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The expression of C-MYC in gastric adenocarcinoma is associated with PD-L1 and FOXP3 expression: C-MYC overexpression is a good prognostic factor

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ABSTRACT

Background: C-MYC appears to initiate and maintain tumorigenesis through modulation of immune regulatory molecules such as PD-L1. The aim of our research was to evaluate the clinical implication of C-MYC expression in gastric adenocarcinoma in relation to the expression of the immune regulatory molecules PD-L1 and FOXP3.
Methods: Tissue samples were acquired from 182 cases of gastric adenocarcinoma that were surgically resected at Kyung Hee University Hospital at Gangdong from 2006 to 2012. Immunohistochemical staining for C-MYC, PD-L1, CD8 and FOXP3 was done.
Results: C-MYC overexpression showed a significant correlation with smaller tumor size, lower T category, lower N category, lower recurrence rate, and less lymphatic invasion. And C-MYC overexpression was negatively correlated with PD-L1 expression. The tumoral FOXP3 was positively correlated with C-MYC overexpression and Tregs count. PD-L1 expression was positively correlated with Tregs, CD8 + T cells, and tumor infiltrating lymphocytes (TIL). Tregs count was positively correlated with CD8 + T cells and TIL. CD8 + T cells was positively correlated with TIL.
Conclusion: We discovered that the immune regulatory effect of C-MYC and PD-L1, and the tumor suppressor function of tumoral FOXP3 had a significant influence on the tumor microenvironment (Tregs, CD8 + T cells, and tumor infiltrating lymphocytes) in a complex manner. The C-MYC overexpression is a good prognostic factor in gastric adenocarcinoma.

1. Introduction

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related deaths [1]. Gastric cancers are characterized by genetic and epigenetic changes that affect the expression of oncogenes, tumor suppressor genes, and DNA mismatch repair genes. Consequently, deregulation of cellular proliferation, adhesion, differentiation, and signal-transduction are related to tumorigenesis and the progression of gastric adenocarcinoma [2].

The C-MYC protein, which is encoded by the *C-MYC* gene, functions as a transcription factor and regulates the expression of genes involved in proliferation, differentiation, metabolism, survival, and apoptosis [3]. C-MYC has been shown to promote tumorigenesis in various malignant tumors [4] and examples of malignant tumors that overexpress C-MYC are gastric adenocarcinoma, esophageal squamous cell carcinoma, and soft tissue leiomyosarcoma, all of which are characterized by poor survival rates [5–7]. Several studies have investigated the clinical implications of C-MYC overexpression in gastrointestinal malignancies with conflicting findings. Ninomiya et al. showed that patients with C-MYC protein-positive gastric carcinomas had a significantly poorer prognosis than those with C-MYC-protein-negative gastric carcinomas, and that C-MYC levels correlated well with the recurrence of cancer by peritoneal dissemination [7]. De Souza et al. reported that C-MYC immunoreactivity and increased mRNA expression were associated with deeper tumor extension and presence of metastasis in gastric cancer [8]. In contrast, Onoda et al. reported that C-MYC expression was more frequent and higher in early gastric cancers than in advanced lesions [9]. Toon et al. reported that patients with colorectal cancers overexpressing C-MYC had better 5-year survival than patients with colorectal cancers not overexpressing C-MYC [10]. Lee

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et al. also showed that patients with colorectal cancer co-expressing C-MYC and β-catenin had a favorable prognosis [11]. Smith et al. reported that overexpression of C-MYC in colorectal carcinoma was associated with reduced mortality. They hypothesized that C-MYC deregulation leads to increased apoptotic death, and that this response may be modulated by downstream events such as point mutations of the *p53* gene [12]. These conflicting results regarding the expression of C-MYC raise questions about the function of C-MYC.

Recently, Casey et al. reported that C-MYC appears to initiate and maintain tumorigenesis through modulation of immune regulatory molecules such as PD-L1 [13]. PD-L1 is expressed by immunocompetent cells such as T cells, B cells, dendritic cells (DCS), and macrophages [14]. Overexpression of PD-L1 in tumors has been shown to inhibit T-cell activation and proliferation, leading to diminished immune responses and the impairment of protective immunity against cancer [15]. Expression of PD-L1 on cancer cells leads to evasion from the immune response, permitting cancer progression and metastasis [16]. Investigation of C-MYC as an immune regulator is a new avenue of research in tumor immunology. Sun et al. recently reported that GCMSCs (gastric cancer mesenchymal stem cell)-derived IL-8 induced PD-L1 expression in gastric cancer cells via the STAT3/ mTOR-C-MYC signaling pathways [17]. However, the immune regulatory functions of C-MYC in gastric adenocarcinoma and the association between C-MYC expression and PD-L1 expression have not been thoroughly investigated. We also investigated the function of the immune-related protein, FOXP3, which is a forkhead helix transcription factor that appears to function as a master regulator of the development and control of regulatory T cells (Tregs) [18]. FOXP3 is regarded as a specific and reliable surface marker of Tregs [19]. Tregs have been found in a number of human tumors and are considered a biomarker and prognostic factor for human malignant tumors. Recent studies have indicated that FOXP3 plays an important role in tumor development in addition to its association with Tregs function in the immune system [20,21]. The FOXP3 protein has dual functions as an immune-related protein and tumor suppressor in several malignancies [22]. Interestingly, FOXP3 is known to transcriptionally repress C-MYC in some cancers [23].

The aims of our research were to evaluate the clinical implication of C-MYC expression in gastric adenocarcinoma and determine its relationship with the expression of the immune regulatory molecules PD-L1 and FOXP3 and also with tumor microenvironment (tumor infiltrating lymphocytes and CD8 + T cells)

2. Materials and methods

2.1. Patients and tissue samples

Tissue samples were acquired from 182 cases of gastric adenocarcinoma that were surgically resected at Kyung Hee University Hospital at Gangdong from 2006 to 2012. All patients underwent surgery without neoadjuvant chemotherapy. For each case, two investigators (K.Y. Won and S.J. Lim) reviewed all the original hematoxylin and eosin-stained sections. Clinicopathological variables including age, sex, tumor type, histologic grade, tumor size, primary tumor (pT), nodal (pN) metastasis, recurrence, lymphatic invasion, vascular invasion, and neural invasion were evaluated. Mean follow-up duration was 72.5 months (range, 1–120 months). Among a total of 182 patients, 31 patients (16.9%) had died of the disease and 133 (72.7%) remained alive on the day when the study was initiated. Patient ages ranged from 34 to 93 years (median age, 63.5 years). This study was approved by the Institutional Review Board of Kyung Hee University Hospital at Gangdong (IRB 2016-07-006-001).

2.2. Tissue microarray (TMA) construction

Hematoxylin eosin-stained sections of formalin-fixed paraffin embedded tumor tissue blocks were screened to identify representative viable areas of gastric adenocarcinoma. Corresponding areas on the tissue block were then marked for tissue core punches. Tissue microarrays (TMA) were assembled using a commercially available manual tissue microarrayer (Quick-Ray; UNITMA Co., Ltd, Seoul, Korea). Briefly, three representative tumor cores with diameters of 2.0 mm were punched from each tumor tissue block, and each was arrayed into three recipient paraffin blocks, respectively. We arrayed three cores per case to increase the concordance rate between the TMA immunohistochemistry results and those of whole sections. Each of the tissue microarray blocks also contained four normal gastric tissue cores. H & E staining was performed for each block to verify tumor cell content. Cases with only stromal tissue or insufficient carcinoma tissue in the cores were excluded from the analysis. Serially sectioned slides were produced, and H & E staining was performed.

2.3. Immunohistochemical staining

Immunohistochemistry was performed on 4 μm tissue sections from each TMA block using the Bond Polymer Intense Detection system (Vision BioSystems, Victoria, Australia) according to the manufacturer's instructions with minor modifications. In brief, 4 μm sections of formalin-fixed, paraffin-embedded tissue were deparaffinized with Bond Dewax Solution (Vision BioSystems), and antigen retrieval was performed by treating the sections with Bond ER solution (Vision BioSystems) for 30 min at 100 °C. Endogenous peroxidases were quenched by incubating the tissue with hydrogen peroxide for 5 min. Sections were incubated for 15 min at ambient temperature with primary polyclonal antibodies to C-MYC (1:500, ab69987, Abcam, USA), PD-L1 (1:200, E1L3N, Cell Signaling, Danvers, USA), FOXP3 (1:100, PCH101, eBioscience, Cambridge, UK), and CD8 (1:50, 1A5, Novocastra, Newcastle, UK) using a biotin-free polymeric horseradish peroxidase-linker antibody conjugate system and a Bond-max automatic slide stainer (Vision BioSystems). Nuclei were counterstained with hematoxylin. The negative control was treated in an identical manner except mouse IgG was used instead of primary antibody.

2.4. Evaluation of immunohistochemical staining

C-MYC expression was observed in the nuclei of gastric adenocarcinoma cells (Fig. 1). C-MYC expression was interpreted by the authors (K.Y. Won and S.J. Lim) as positive (overexpressed) or negative (not overexpressed). C-MYC nuclear staining in greater than 50% of tumor cells was considered positive C-MYC staining. Faint staining (staining of less than 50% of cells) was considered negative C-MYC staining [24]. PD-L1 was expressed in the membrane of carcinoma cells (Fig. 2). PD-L1 expression was considered positive if ≥ 10% of viable cancer cells exhibited membrane staining of any intensity [25]. Tumoral FOXP3 expression was observed in the nuclei and cytoplasm of carcinoma cells (Fig. 3). Sections with nuclear or cytoplasmic staining of at least 20% of cells were considered positive for FOXP3 expression [26]. FOXP3 expression in Tregs appeared as nuclear staining (Fig. 3). The number of FOXP3-expressing Tregs in the tumoral epithelium and stroma was counted in three separate high power fields (HPF, ×400 magnification), and average scores were correlated with clinicopathological variables. We defined high expression of FOXP3 as ≥ 25 FOXP3-positive cells/HPF [27]. CD8 + T-cell density was determined by calculating the average amount of immunoreactive cells/HPF. To assess CD8 + T-cell infiltration in the specimens, positively stained cells were manually counted in 3 separate fields under HPF. The average of infiltrated CD8 + T cells was 45/HPF. Therefore, we defined high as ≥45/HPF and low as < 45/HPF. For the evaluation of tumor infiltrating lymphocytes (TIL), both areas of stroma infiltrated by lymphocytes (proportional score) and intensity of lymphatic infiltration (intensity score) were taken into consideration. Proportional scores were defined as 3, 2, 1, and 0 if the area of stroma with

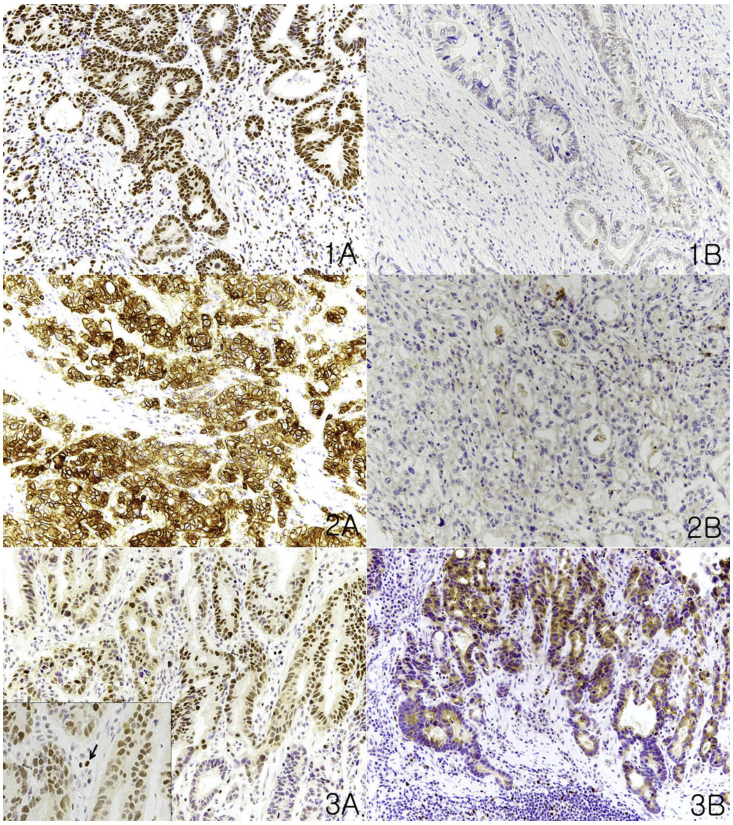

Fig. 1. (1A, 1B). Representative photographs of C-MYC expression in gastric adenocarcinomas.
A. Positive C-MYC expression is seen in the nuclei of gastric adenocarcinoma cells (original magnification, ×200). B. Another case of gastric adenocarcinoma was negative for C-MYC expression (original magnification, ×200).
(2A, 2B). Representative photographs of PD-L1 expression in gastric adenocarcinomas.
A. In this case, the membranes of gastric adenocarcinoma cells were positive for PD-L1 expression (original magnification, ×200). B. Example of a gastric adenocarcinoma negative for PD-L1 expression (original magnification, ×200).
(3A, 3B). Representative photographs of tumoral FOXP3 expression in gastric adenocarcinomas. FOXP3 expression was observed in the nuclei and cytoplasm of carcinoma cells. A. Positive FOXP3 expression was mainly seen in the nuclei of gastric adenocarcinoma cells (original magnification, ×200). B. Another case of gastric adenocarcinoma showed strong FOXP3 staining mainly in the cytoplasm of gastric adenocarcinoma cells. FOXP3 expression in Tregs appeared as nuclear staining. Scattered FOXP3-positive lymphoid cells (Tregs) were identified in the tumor stroma (inset black arrow, Fig. 3A) (original magnification, ×200).

Table 1
Correlation between tumoral FOXP3 expression, C-MYC expression and clinicopathological variables in 182 gastric adenocarcinomas.

		Tumoral FOXP3			C-MYC		
		negative	positive	p value	negative	positive	p value
Tumor size							
	≤ 3 cm	27 (42.9)	36 (57.1)	0.088	12 (19.0)	51 (81.0)	< 0.0001*
	> 3 cm	65 (54.6)	54 (45.4)		76 (63.9)	43 (36.1)	
Histologic type							
	mixed	15 (68.2)	7 (31.8)	0.061	6 (27.3)	16 (72.7)	0.029*
	tubular	77 (48.1)	83 (51.9)		82 (51.2)	78 (48.8)	
Histologic grade							
	well / moderately	43 (46.2)	50 (53.8)	0.166	43 (46.2)	50 (53.8)	0.36
	poorly	48 (54.5)	40 (45.5)		44 (50.0)	44 (50.0)	
Primary tumor category (T)							
	I / II	45 (44.6)	56 (55.4)	0.049*	30 (29.7)	71 (70.3)	< 0.0001*
	III / IV	47 (58.0)	34 (42.0)		58 (71.6)	23 (28.4)	
Lymph node metastasis (N)							
	Absent	41 (41.0)	59 (59.0)	0.003*	35 (35.0)	65 (65.0)	< 0.0001*
	Present	51 (62.2)	31 (37.8)		53 (64.6)	29 (35.4)	
Recurrence							
	Absent	60 (43.5)	78 (56.5)	< 0.0001*	54 (39.1)	84 (60.9)	< 0.0001*
	Present	27 (77.1)	8 (22.9)		27 (77.1)	8 (22.9)	
Lymphatic invasion							
	Absent	37 (41.1)	53 (58.9)	0.009*	30 (33.3)	60 (66.7)	< 0.0001*
	Present	55 (59.8)	37 (40.2)		58 (63.0)	34 (37.0)	
Vascular invasion							
	Absent	86 (50.6)	84 (49.4)	0.601	79 (46.5)	91 (53.5)	0.052
	Present	6 (50.0)	6 (50.0)		9 (75.0)	3 (25.0)	
Neural invasion							
	Absent	78 (48.4)	83 (51.6)	0.09	75 (46.6)	86 (53.4)	0.138
	Present	14 (66.7)	7 (33.3)		13 (61.9)	8 (38.1)	

NOTE. Values are n (%). *Significantly different by the chi-squared test.

lymphoplasmacytic infiltration around invasive tumor cell nests was > 50, > 10–50, or ≤10%, and absent, respectively. Intensity scores were defined as 2, 1, and 0, if the intensity of lymphatic infiltration was marked, mild, and absent, respectively. Lymphocyte infiltration surrounding non-invasive tumor cells was not taken into account. Proportional and intensity scores were summed for each tumor, and the TIL score was classified as high if the sum was 3–5, whereas the TIL score was classified as low if the sum was 0–2 [28].

Table 2
Correlation between PD-L1 expression, Tregs and clinicopathological variables in 182 gastric adenocarcinomas.

		PD-L1 negative	positive	p value	Tregs < 25/HPFs	≥ 25/HPFs	p value
Tumor size							
	≤ 3 cm	44 (78.6)	12 (21.4)	0.003*	35 (55.6)	28 (44.4)	0.274
	> 3 cm	66 (56.4)	51 (43.6)		73 (61.3)	46 (38.7)	
Histologic type							
	mixed	13 (65.0)	7 (35.0)	0.549	11 (50.0)	11 (50.0)	0.234
	tubular	97 (63.4)	56 (36.6)		97 (60.6)	63 (39.4)	
Histologic grade							
	well / moderately	62 (69.7)	27 (30.3)	0.073	54 (58.1)	39 (41.9)	0.382
	poorly	48 (57.8)	35 (42.2)		54 (61.4)	34 (38.6)	
Primary tumor category (T)							
	I / II	67 (71.3)	27 (28.7)	0.016*	58 (57.4)	43 (42.6)	0.332
	III / IV	43 (54.4)	36 (45.6)		50 (61.7)	31 (38.3)	
Lymph node metastasis (N)							
	Absent	63 (67.7)	30 (32.3)	0.143	56 (56.0)	44 (44.0)	0.195
	Present	47 (58.8)	33 (41.2)		52 (63.4)	30 (36.6)	
Recurrence							
	Absent	81 (62.3)	49 (37.7)	0.357	76 (55.1)	62 (44.9)	0.104
	Present	23 (67.6)	11 (32.4)		24 (68.6)	11 (31.4)	
Lymphatic invasion							
	Absent	55 (66.3)	28 (33.7)	0.293	47 (52.2)	43 (47.8)	0.037*
	Present	55 (61.1)	35 (38.9)		61 (66.3)	31 (33.7)	
Vascular invasion							
	Absent	104 (64.2)	58 (35.8)	0.366	99 (58.2)	71 (41.8)	0.203
	Present	6 (54.5)	5 (45.5)		9 (75.0)	3 (25.0)	
Neural invasion							
	Absent	93 (60.8)	60 (39.2)	0.026*	93 (57.8)	68 (42.2)	0.168
	Present	17 (85.0)	3 (15.0)		15 (71.4)	6 (28.6)	

NOTE. Values are n (%). *Significantly different by the chi-squared test.

Table 3
Correlations among Tumoral FOXP3, C-MYC, PD-L1, Tregs, CD8, and TIL in 182 gastric adenocarcinoma.

		Tumor FOXP3	C-MYC	PD-L1	Tregs	CD8	TIL
Tumor FOXP3 (positive vs negative)	Correlation coefficient		0.165	0.049	0.210	−0.056	−0.066
	Significance		0.026*	0.521	0.004*	0.474	0.375
C-MYC (positive vs negative)		0.165		−0.241	0.129	0.031	0.133
		0.026*		0.001*	0.082	0.687	0.074
PD-L1 (positive vs negative)		0.049	−0.241		0.193	0.280	0.217
		0.521	0.001*		0.011*	0.000*	0.004*
Tregs (< 25/HPF ≥25/HPF)		0.210	0.129	0.193		0.171	0.152
		0.004*	0.082	0.011*		0.027*	0.040*
CD8 (< 45/HPF ≥45/HPF)		−0.056	0.031	0.280	0.171		0.266
		0.474	0.687	0.000*	0.027*		0.001*
TIL (high vs low)		−0.066	0.133	0.217	0.152	0.266	
		0.375	0.074	0.004*	0.040*	0.001*	

NOTE. *Significantly different by the Spearman correlation analysis, TIL: tumor infiltrating lymphocytes.

All slides were evaluated independently by two investigators without knowledge of patient identity or clinical outcome. Negative controls were obtained by omitting the primary antibody.

2.5. Statistical analysis

Pearson's chi-square test was used to evaluate the associations between C-MYC expression and tumoral FOXP3 and PD-L1 expression, as well as the number of Tregs and several clinicopathological variables. Spearman correlation analysis was used to study the interrelationship between C-MYC, tumoral FOXP3, PD-L1, Treg count, CD8 + T cells, and TIL. The Kaplan-Meier method was used to determine the probability of disease-free and overall survival, and data were analyzed using the log-rank test. Multivariate analysis was performed using the Cox regression model to study the relationship of different variables to overall survival. Overall survival was defined as survival from the date of surgery to the date of death due to cancer. A p-value < 0.05 was considered significant. Statistical analyses were performed using the SPSS software package (version 15.0; SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Relationships between C-MYC expression and tumoral FOXP3 expression, as well as other clinicopathological variables (Table 1)

Ninety-four gastric adenocarcinomas (51.6%) were positive for C-MYC expression, and 49.2% (90/182) of gastric adenocarcinomas were positive for FOXP3 expression. Most normal gastric mucosal epithelial cells were positive for C-MYC expression but negative for FOXP3 expression. As shown in Table 1, positive C-MYC expression was significantly correlated with smaller tumor size, a lower T category, a lower N category, a lower recurrence rate, and less lymphatic invasion. Positive tumoral FOXP3 expression was significantly related with a lower T category, lower N category, lower recurrence rate, and less lymphatic invasion.

Table 4
Univariate analysis of clinicopathological variables for overall survival rate in 182 gastric adenocarcinomas.

Variables	Disease-free survival (P value)	Overall survival (P value)
Tumor size (< 3.0 cm vs. ≥ 3.0 cm)	< 0.0001*	0.020*
Tumor type (tubular vs. mixed)	0.836	0.890
Histologic grade (well to mod vs. poor)	0.091	0.420
Primary tumor (T) (I,II vs. III,IV)	< 0.0001*	< 0.0001*
Lymph node metastasis	< 0.0001*	< 0.0001*
Recurrence	N.A	< 0.0001*
Lymphatic invasion	< 0.0001*	< 0.0001*
Vascular invasion	0.070	0.218
Neural invasion	0.053	0.296
Tumoral FOXP3 expression	0.001*	0.009*
C-MYC expression	< 0.0001*	< 0.0001*
PD-L1 expression	0.602	0.772
Tregs count (< 25/HPFs vs. ≥ 25/HPFs)	0.151	0.139

* Statistically significant, N.A: not applicable.

3.2. Relationships between PD-L1 expression and Tregs, as well as other clinicopathological variables (Table 2)

Sixty-three gastric adenocarcinomas (36.4%) expressed PD-L1. Infiltrating FOXP3-expressing Tregs (≥25 FOXP3 positive cells/HPF) were observed in 40.4% (74/182) of cases. Normal gastric mucosal epithelial cells were negative for PD-L1 expression. Positive PD-L1 expression was significantly related to larger tumor size and a higher T category and inversely related to less neural invasion. Number of infiltrating FOXP3-expressing Tregs (≥25 FOXP3 positive cells/HPF) was significantly related to reduced lymphatic invasion.

3.3. Interrelationships between C-MYC expression, tumoral FOXP3 expression, PD-L1 expression, CD8 + T cells, Tumor infiltrating lymphocytes (TIL), and Tregs in gastric adenocarcinoma (Table 3)

C-MYC overexpression was negatively correlated with PD-L1 expression. Tumoral FOXP3 was positively correlated with C-MYC overexpression and Tregs count. PD-L1 expression was positively correlated with Tregs, CD8 + T cells, and tumor infiltrating lymphocytes (TIL). Tregs count was positively correlated with CD8 + T cells and TIL. CD8 + T cells were positively correlated with TIL.

3.4. Overall and disease-free survival rates according to C-MYC, tumoral FOXP3, and PD-L1 expression and Tregs in gastric adenocarcinoma patients (Table 4 Fig. 4)

Adequate clinical follow-up information was available for all 182 patients. As shown in Table 4, univariate analyses revealed an association between overall survival and C-MYC expression ($p < 0.0001$), tumoral FOXP3 expression ($p = 0.009$), larger tumor size ($p = 0.020$), higher primary tumor category ($p < 0.0001$), lymph node metastasis ($p < 0.0001$), recurrence ($p < 0.0001$), and lymphatic invasion ($p < 0.0001$). Univariate analyses also revealed an association between disease-free survival and C-MYC expression ($p < 0.0001$), tumoral FOXP3 expression ($p = 0.001$), larger tumor size ($p < 0.0001$), higher primary tumor category ($p < 0.0001$), lymph node metastasis ($p < 0.0001$), and lymphatic invasion ($p < 0.0001$). Cox multivariate analysis showed that recurrence ($p < 0.0001$) was an independent prognostic factor.

4. Discussion

In this study, through clinicopathological analysis, we observed that positive C-MYC expression was significantly correlated with smaller tumor size, a lower T category, a lower N category, a lower recurrence rate, and less lymphatic invasion. In addition, C-MYC overexpression in gastric adenocarcinoma patients was associated with better disease-free survival and overall survival than lack of expression of C-MYC. These findings suggest that C-MYC overexpression in gastric adenocarcinoma patients is a good prognostic factor.

To determine why C-MYC overexpression is a good prognostic factor in gastric adenocarcinoma, we investigated the association between C-MYC expression and that of the immune regulatory proteins PD-L1 and FOXP3. We found that C-MYC overexpression was correlated with decreased PD-L1 expression and increased tumoral FOXP3 expression in gastric adenocarcinoma. In a previous study, we showed that FOXP3 has a tumor suppressor function in gastric adenocarcinoma [22]. In the current study, tumoral FOXP3 expression was significantly positively correlated with C-MYC expression and C-MYC expression was inversely correlated with PD-L1 expression. C-MYC functions as a transcription factor to regulate the expression of a multitude of gene products involved in cell proliferation, growth, differentiation, and apoptosis [29,30]. In addition to these classical functions, C-MYC regulates host immune-mediated mechanisms. In particular, C-MYC appears to initiate and maintain tumorigenesis in part through regulation of the expression of the immune regulatory molecules CD47 and PD-L1 [13]. Recently, Sun et al. found that GCMSC (gastric cancer mesenchymal stem

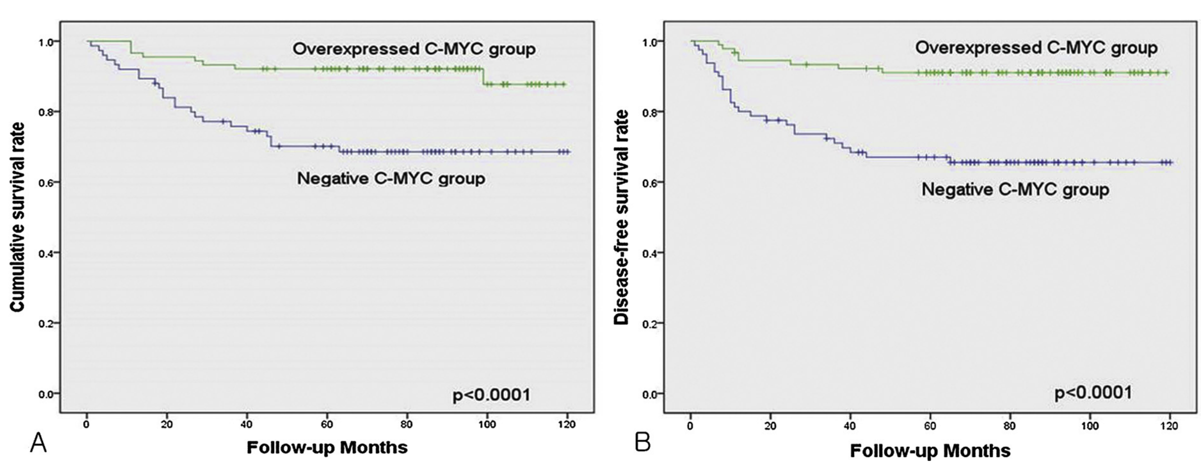

Fig. 4. (A, B). Analysis of overall survival and disease-free survival rates according to tumoral C-MYC expression in patients with gastric adenocarcinoma.
A. The C-MYC-positive group ($n = 94$) showed a significantly higher cumulative survival rate than the C-MYC-negative group ($n = 88$) ($p < 0.0001$) B. The C-MYC-positive group ($n = 94$) showed a significantly higher disease-free survival rate than the C-MYC-negative group ($n = 88$) ($p < 0.0001$).

cells) could up-regulate C-MYC and PD-L1. At the same time, PD-L1 level decreased after the inhibition of C-MYC. So, they thought that C-MYC played an important role in GCMSC-mediated PD-L1 up-regulation in gastric cancer cells [17]. These findings are different from our present results. We found that C-MYC overexpression was inversely correlated with PD-L1 expression in gastric adenocarcinoma. The mechanisms of PD-L1 regulation were multifaceted in a highly complex manner. So, the discrepancy between PD-L1 and C-MYC expression in whole tissue samples containing many microenvironmental elements compared to each cell line remain. Further, there are only a few studies about the relationship between C-MYC and PD-L1 expression, which needs further study.

Based on our present results, we suggest that C-MYC overexpression is significantly related to decreased PD-L1 expression and an increase in tumoral FOXP3 expression in gastric adenocarcinoma, thereby regulating complex immune reactions in the tumor microenvironment. This mechanism explains why C-MYC overexpression in gastric adenocarcinoma is a favorable clinicopathological factor.

Additionally, we discovered a relationship between infiltrated Tregs in the tumor stroma and C-MYC, PD-L1, and FOXP3 expression in gastric adenocarcinoma cells. PD-L1 expression was positively correlated with the number of infiltrating Tregs. FOXP3-expressing regulatory T cells (Tregs), which suppress aberrant immune responses against self-antigens, also suppress anti-tumor immune responses. Thus, infiltration of a large number of Treg cells into tumor tissues is often associated with a poor prognosis due to decreased anti-tumor immunity [31]. Overall, C-MYC overexpression may suppress the expression of PD-L1, and reduced PD-L1 expression could suppress the infiltration of Tregs into the tumor stroma in gastric adenocarcinoma. Que et al. reported that PD-L1 and FOXP3+ Tregs may work synergistically in promoting immune evasion by soft tissue sarcomas. PD-L1 was found to up-regulate FOXP3 expression and modulate signaling molecules critical for conversion of naïve T cells to Tregs. These authors reported that the combined strategy of blocking PD-L1/PD-1 and simultaneously depleting of Tregs may enhance therapeutic efficacy [32]. Similar to the study of Que et al., we observed that PD-L1 expression was positively correlated with the number of infiltrating Tregs. Decreased expression of PD-L1 and a decrease in number of infiltrating Tregs may have resulted in synergistic anti-tumor activity in patients with gastric adenocarcinoma.

We studied the relationship between microenvironmental elements (tumor infiltrating lymphocytes and especially CD8 + T cells) and C-MYC and PD-L1, as well as Tregs. As a result, we found that PD-L1 expression was significantly associated with increased TIL and CD8 + T cells. C-MYC and tumoral FOXP3 expression showed no correlation with TIL and CD8 + T cells. IFN-γ secreted by CD8 + T cells also rapidly induced PD-L1 upregulation in co-cultured hepatocellular carcinoma cell lines. These data indicate that specific T lymphocyte infiltration in tumors can produce factors driving PD-L1 expression as a negative feedback mechanism, leading to what could be considered an adaptive immune resistance mechanism by the tumor [33]. Our study also showed that increased CD8 + T cells and TIL were positively correlated with tumor PD-L1 expression. These findings indicate that cytokines induced from elements in the tumor microenvironment influence the expression of PD-L1 expression in tumor cells. Based on these findings, PD-L1 expression seems to be influenced by many tumor microenvironmental factors.

Taken together, we suggest a microenvironmental immune regulatory function for C-MYC via its effects on PD-L1 and FOXP3 expression in gastric adenocarcinoma. Especially, we discovered that the immune regulatory effect of C-MYC and PD-L1 and tumor suppressor function of tumoral FOXP3 had a significant influence on the tumor microenvironment (Tregs, CD8 + T cells, and other tumor infiltrating lymphocytes) in complex manner. And the C-MYC overexpression is a good prognostic factor in gastric adenocarcinoma, likely due to its regulation of the microenvironmental immune system. The C-MYC-associated changes in expression of PD-L1 and FOXP3 in gastric adenocarcinoma reported here may form the basis for new therapeutic strategies.

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