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Investigations on the clinical significance of FOXP3 protein expression in cervical oesophageal cancer and the number of FOXP3+ tumour-infiltrating lymphocytes

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Abstract

Objectives: Investigations of FOXP3 protein expression in cervical oesophageal cancer cells and the number of FOXP3 + lymphocytes infiltrating tumour tissue were undertaken.

Methods: FOXP3 protein expression and FOXP3 + tumour-infiltrating lymphocytes were studied immunohistochemically, in cervical oesophageal cancer tissue samples from 42 cases and paracancerous tissue samples from 30 of these cases.

Results: The percentage of parenchymal cells expressing FOXP3 protein was significantly higher in cancer tissue (42.9%, 18/42) than in paracancerous tissue 6.67% (two of 30). FOXP3 + lymphocyte infiltration was significantly more frequent in cancer (38.1%, 16/42) than in paracancerous (13.33%, four of 30) tissue. FOXP3 protein expression in cancer parenchymal cells in patients with lymph node metastasis was significantly greater than expression in those without lymph node metastasis. FOXP3 protein expression was significantly higher in cancer tissue samples from clinical stage III or IV than those from stage I or II disease. FOXP3 + lymphocyte infiltration of tumours was significantly greater in patients with lymph node metastasis than in those without metastasis.

Conclusions: Abnormal FOXP3 expression in cervical oesophageal cancer parenchyma and FOXP3 + lymphocyte infiltration might be closely related to metastasis of this cancer by promoting immune escape of the tumour. FOXP3 might be a potential marker for the assessment of postoperative metastasis in cervical oesophageal cancer.

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Introduction

The annual incidence rate of cervical oesophageal cancer in China is 0.35/100 000, accounting for 5.9–10.0% of the total number of cases of oesophageal cancer. As the location of disease is hidden and diagnosis is difficult, the prognosis for patients with cervical oesophageal cancer is usually poor. Studies have shown that the 3- and 5-year survival rates of cervical oesophageal cancer after comprehensive treatment were 56.9 and 46.3%, respectively, and rates for cases treated with surgery only were as low as 34.5 and 21.5%, respectively.[1–3] Forkhead box P3 (FOXP3) is a member of the forkhead transcription factor family.[4,5] It was initially found to be specifically expressed in CD4 + CD25 + regulatory T (Treg) cells, regulating the development and immunosuppressive effects of these cells. Thus, FOXP3 plays an important role in tumour immune escape.[6–8] Studies have found that some tumour cells expressing FOXP3 at a high level have immunosuppressive effects similar to those of Treg cells; through such an effect or other, unknown mechanisms, the immune killing of tumour cells (such as pancreatic cancer cells and breast cancer cells) may be directly or indirectly inhibited, resulting in escape from immune surveillance.[9–11] These findings have provided new directions for studying of the mechanism of tumour immune escape. However, there have been few studies on squamous cell carcinoma, especially of the head and neck.[12]

In the present study, immunohistochemical methods were used to observe FOXP3 expression levels in cancer cells and lymphocytes from cervical oesophageal cancer tissue samples. The relationship between FOXP3 expression and clinical and pathological features of cervical oesophageal cancer was analysed, to investigate the biological significance of FOXP3 expression in the immune resistance of tumour cells in this cancer.

Materials and methods

Specimens

Dissected specimens were collected from 42 patients with cervical oesophageal cancer: 31 males and 11 females, aged 40–71 (median age, 59.5 years). The patients had been treated in Shandong Provincial Hospital, Jinan, China between January 2007 and December 2011. Verbal consent for the use of specimens in this study was obtained from the patients or their carers. Additionally, morphologically normal paracancerous tissues (taken from areas >2 cm from the tumour junction, ~5 cm from tumour tissue) were sampled from 30 of the 42 cases, to provide a normal control group of specimens.[13,14] All specimens were obtained during the patient's operation and were fixed in precooled 10% neutral formalin for immunohistochemical study.

None of the patients had previously undergone chemotherapy or biological therapy (defined as immunotherapy, biotherapy or biological response-modifier therapy) for their oesophageal cancer or for any other condition, and surgery was the first-line treatment for their oesophageal cancer. Preoperative radiotherapy, chemotherapy or immunotherapy were not performed. Routine imaging examinations were carried

out. Postoperative pathological diagnosis indicated squamous cell carcinoma in all cases. Clinical staging and classification were performed according to the 2009 tumour–node–metastasis (TNM) staging standard for oesophageal cancer, specified by the International Union Against Cancer.[15] For immunohistochemical analysis (see below) tissue specimens were fixed in 10% formalin solution, embedded in paraffin wax, sectioned, stained with haematoxylin and eosin, then observed under a light microscope. Sections (4 μm thick) were placed in xylene for dewaxing, then dehydrated through a serial gradient alcohol of 100, 85 and 75% to hydration.

Detection of FOXP3 protein expression in parenchymal cells

Reagents used were a solution of mouse antihuman FOXP3 monoclonal antibody (Abcam, Cambridge, MA, USA) and the complete ChemMate Envision^{TM} rabbit/mouse universal immunohistochemistry kit (Dako, Glostrup, Denmark). A two-step immunohistochemical staining method was used, following the instructions of the reagent manufacturers. Diaminobenzidine was used to produce a brown colour and the sections were counterstained with haematoxylin. Antigen repair was achieved by heating. Phosphate-buffered saline replaced primary antibody in the negative control.

A brownish yellow or brown nucleus indicated positive FOXP3 staining. FOXP3 expression was graded semiquantitatively according to staining intensity and percentage of positive cells. Staining intensity was scored as 0 (achromatic), 1 (light yellow), 2 (brownish yellow) or 3 (brown). The percentage of positive cells was scored as 0 ($\leq$10%), 1 (11–25%), 2 (26–50%), 3 (51–75%) or 4 ($\geq$76%). The final score was the product of the two scores; 0 represented a negative result and $\geq$1 a positive result.

Detection of FOXP3 protein expression in lymphocytes

Reagents used were rabbit antihuman CD3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse antihuman FOXP3 monoclonal antibody solution (Abcam) and the DouSP^{TM} immunohistochemical double staining kit (Fuzhou Maixin Company, Fuzhou, Fujian, China). The DouSP^{TM} antigen was repaired by treatment with citrate at high temperature and high pressure, combined with pepsin digestion. CD3/FOXP3 co-expression was detected using double staining.[13] Reagents were used according to the instructions provided by their manufacturers.

A positive result for FOXP3 was blue–black staining of the nucleus; a positive result for FOXP3 and CD3 co-expression was brown–red staining of the cytoplasm and blue–black staining of the nucleus of the same cell. To determine the FOXP3 + lymphocyte count, 10 independent fields of view were examined under a high-power lens (magnification × 400) for each sample, and the mean value was taken. A FOXP3 + lymphocyte count of at least three per field of view indicated a positive result.

Statistical analyses

Continuous data were expressed as mean ± standard deviation; all experimental data were analysed using SPSS® statistical software, version 11.0 (SPSS Inc., Chicago, IL, USA). Mean values of two samples were compared using a group-design *t*-test; mean values of multiple samples in the group design were compared using single-factor analysis of variance (one-way ANOVA). The χ^2-test was used when comparing categorical variables. Correlation analysis was performed using Pearson's correlation

analysis. Statistical significance was accepted at $P < 0.05$.

Results

The FOXP3 proteins were focally and diffusely distributed in the parenchymal cells of cervical oesophageal cancer tissue samples. Focal expression was observed in a small number of cases. Proteins were primarily expressed in oesophageal epithelial mucosae and cervical oesophageal cancer cells, and located in the nucleus, as shown in Figure 1. FOXP3 expression rates in parenchymal cells of cervical oesophageal cancer and paracancerous tissues were 42.9% (18/42) and 6.67% (two of 30) respectively; this difference was statistically significant ($\chi^2 = 10.324$, $P < 0.01$).

The FOXP3 + lymphocytes had infiltrated interstitial tissues (Figure 2) in 16 of 42 (38.1%) specimens of cervical oesophageal cancer tissue and four of 30 (13.33%) specimens of paracancerous tissue; this difference was statistically significant ($\chi^2 = 15.376$, $P < 0.01$). The frequency of FOXP3 + lymphocyte infiltration in tumour interstitial tissues was not significantly associated with FOXP3 protein expression levels in cancer parenchyma.

As shown in Table 1, the FOXP3 protein expression in parenchymal cells of cervical oesophageal cancer specimens from the group with peripheral lymph node metastasis was significantly higher than in the group without lymph node metastasis ($P < 0.05$). The FOXP3 protein expression level in specimens from patients with clinical stage III or IV disease was significantly higher than that in specimens from stage I or II disease. FOXP3 protein expression levels in tumour parenchymal cells from patients of different ages, sexes and histological classifications and groupings did not differ significantly. Infiltration of FOXP3 + lymphocytes into tumour interstitial tissues of the group with lymph node metastasis was significantly greater than that in the group without lymph node metastasis ($P < 0.05$). However, FOXP3 + lymphocyte infiltration did not differ significantly with age, sex, histological grade or TNM stage.

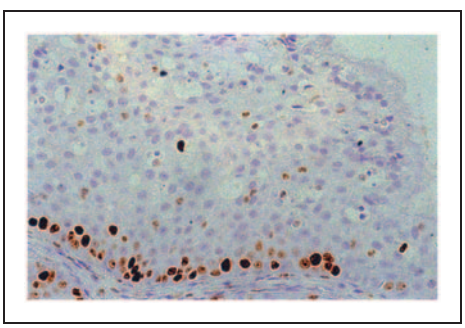

Figure 1. FOXP3 protein expression in parenchymal cells of cervical oesophageal cancer tissue samples by immunohistochemistry. There is strong FOXP3 staining (dark brown). Original magnification × 400. The colour version of this figure is available at: http://imr.sagepub.com

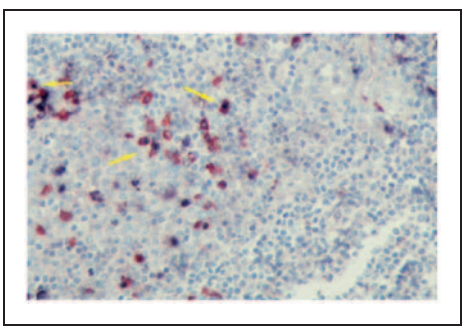

Figure 2. Infiltrating FOXP3 + lymphocytes in tumour interstitial tissues revealed by immunohistochemical double staining. The infiltrating FOXP3 + lymphocytes, indicated by yellow arrows, are double stained for CD3 (red, cytoplasm) and FOXP3 (blue, nucleus). Original magnification × 400. The colour version of this figure is available at: http://imr.sagepub.com

Table 1. Clinicopathological parameters of patients with cervical oesophageal cancer and their correlation with FOXP3 expression

Clinicopathological variable	*n*	FOXP3 protein expression			FOXP3 + lymphocyte infiltration		
		Positive	Negative	Statistical significance	Positive	Negative	Statistical significance
Age, years				$P = 0.632$			$P = 1.563$
≤60	20	8	12		7	13	
≥61	22	10	12		9	13	
Sex				$P = 0.356$			$P = 1.752$
Male	34	14	20		13	21	
Female	8	3	5		3	5	
Histological grade				$P = 0.127$			0.864
I	14	6	8		5	9	
II	19	8	11		7	12	
III	9	4	5		4	5	
Clinical (TNM) stage				$P = 0.038$			0.542
I or II	23	7	16		9	14	
III or IV	19	11	8		7	12	
Lymph node metastasis				$P = 0.024$			
Absent	23	7	16		6	17	$P = 0.042$
Present	19	11	8		10	9	

Data presented as *n* patients.
The χ^2-test was used for statistical analysis.
TNM, tumour, node, metastasis classification system.[15]

Discussion

Research has shown that CD4 + CD25 + regulatory T cells play a key role in tumour immune escape in, for example, melanoma, liver cancer and lung cancer.[16–18] FOXP3 is one of the most specific markers of CD4 + CD25 + Treg cells, in which it regulates immunosuppressive functions. Most studies have therefore focused on FOXP3 expression and regulation in CD4 + CD25 + Treg cells themselves.[19,20] FOXP3 performs critical functions in controlling local immune responses and promoting the immune escape of tumours. In the present work, the rate of FOXP3 + lymphocyte infiltration into interstitial tissues of cervical oesophageal cancer was 38.1% (16/42 samples), which was significantly higher than that in paracancerous tissues (13.33%, four of 30 samples). Further analysis found that FOXP3 + lymphocyte infiltration into cancer interstitium in the group with lymph node metastasis was significantly greater than that in the group without lymph node metastasis. This suggested that FOXP3 + lymphocytes may be an important marker of the biological behaviour of cervical oesophageal cancer. Increased numbers of FOXP3 + lymphocytes in the tumour microenvironment may stimulate negative regulation of immune responses, suppressing the immune response to tumour cells and promoting tumour invasion and metastasis. Research has consistently found that numbers of FOXP3 + regulatory T cells are significantly increased in local tumours such as breast, pancreatic and prostate cancer.[21–23] Such increases may be related to the cytokines secreted by tumour cells.

Expression of FOXP3 by tumour cells themselves has been reported, but because of

the small number of such studies the function of FOXP3 in tumour cells is still disputed. Some findings are contradictory, even in the same tumour. Hinz et al.[9] found that FOXP3 was expressed in pancreatic cancer cells: FOXP3 + pancreatic cancer cells had functions similar to CD4 + CD25 + Treg cells and could promote immune escape of the tumour. Subsequent investigations showed that FOXP3 was also expressed in gastric cancer cells, and was closely associated with lymph node metastasis of gastric cancer.[24] In the present study, the FOXP3 protein expression rate was significantly higher in parenchymal cells of cervical oesophageal cancer than in parenchymal cells from paracancerous tissue samples, and was also significantly higher in the group with peripheral lymph node metastasis than in the group without lymph node metastasis. FOXP3 protein expression levels in specimens from clinical (TNM) stage III or IV disease were significantly higher than in those from stage I or II disease, in our study. However, there have been contradictory reports about the expression and functions of FOXP3 in breast cancer cells. Matsuura et al.[10] found that the FOXP3 mRNA and protein were highly expressed in tumour tissue from breast cancer patients, and that expression was closely associated with tumour recurrence and overall survival rate. We suggest that that FOXP3 could be a new prognostic factor for breast cancer. However, Gupta et al.[25] reported the opposite results, finding that FOXP3 was expressed at a high level (80%) in normal breast tissue but at a low level in tumour tissue samples (20%). These findings indicate that the role of FOXP3 in the malignant cells is complex. Moreover, signalling pathways involved in the regulation of FOXP3 expression in tumour cells remain to be clarified. Thus, further investigations are necessary.

In conclusion, the results of our study suggest that FOXP3 is highly expressed in cervical oesophageal cancer tissue, and that it may be expressed not only in CD4 + CD25 + Treg cells but also in a variety of tumour cells. However, the significance of FOXP3 expression, its regulatory mechanism and its association with the occurrence, development and prognosis of cervical oesophageal cancer are not yet fully understood. The results of the present study suggest that the discovery of new targets in cervical oesophageal cells has considerable clinical significance.

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Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

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