Showa Univ J Med Sci **25**(2), 133～142, June 2013

Original

Clinicopathological Significance of FOXP3 Expression in Esophageal Squamous Cell Carcinoma

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Abstract: The expression of transcription factor forkhead box protein 3 (FOXP3), a master control gene for regulatory T cells, has been reported to influence patient survival. However, there have been few reports of the relationship between FOXP3 positive cells and esophageal squamous cell carcinoma (ESCC). The aim of this study was to clarify the prognostic value of FOXP3 expression in ESCC. Ninety-five patients who were diagnosed with primary ESCC and underwent subtotal esophagectomy during 2009 and 2010 were retrospectively analyzed. Deepest sections from each tumor were selected for immunohistochemistry and the number of FOXP3 positive cells was counted. The median number was used as a cutoff to divide into FOXP3 positive and FOXP3 negative subgroups. Relationships between FOXP3 expression and clinicopathological features, disease-free survival (DFS) and overall survival (OS) were determined. Statistical values of $p < 0.05$ were considered significant. FOXP3 positive cells were found in all 95 cases and the number of FOXP3 positive cells was significantly higher in the peri-tumor compartment than in the intra-tumor compartment ($p = 0.0006$). For this reason, the peri-tumor compartment numbers were used for all of the association studies. Results showed that the FOXP3 positive group had a significantly larger mean tumor size (43.8 ± 4.1 mm vs 29.1 ± 4.0 mm, $p = 0.0055$), and the FOXP3 negative group had a significantly higher percentage of deep invasion (T2, T3, T4) ($p = 0.0399$). There was no significant association for DFS, however, for OS the FOXP3 positive group demonstrated a significantly better prognosis ($p = 0.0024$). Multivariate analysis showed that peri-tumor FOXP3 expression is an independent prognostic factor for OS ($p = 0.0035$). Peri-tumoral FOXP3 expression is an independent and favorable prognostic factor for ESCC.

Key words: FOXP3, esophageal cancer, squamous cell carcinoma, immunohistochemistry, prognostic factor

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Introduction

Tumor-infiltrating lymphocytes (TILs) may play an important role in the host immune response to cancer in the tumor microenvironment. Many studies have shown a clear association between TILs and patient survival in several types of human cancers. Regulatory T cells, in particular, have been reported to inhibit the antitumor response in mice[1,2] and the depletion of regulatory T cells can result in an effective antitumor response[3,4]. Additional studies report that the regulatory T cell population is increased in peripheral blood and tumor tissues in patients with different types of human cancer[5-8].

In 2003, the transcription factor forkhead box protein 3 (FOXP3) was reported to be a master control gene for thymically derived naturally occurring regulatory T cells[9]. Since then, many studies have shown an association between a high density of tumor-infiltrating FOXP3 positive cells and poor prognosis in various human cancers. These include ovarian[7], pancreatic[10], lung[11], and hepatocellular carcinomas[12]. In contrast, other studies have found that a high density of tumor-infiltrating FOXP3 positive cells may be associated with improved patient survival in colorectal cancer[13].

There have been few reports describing the relationship between tumor-infiltrating FOXP3 positive cells and esophageal squamous cell carcinoma (ESCC) and the issue remains controversial. Yoshioka *et al* suggested that the number of tumor-infiltrating FOXP3 positive regulatory T cells is not a predictive factor for patient survival in ESCC[14], whereas Xue *et al* reported that the expression of FOXP3 in ESCC closely correlates to lymphatic invasion and pathological stage. They concluded that FOXP3 expression might play an important role in cancer progression[15]. The aim of this study was to clarify the prognostic value of FOXP3 expression in ESCC.

Methods

Patients and specimens

A series of 100 consecutive unselected patients who were diagnosed with primary ESCC and underwent video-assisted thoracic subtotal esophagectomy during 2009 and 2010 at the Department of Surgery, Division of General and Gastroenterological Surgery, Showa University School of Medicine, were retrospectively analyzed. Five cases that underwent subtotal esophagectomy after endoscopic submucosal dissection (ESD) were excluded from the current study. All of the surgically obtained tumor tissues were stained using hematoxylin-eosin and the histopathologic classification and grade were determined according to the Japanese Classification of Esophageal Cancer (10th edition, April 2008, The Japan Esophageal Society). The clinicopathological data for each patient was recorded and filed at the Department of Pathology, Showa University School of Medicine.

Follow-Up

The most recent collection of data to determine disease-free survival (DFS) and overall survival (OS) was performed on July 31, 2012. DFS was defined as the interval between the date

of surgery and recurrence or most recent follow-up. OS was defined as the interval between the date of surgery and death. The diagnosis of recurrence was based on an elevated serum SCC and typical imaging appearance. Patients with confirmed recurrence received further treatment, such as surgery, radiotherapy, chemotherapy (CT) or chemoradiotherapy (CRT).

Immunohistochemistry

For immunohistochemistry analysis, one of the deepest sections from each tumor was selected for evaluation. Formalin-fixed and paraffin-embedded specimens were deparaffinised and dehydrated. Monoclonal FOXP3 antibody (clone 236A/E7; Abcam, Cambridge, UK; dilution 1 : 100) was used. Staining was performed using a fully automated Ventana Benchmark XT slide stainer (Ventana Medical Systems, Inc., Tucson, Arizona, USA).

Evaluation of FOXP3 positive cells

The number of FOXP3 positive cells was counted in each specimen using an optical microscope (Olympus Optical Co. Ltd., Tokyo, Japan). Two scorers were blinded to the clinical information and the mean number of FOXP3 positive cells was determined for each slide. Cells were counted in two different compartments in each tumor: within the intra-tumor compartment, defined as tumor cell nests; and within the peri-tumor compartment, defined as the stromal region surrounding the tumor cell nests within a distance of 5 mm. In each compartment, FOXP3 positive cells were counted at a magnification of 400 ×. The median number of FOXP3 positive cells in each compartment was used as a cutoff to divide into FOXP3 positive group and FOXP3 negative subgroups.

Statistical analysis

Statistical analysis using the Student's t test or the Mann-Whitney U test was performed where appropriate. The Kaplan-Meier method was used to estimate OS and DFS, and differences were compared using the log-rank test. Multivariate analysis of prognostic factors was performed using the Cox proportional hazard model. Values of $p < 0.05$ were considered statistically significant.

Results

Characteristics

Patient characteristics are shown in Table 1. The study population consisted of 80 males and 15 females, and the mean age was 64.6 years (range 41 to 90 years). There were 60 cases that received CT using either 5-fluorouracil and Nedaplatin alone, or CRT before the operation. Fifteen patients had not received any induction therapy.

Immunohistochemistry staining

Examples of immunohistochemistry staining are shown in Fig. 1. We found FOXP3 positive cells in all cases. As a control, we also examined a distant compartment. This was defined as a

Table 1. Patient characteristics and clinicopathological parameters

Characteristics	n = 95
Age	
Mean value ± SD	64.6 ± 0.9
Range	4.1–9.0
Gender	
Male	80
Female	15
Tumor size (mm)	
Mean value ± SD	36.5 ± 2.9
Range	0–165
Tumor differentiation	
Well	25
Moderately	55
Poorly	15
Induction therapy	
Present	60
Absent	35
Lymphatic invasion	
Present	43
Absent	52
Vascular invasion	
Present	49
Absent	46
T classification	
T0	6
T1a	9
T1b	33
T2	10
T3	32
T4	5
N classification	
N0	48
N1	11
N2	27
N3	8
N4	1
Pathological stage	
0	15
I	20
II	21
III	34
IV	5

SD, standard deviation

normal epithelial stromal compartment sufficiently distant from the tumor nests. Both intra- and peri-tumor compartments expressed significantly higher numbers of FOXP3 positive cells than the distant compartments ($p < 0.0001$) (Table 2), and the number of FOXP3 positive cells in the peri-tumor compartment was significantly higher than in the intra-tumor compartment ($p = 0.0002$).

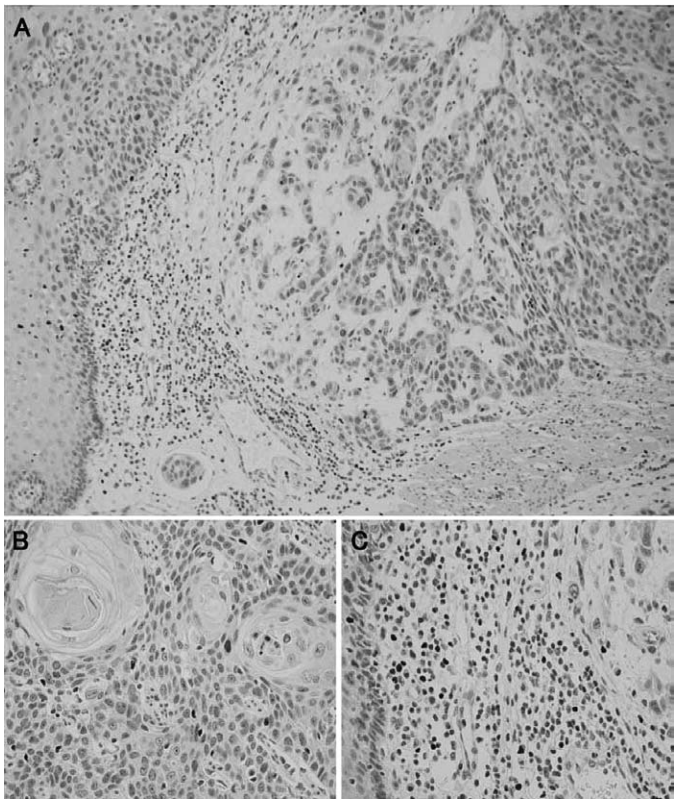

Fig. 1. Representative immunohistochemical staining
A) Tumor nests (intra-tumor compartment) and surrounding stroma (peri-tumor compartment) (40 × magnification).
B) FOXP3 positive cells in the intra-tumor compartment (400 × magnification).
C) FOXP3 positive cells in the peri-tumor compartment (400 × magnification).

Table 2. The number of FOXP3 positive cells counted in each compartment. P-value is estimated by the Wilcoxon matched-pairs signed-rank test.

Compartment	Range	Mean ± SD	Median	p-value
Intra-tumor	1-233	63.6 ± 5.1	50	
Peri-tumor	5-413	88.9 ± 7.6	70	0.0002
Distant stroma	0-161	27.4 ± 2.5	20	< 0.0001

SD ; standard deviation

Relationship between FOXP3 expression and patient characteristics

The relationships between FOXP3 expression and the clinicopathological characteristics of the patients are shown in Table 3. In the peri-tumor compartment, the FOXP3 positive group had a significantly larger mean tumor size (43.8 ± 4.1 mm) than the FOXP3 negative group ($p = 0.0055$), whereas there was no significant association between tumor size and FOXP3 subgroup in the intra-tumor compartment. Also, in the peri-tumor compartment, the FOXP3 negative group had a high percentage of deep invasive tumors (T2, T3, T4) compared to the FOXP3

Table 3. Relationship between clinicopathological features and FOXP3 expression in intra- and peri-tumor compartments

Characteristics	Intra-tumor FOXP3		p-value	Peri-tumor FOXP3		p-value
	Negative (n = 48)	Positive (n = 47)		Negative (n = 49)	Positive (n = 46)	
Age	64.8 ± 1.4	64.5 ± 1.4	0.7459	65.8 ± 1.3	64.2 ± 1.4	0.6955
Gender						
Male	40	40	0.8126	43	37	0.3273
Female	8	7		6	9	
Tumor size (mm)	34.6±4.0	38.5±4.3	0.551	29.7±4.0	43.8±4.1	< 0.01
Tumor differentiation						
Well	16	9	0.1314	12	13	0.4355
Moderately	23	32		27	28	
Poorly	9	6		10	5	
Induction therapy						
Present	32	28	0.4735	34	26	0.1934
Absent	16	19		15	20	
Lymphatic invasion						
Present	24	19	0.3482	21	22	0.6267
Absent	24	28		28	24	
Vascular invasion						
Present	27	22	0.3569	24	25	0.6007
Absent	21	25		25	21	
T classification						
T0, T1a, T1b	23	25	0.6071	20	28	< 0.05
T2, T3, T4	25	22		29	18	
N classification						
N0	29	19	0.0506	25	23	0.9208
N1, N2, N3, N4	19	28		24	23	
Stage						
0, Ⅰ, Ⅱ	29	27	0.7686	27	29	0.4312
Ⅲ, Ⅳ	19	20		22	17	

positive group (p = 0.0399). Other clinicopathological parameters such as patient's age, sex, tumor differentiation, lymphatic and vascular invasion, lymph node metastasis and pathological stage showed no significant differences between subgroups in both the intra- and peri-tumor compartments.

Disease-free survival and overall survival analysis

Fig. 2 shows DFS for both FOXP3 expression subgroups. There were no significant differences in DFS in either compartment. In contrast, Fig. 3 shows that in OS, the FOXP3 positive group had a significantly better prognosis compared to the FOXP3 negative group (p = 0.0024).

Multivariate analysis

Table 4 shows the multivariate analysis which indicates that only peri-tumor FOXP3 expression is an independent prognostic factor for OS (p = 0.0035). Other factors, such as age, gender,

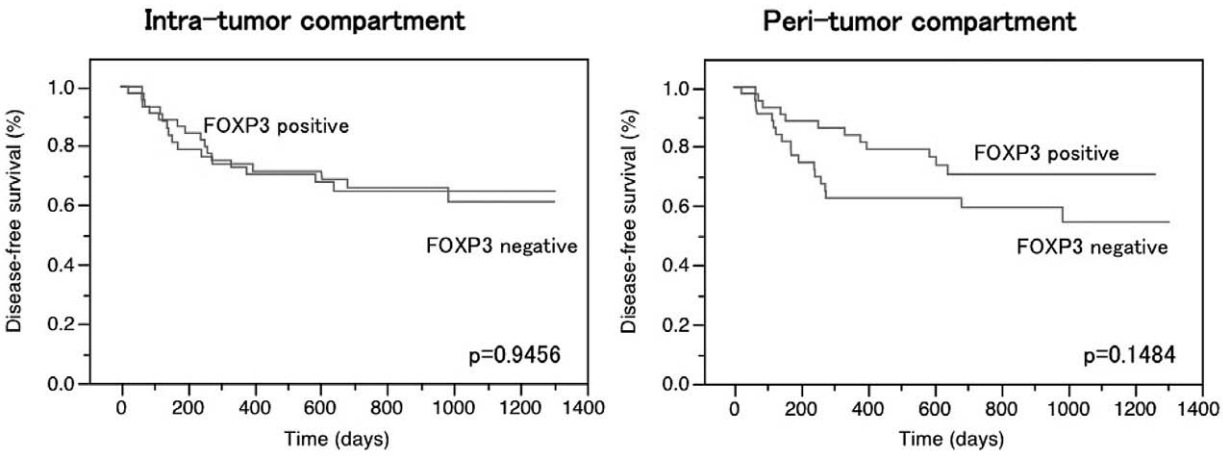

Fig. 2. Disease-free survival (DFS) rates for intra- and peri-tumoral FOXP3 expression were estimated by the Kaplan-Meier method. The log-rank test was applied to compare between groups.

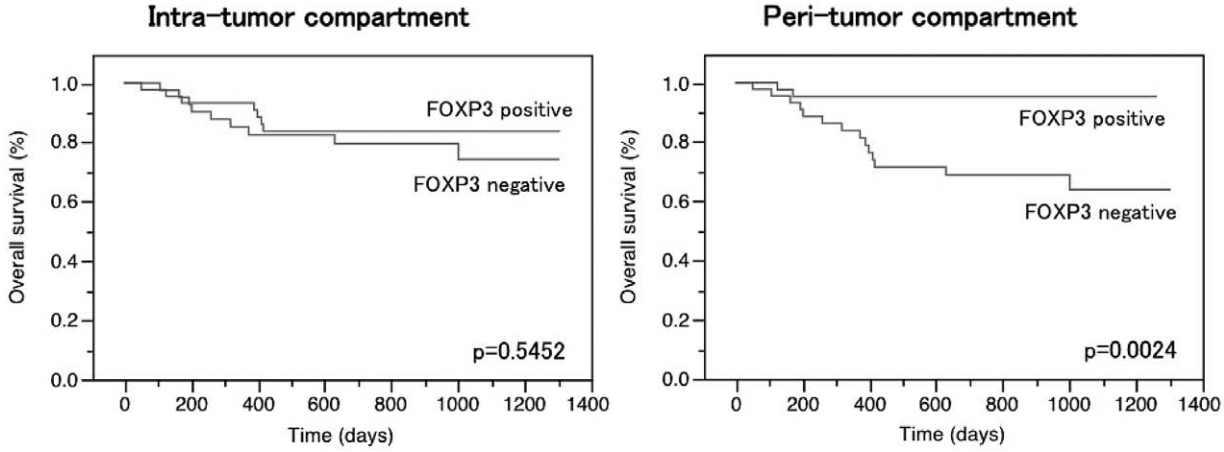

Fig. 3. Overall survival (OS) rates for intra- and peri-tumoral FOXP3 expression were estimated by the Kaplan-Meier method. The log-rank test was applied to compare between groups.

tumor size, depth of invasion, lymphatic and vascular invasion, lymph node metastasis, pathological stage, presence or absence of induction therapy, and intra-tumor FOXP3 expression had no significant association with prognosis.

Discussion

There are many studies evaluating FOXP3 expression by immunohistochemistry in different tissue compartments. Sahar *et al* evaluated FOXP3 expression in intra-tumor, peri-tumor and distant stromal compartments in human breast cancer, and reported that FOXP3 positive cells were seen more often in distant stromal compartments[16]. Another study evaluated FOXP3 expression in intra-tumor and peri-tumor regions in hepatocellular carcinoma and reported that the number of FOXP3 positive T cells in the intra-tumor region was significantly higher than that in the peri-tumor region[17]. In contrast, our study showed a significantly higher number of FOXP posi-

Table 4. Multivariate analyses and clinicopathological features

Variables	Hazard ratio	95% CI	p-value
Age (≧65 vs < 65) (years)	0.333	0.088-1.047	0.0603
Gender (male vs female)	0.884	0.195-6.198	0.8835
Tumor size (≧30 mm vs < 30 mm)	1.424	0.453-4.586	0.5438
Induction therapy (present vs absent)	1.050	0.215-5.729	0.9514
Lymphatic invasion (present vs absent)	1.057	0.243-5.000	0.9416
Vascular invasion (present vs absent)	0.938	0.292-3.101	0.9141
Depth of invasion (T0/T1a/T1b vs T2/T3/T4)	0.702	0.083-13.237	0.7735
Lymph node metastasis (present vs absent)	0.632	0.070-5.715	0.6903
Pathological stage (0/I/II vs III/IV)	0.297	0.010-4.740	0.4115
Intra-tumor FOXP3 (negative vs positive)	0.790	0.244-2.542	0.6889
Peri-tumor FOXP3 (negative vs positive)	7.991	1.877-56.516	0.0035

CI, confdence interval.

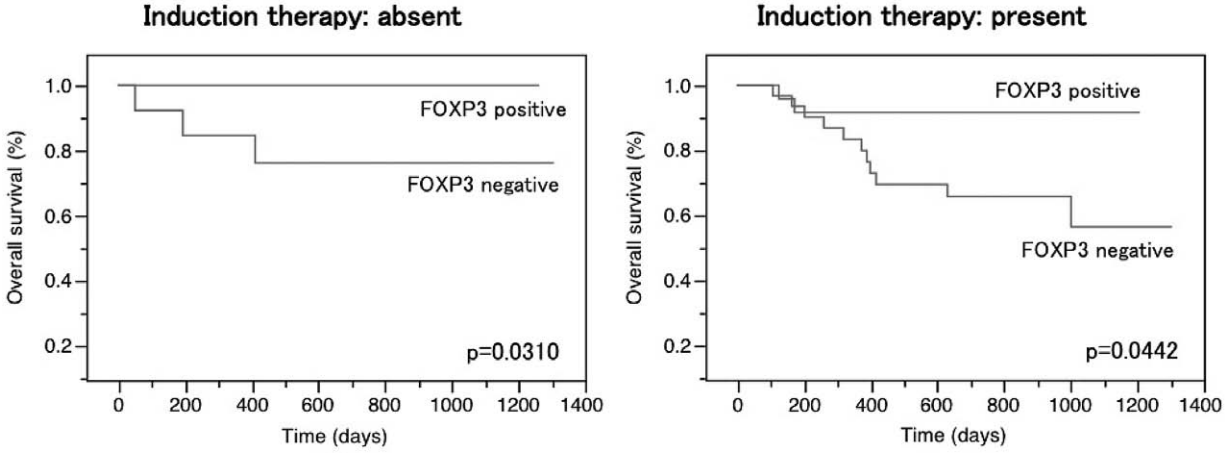

Fig. 4. The FOXP3 positive group had a significantly better prognosis regardless of the absence or presence of induction therapy.

tive cells in the peri-tumor compartment. This difference may result from differences in methods and evaluation strategies. Previously, only one study has evaluated FOXP3 expression in fresh esophageal squamous cell carcinoma tissue[15], however that study did not discriminate between compartments. Our study is the first study to evaluate FOXP3 expression in different tumor microenvironments in ESCC and found higher numbers of FOXP3 positive cells in the peri-tumor compartment.

Previous studies indicate that FOXP3 expression is significantly decreased after chemotherapy[18]. Our study includes 60 patients who underwent either CT or CRT induction therapy. Our study also showed significantly lower FOXP3 positive expression in the induction therapy group, supporting the previous study. Our additional analysis indicates that prognosis for the FOXP3 positive group was significantly better than for the FOXP3 negative group regardless of the absence or presence of induction therapy (Fig. 4). However, the mechanism of how induc-

tion therapy affects the expression of FOXP3 is still unclear and further study is necessary.

We also analyzed how differences in induction therapy, such as CT or CRT, affected FOXP3 expression. There were 36 cases of CT, 21 cases of CRT and 3 cases in which the therapy regimen was unknown. Comparing CT and CRT, the CT group had a significantly higher percentage of FOXP3 positive cases and CRT had significantly higher percentage of FOXP3 negative cases in both the intra-tumor ($p = 0.0008$) and peri-tumor ($p = 0.0003$) compartments. However, the differences between CT or CRT did not affect DFS ($p = 0.3570$) or OS ($p = 0.1322$). This result may indicate that CRT has a greater regulatory effect on FOXP3 expression than CT, but still further study is necessary.

With respect to the relationships between FOXP3 expression and clinicopathological characteristics, our study showed significance in tumor size and the depth of invasion (Table 3). In the peri-tumor compartment, the FOXP3 negative group had a high percentage of deep invasive (T2, T3, T4) tumors and poorer prognosis in OS, but contrary to our expectations, it had a smaller mean tumor size. We speculate that the reason for this discrepancy is that we did not exclude some cases of so-called superficial spread-type ESCC in this study, and this is reflected in the tumor size.

The log-rank test revealed that ESCC patients with high numbers of FOXP3 positive T cells in the peri-tumor compartment had a better prognosis. A previous study showed a similar result, but multivariate analysis showed no significance[14]. In contrast, our multivariate analysis indicates that the density of peri-tumoral FOXP3 positive T cells may be an independent prognostic factor for OS in ESCC. Our literature search indicates that this is the only study to report FOXP3 expression as an improved and independent prognostic factor for esophageal squamous cell carcinoma.

Although further study is necessary, FOXP3 expression can be used as an independent prognostic factor for ESCC; high numbers of FOXP3 positive T cells in the peri-tumor compartment predict a better prognosis.

Acknowledgements

The authors gratefully acknowledge the Showa University Research Grant for Young Researchers which supported this study.

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[Received January 11, 2013 : Accepted february 5, 2013]