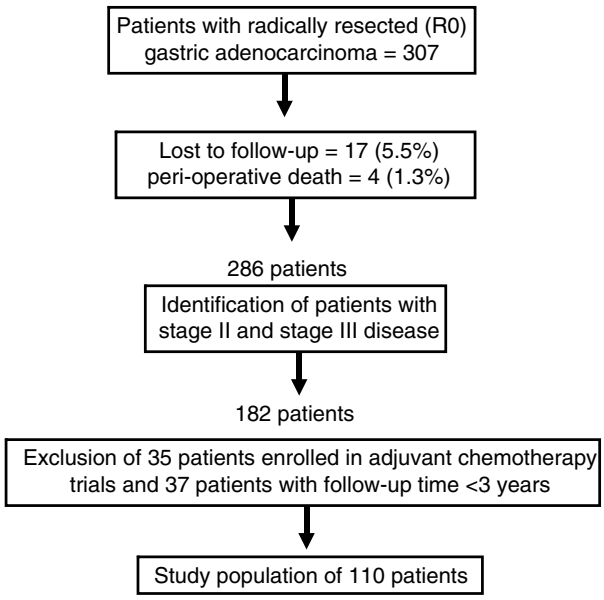

Fig. 1 – Diagram showing the flow of the patients in this study according to the newly proposed Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) criteria for tumour marker publications.

CD4+ CD25+ and/or FOXP3+ Treg cell have been identified in the peripheral blood,[15–17] ascitic fluid,[15] primary tumours and draining lymph nodes[16,18,19] of gastric cancer patients. A higher percentage of Treg cells have been found in the peripheral blood of gastric cancer patients than healthy controls.[15–17] The presence of these cells may thus represent an unfavourable feature. Despite radical surgery, a significant proportion of patients with locally advanced gastric cancer relapse and die. Also, the role of adjuvant chemotherapy for these patients is controversial.[20] The principal aim of this study was to establish whether intratumoural FOXP3-positive T cell count adds prognostic information after considering traditional clinico-pathologic features of patients with radically resected, stage II–III gastric cancer.

2. Materials and methods

2.1. *Patients*

Tissues from gastric adenocarcinomas used in this retrospective study represent a consecutive series of patients from the

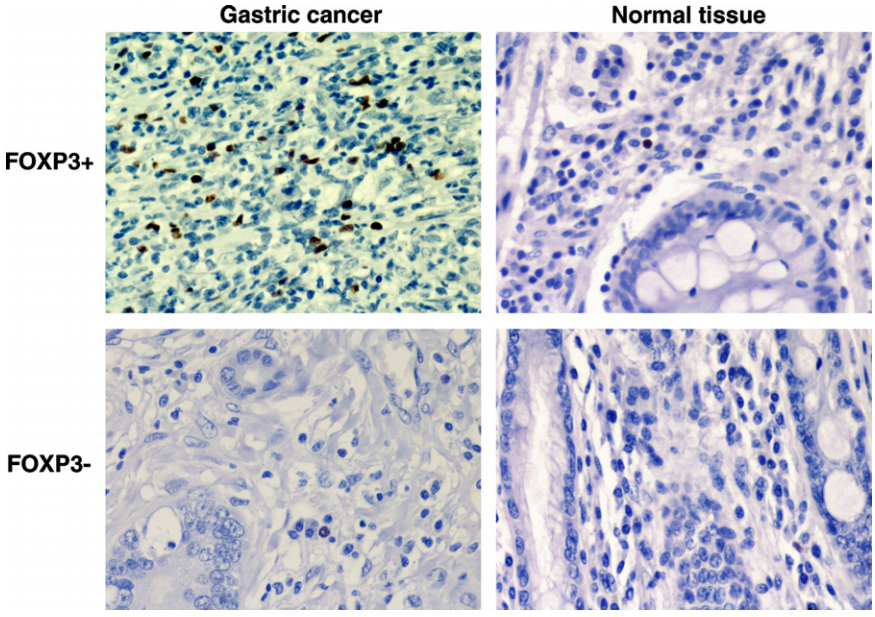

Fig. 2 – Immunohistochemical detection of regulatory T cells (Tregs).

referral population of a regionally-based cancer service and they were collected from patients undergoing surgery at the Azienda Ospedale San Salvatore (Pesaro, Italy). From 1997 to 2006, 307 patients underwent radical surgery (R0) for gastric adenocarcinoma. This study targeted a high-risk population of stage II–III patients. In planning the study we incorporated the newly-proposed Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) criteria[21] for tumour marker publications and we have included a diagram showing the flow of the patients in this study (Fig. 1). One hundred and eleven patients formed the study population representing a homogenous group with stage II–III disease, which was treated with R0 surgery and D1–D2 lymphoadenectomy (>10 retrieved lymph nodes).[22] A minimum 3-year follow-up was required. Follow-up procedures consisted of interim history, physical examination, haematologic studies, carcinoembryonic antigen levels, and diagnostic imaging (chest X-ray and abdominal ultrasonography) every 4 months in the first year and every 6 months thereafter. Patients underwent upper endoscopy 6 months after surgery and every 12 months thereafter. Whole-body computed tomography was done for corroborative evidence of relapse. The recurrences of gastric carcinoma had to be confirmed by cytology biopsy or surgery. The 1997 revision of the AJCC manual was used for the classification of each case. None of the patients received chemotherapy or radiation therapy before or after surgery as a part of an adjuvant programme. The study was conducted in a blinded fashion so that patients' outcomes were unknown to investigators performing immunohistochemistry analyses.

2.2. *Immunohistochemical labelling of FOXP3-positive Tregs*

Tissue samples were fixed in 10% neutral buffered formaldehyde and embedded in paraffin. Routine haematoxylin and eosin staining was performed on the sections for histopathologic evaluation. Based on the quality of the morphologic preservation of all available haematoxylin and eosin stained slides of the surgical specimen sections, we selected one paraffin block for each case. Consecutive 3 μm sections were re-cut from each study block for immunohistrochemistry experiments.

Immunohistochemistry was performed by the streptoavidin-biotin method. Tissues were dewaxed followed by antigen retrieval by microwaving in 50 mmol/L Tris/2 mmol/L EDTA (pH 9.0). Immunohistochemistry to label FOXP3-positive Tregs was performed using a murine monoclonal antibody against FOXP3 protein (clone: 236A/E7, eBioscience, Inc.)[14] at a 1:100 dilution. Sections were incubated with LSAB2 (Dakocytomation). 3-3′-diaminobenzidine (DAB) was used for colour development and haematoxylin was used for counterstaining. Positive-staining controls were carried out in parallel with paraffin tonsil sections using 236A/E7. Negative control slides processed without primary antibody were included for each staining. Previously, an extensive characterisation of this antibody has been published and, after comparison with the available commercial reagents, 236A/E7 was the best currently available to study FOXP3 expression by immunohistochemistry.[14]

For each immunolabelled slide, 10 HPF digital images of the deepest infiltrative tumour areas were chosen by two pathologists (GP and CR) after consensus. In 64 paired cases, non-neoplastic gastric mucosa was available for comparative analysis. The absolute number of FOXP3-positive lymphocytes was determined in 10 HPF digital images using image analysis software (Arkon software, Nikon Instrument). The number of immunostained FOXP3 cells for each tumour was determined by the average of the 10 HPF digital image cell counts. Slides were examined without knowledge of the corresponding clinico-pathologic data (see Fig. 2).

2.3. *Statistical analyses*

Given that there are no widely accepted standard cut-points to define clinical outcomes for the number of Tregs in this setting, and consistent with previous studies,[10,20] we selected the median intratumoural FOXP3+ T cell count in the entire group to define possible risk factors. We assessed the association between Tregs numbers and clinico-pathologic features,

Table 1 – Characteristics of the 110 gastric cancer patients

Age (years)	
Median (minimum–maximum)	72 (36–88)
Gender: number (%)	
Male	53 (48%)
Female	57 (52%)
Gastrectomy: number (%)	
Total	79 (72%)
Partial	31 (28%)
Histology: number (%)	
Diffuse	27 (25%)
Intestinal	68 (62%)
Mixed	15 (14%)
Stage: number (%)	
2	46 (42%)
3	64 (58%)
Grading: number (%)	
Well differentiated	5 (5%)
Moderately differentiated	59 (54%)
Poorly differentiated	46 (42%)
Lymph node status: number (%)	
Positive	79 (72%)
Negative	31 (28%)
Percentage of positive nodes	
Median (minimum–maximum)	9.3 (0–87.5)
VELIPI status[a]: number (%)	
Positive	54 (49%)
Negative	56 (51%)
Relapse: number (%)	
No	62 (56%)
Yes	48 (44%)
Death: number (%)	
No	62 (56%)
Yes	48 (44%)

a VELIPI: Histology showing the presence of tumour vascular, lymphatic or perineural invasion.

relapse-free survival (RFS) or overall survival (OS). Patient characteristics investigated were: age, gender, history of total or partial gastrectomy, stage of gastric cancer (2 or 3), presence of VELIPI, presence of positive lymph nodes, histotype (diffuse, intestinal, mixed), and tumour grading (well differentiated, moderately differentiated and poorly differentiated). Chi-square tests for categorical variables and the two-sample *t* test for continuous variables were used to investigate differences in patient characteristics between those with low and high FOXP3 counts.

To explore the association between OS and RFS with FOXP3 count, stratified log-rank tests were used. Cox proportional hazards models were employed to further explore these relationships, controlling for the impact of other potential prognostic factors (defined above). A stepwise selection procedure was applied: a variable was entered with $P \leqslant 0.25$ and remained in the model if $P \leqslant 0.15$. Only factors significant at the (approximate) two-sided 0.05 level were included in the final model. OS and RFS were defined as the time from surgery to death and relapse, respectively. Patients who were alive at the time of the data analysis were censored. All analyses were performed using SAS 9.1 (Cary, NC).

3. Results

The characteristics of the 110 studied patients are shown in Table 1. In this study, 48 patients (44%) died with positive cytological or histological confirmation of recurrent disease. All 48 relapsed patients underwent 5-fluorouracil/cisplatin-based palliative chemotherapy.

In the 110 cases, the median for the FOXP3-positive lymphocyte count was 6; (minimum–maximum, 0–49.5). The number of cases with median FOXP3-positive cells <6 and $\geqslant$6 was 52 (47%) and 58 (53%), respectively. In 64 cases (58%) with assessable paired normal and tumour tissue, the median FOXP3-positive lymphocytes count was 0.2 (minimun–maximum, 0–6.7) and 7.7 (minimun–maximum, 0–49.5), respectively ($P < 0.0001$, paired *t* test).

Table 2 – Association of median FOXP3 positive cells (<6 and $\geqslant$6) and patient characteristics

Characteristic	FOXP3 < 6 N = 52	FOXP3 $\geqslant$ 6 N = 58	P-value[a]
Gender; number (%)			
Male	31 (58%)	22 (42%)	0.0231
Female	21 (37%)	36 (63%)	
Age, years; mean (standard deviation)	69.1 (11.7)	70.1 (9.7)	0.6248
Gastrectomy; number (%)			
Total	34 (43%)	45 (57%)	0.1556
Partial	18 (58%)	13 (42%)	
Stage; number (%)			
2	24 (52%)	22 (48%)	0.3827
3	28 (44%)	36 (56%)	
VELIPI[b]; number (%)			
Present	20 (37%)	34 (63%)	0.0347
Absent	32 (57%)	24 (43%)	
Lymph Node Status; number (%)			
Positive	36 (46%)	43 (54%)	0.5679
Negative	16 (52%)	15 (48%)	
Histotype; number (%)			
Diffuse	15 (56%)	12 (44%)	0.6014
Intestinal	30 (44%)	38 (56%)	
Mixed	7 (47%)	8 (53%)	
Grading; number (%)			
Well differentiated	2 (40%)	3 (60%)	0.7138
Moderately differentiated	30 (51%)	29 (49%)	
Poorly differentiated	20 (43%)	26 (57%)	
Overall Survival			
Death; number (%)	17 (35%)	31 (65%)	0.0023
Months: median	Undefined	61.3	
(95% CI)	(undefined, undefined)	(21.9, undefined)	
Relapse-Free Survival			
Relapse; number (%)	17 (35%)	31 (65%)	0.0082
Months: median	Undefined	54.5	
(95% CI)	(undefined, undefined)	(12.5, undefined)	

a Two-sample *t*-test for continuous variables, chi-square test for discrete variables, log-rank test for time-to-event variables.
b VELIPI: Histology showing the presence of tumour vascular or lymphatic or perineural invasion.

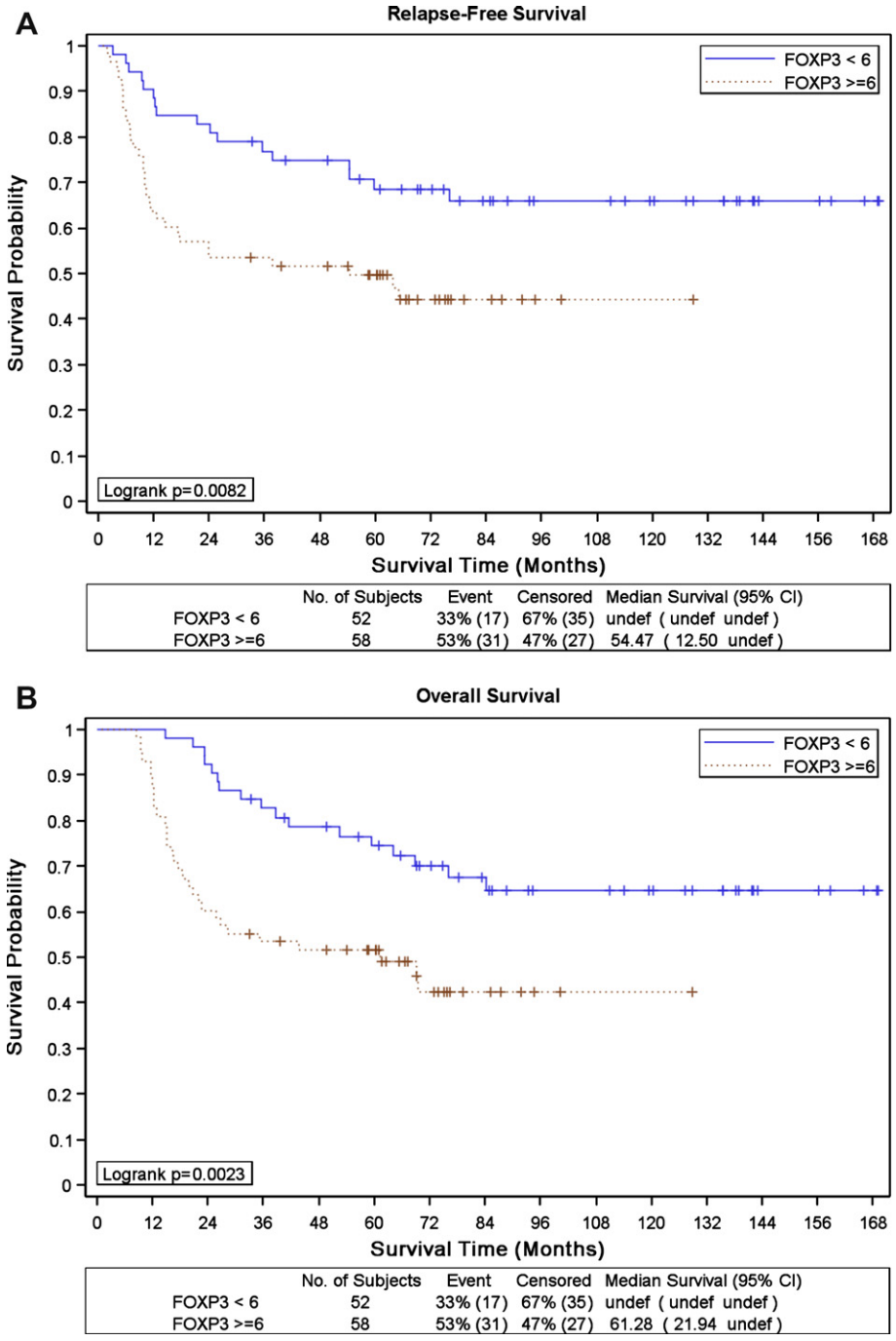

Fig. 3 – Kaplan–Meier curves for relapse-free survival (A) and overall survival (B) of the 110 patients stratified by low (< 6) or high (⩾ 6) numbers of FOXP3-positive regulatory T cells (Tregs).

As shown in Table 2, there was a significant association between FOXP3-positive cell count ⩾6 and vascular/lymphatic/perineural invasion and gender. There was also a statistically significant association between gender and FOXP3. Kaplan–Meier curves showed unfavourable RFS (Fig. 3A) and OS (Fig. 3B) in patients with tumours containing ⩾6 FOXP3-positive cells ($P < 0.01$). Among clinico-pathologic features (Table 2), which entered the multivariate analyses (Table 3), adverse RFS was associated with poorly differentiated tumours, stage III disease, presence of VELIPI and FOXP3-positive cell count ⩾6, while adverse OS was associated with poorly differentiated tumours, stage III disease, presence of VELIPI and FOXP3-positive cell count ⩾6.

4. Discussion

Our study shows a significant increase in the FOXP3-positive T cell number in gastric adenocarcinomas as compared with

Table 3 – Multivariate analysis of overall survival and relapse-free survival with FOXP3 positive cells, controlling for other factors

Variable	Overall survival			Relapse-free survival		
	HR	95% CI	P-value	HR	95% CI	P-value
FOXP3+ Cells ⩾ 6	2.34	1.27, 4.28	0.0061	2.00	1.10, 3.65	0.0241
Grading 3	2.21	1.22, 3.98	0.0085	2.15	1.19, 3.89	0.0110
Stage 3	2.40	1.23, 4.67	0.0102	2.34	1.20, 4.57	0.0130
Presence of VELIPI	1.80	0.99, 3.28	0.0525	1.96	1.08, 3.55	0.0263

HR: hazard ratio.
95%CI: 95% confidence interval.
VELIPI: vascular, lymphatic or perineural invasion in the tumour.

surrounding normal tissue. This difference has also been observed in other solid tumours, e.g. hepatocellular carcinoma, colorectal, breast, pancreatic, non-small cell lung cancer[6–12,23–25] and likely reflects immunological tumour–host interactions at the tumour site. Studies in prostate,[26] ovarian[8] and gastric[27] carcinomas showed Tregs recruitment into tumours via the CCL22-CCR4 axis. Intratumoural Tregs likely contain not only naturally occurring Tregs, but also peripherally antigen-induced Treg cells[18] possibly derived from CD4+CD25– T cells converted into Tregs within the immunosuppresive tumour microenvironment.[28] The recruitment of Tregs into tumours likely represents one of the mechanisms by which malignant cells evade host immune response. Treg cells isolated from ascites or tumours of patients with ovarian cancer equally suppressed tumour-associated antigen (i.e. Her-2)-specific immunity *in vitro* and in a mouse xenotransplantation model[8] providing compelling evidence for Treg-mediated local suppression of antitumour immunity. Similar findings have also been reported in head and neck[29] and cervical cancer.[30] Furthermore, once activated, Tregs can suppress tumour antigen-independent immune responses through bystander mechanisms, and they can also inhibit the function of dendritic cells, NK cells, B cells and other immune cells.[31–33] Less intuitive is the observation of the gender difference in intratumoural FOXP3 positive cells with preponderance in women. Among possible explanations is the contribution of sex hormones in the modulation of Tregs.[34]

Treg-mediated local immune evasion underpins the second finding of our study, i.e. the positive association between higher FOXP3-positive T cells and the presence of VELIPI. The association between intratumoural FOXP3-positive T cells and vascular invasion has been observed in other solid tumours such as breast cancer[35] and hepatocellular carcinoma.[6] Notably, VELIPI was not recorded in colorectal carcinomas with CD45RO+ T cell infiltration.[2] These findings suggest that an effective antitumour immune response may restrain the invasive potential of tumour cells. Conversely, the immuno-suppressive role of FOXP3 positive cells allowing tumour invasiveness may explain our third and most relevant observation that elevated intratumoural FOXP3-positive T cell numbers conferred unfavourable outcomes in this population of gastric cancer patients. Our homogeneous population of consecutive patients with R0, stage II–III disease allowed a proper investigation of the prognostic role of FOXP3-positive cells. It is likely that such an association may be underestimated when patients display large variability in their stage and management.[19] After controlling for grading, stage and VELIPI in multivariate models for OS and RFS, the effect of FOXP3 count was statistically significant. The pronounced relationship between FOXP3 count and OS may suggest there is a detrimental effect of unfavourable immune status that persists in the course of the disease. In addition, tumour stage showed similar effects on RFS and OS with effects (HR) that are comparable to those reported in similar populations.[36] Molecular factors like p53, c-erbB2, Mib1, Thymidylate Synthase or Herg1 do not seem to characterise sub-groups of high-risk gastric cancer patients.[36] The prognostic role of Tregs may support an immune perspective for improving adjuvant treatment strategies in these patients.[20] We cannot rule out that a broader investigation of the intratumoural immune cell infiltrate, taking into account not only Tregs but also intratumoural effector T cells, may provide even more accurate prognostic information. In fact, in epithelial ovarian cancer, the ratio between intratumoural CD8+ T cells and Treg was a better predictor of patient survival than Treg alone.[37] In colorectal cancer, the type, density and location of immune cells within the tumour sample was even found to be a better predictor of patient survival than traditional histopathological parameters.[3]

From a therapeutic standpoint, strategies can aim at depleting Tregs or reversing their immunosuppressive function. In patients with advanced disease, Treg depleting strategies have included administration of denileukin diftitox[5] and cyclophosphamide.[38] However, peripheral (i.e. at the tumour site) conversion of non-Treg T cell into Tregs may frustrate such a strategy, thus interventions aimed at reversing Treg immunosuppressive function have been advocated as more rationale.[39] Additional efforts should be addressed toward understanding if known anticancer cytotoxic agents can also restore an efficient immune response that contributes to the therapeutic effects of chemotherapy.[40] In early stage patients, the local and systemic Treg-depleting effect of radical surgery supports the possibility of adjuvant tumour-specific vaccination in this setting.[41] Recently, Jeung et al. [42] found in a randomised phase III trial that a chemoimmunotherapy approach after curative surgery may improve the prognosis of gastric cancer patients.

In conclusion, the present findings deserve additional investigation as Tregs may represent an innovative prognostic feature in this lethal disease and, possibly, they could establish a new, intriguing therapeutic target.

Conflict of interest statement

None declared.

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