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Original Contribution

FOXP3 and CD25 double staining antibody cocktails identify regulatory T cells in different types of tumor tissues using tissue microarrays

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ABSTRACT

Regulatory T cells (Tregs) are CD4+ T cells that express CD25 and transcription factor Forkhead box P3 (FOXP3), and Tregs play a central role in regulation of tumor immunity. FOXP3 immunohistochemistry has been widely used to study Tregs in paraffin embedded tissue, and flow cytometry using fresh tissue has been used to identify FOXP3+/CD25+ double positive Tregs. In our study, we validated the FOXP3/CD25 double staining antibody cocktails for detecting Tregs in paraffin-embedded tissue. Tissue microarrays (TMA) included 115 malignant tumors, 3 ovarian mucinous borderline tumors and 15 benign tissues. Digital image analysis was performed using ImageJ software. Our results showed that FOXP3/CD25 double positive lymphocytes, a subset of FOXP3+ lymphocytes, accounted for variable percentage of the total FOXP3+ lymphocytes and they were positively correlated with FOXP3+ cell counts. Tumors from different sites had variable FOXP3+/CD25+ and FOXP3+ lymphocyte counts. Tumors from lung, head & neck and colon had more and renal cell carcinoma had minimal FOXP3+/CD25+ and FOXP3+ lymphocytes. In conclusion, FOXP3/CD25 double staining antibody cocktails can be easily applied to paraffin-embedded tissue, and FOXP3+/CD25+ Tregs count was positively correlated with FOPX3+ Tregs count but they were not interchangeable. We recommend using CD25/FOXP3 double staining for studying Tregs in tumor tissue.

1. Introduction

Regulatory T cells (Tregs), a subpopulation of T cells and usually defined as CD4+CD25+FOXP3+ T cells, play a central role in regulation of immune responses to self-antigens, allergens, infectious agents and tumors [1]. Tregs have the ability to inhibit host versus tumor immunity in the tumor microenvironment by suppressing anti-tumor CD8+ lymphocytes [2-4], and they have been proposed to dampen functions of anti-neoplastic immune cells and thus promote cancer progression [5]. Tregs have high numbers in tumor tissue compared to the surrounding non-tumor tissue [6-9], and Tregs are considered as a prognostic biomarker for human malignant tumors [10,11].

Tregs are characterized by high expression of IL-2 receptor α chain (CD25) and FOXP3. FOXP3 is a forkhead helix transcription factor which functions as a master regulator in the development and control of Tregs and it has been considered as the most specific and reliable surface marker of Tregs [12-14]. Therefore, Tregs are often evaluated by using FOXP3 immunohistochemistry. However, FOXP3+ T lymphocytes are heterogeneous cells, and FOXP3+/CD25+ and FOXP3+/CD25- Tregs may have different functions. It is very important to use more than one marker to identify Tregs subsets in human cancer tissues [15]. However, the method for identifying Tregs with FOXP3/CD25 double staining in the literature was mainly by flow cytometry analysis [16-18], which has limitations of requiring fresh tissue and lacking other important information such as the distribution of Tregs in tumor tissue. Immunohistochemistry for FOXP3/CD25 double stains on paraffin embedded tissue has not been well documented in the literature. Here we report our experience of using FOXP3/CD25 double stain antibody cocktails in different types of tumor tissue. The correlation among FOXP3+/CD25+, FOXP3+ and CD45+ lymphocytes was also analyzed.

2. Materials and methods

This study was approved by the Institutional Review Board (HSC-

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Table 1
Variety tissue types used for TMA.

Organ types	Malignant neoplasms(n)	Borderline or benign tissue (n)	Total
Ovary	14	3	17
Uterus	11	2	13
Breast	9	4	13
Kidney	19	0	19
Head & neck	5	0	5
Colorectal	21	1	22
Pancreas	4	5	9
Lung	9	0	9
Liver	4	1	5
Soft tissue	8	0	8
Uterine cervix	2	0	2
Lymph nodes	3	0	3
Stomach	3	0	3
Skin	1	0	1
Testicle	1	0	1
Heart	0	1	1
Pleura	1	0	1
Placenta	0	1	1
Total	115	18	133

MS-14-1023), and total 133 different types of tissue (See Table 1) were used for tissue microarray (TMA) construction.

2.1. TMA construction

TMA was manually constructed using 2.0 mm punch cores from the paraffin embedded tissue blocks. One Hematoxylin & Eosin (H&E) stained slide was reviewed to confirm the diagnosis for each TMA.

2.2. IHC staining protocol

The 4-μm thick unstained sections on charged slides were used for immunohistochemistry. The FOXP3/CD25 double staining antibody cocktails were made by using rabbit anti-human CD25 (1:100, Abcam, ab128955, Cambridge, MA) and mouse anti-human FOXP3 (1:200, Abcam, ab20034, Cambridge, MA). Mouse anti-human CD45 (1:100,Dako, 00087397, Santa Clara, CA) was used for identifying all lymphocytes. All primary antibodies were diluted in GeneTex antibody diluent (GeneTex, GTX28208, Irvine, CA). For FOXP3/CD25, the double staining secondary antibody was from Biocare Medical with mouse-HRP and rabbit-AP (Biocare Medical, MRCT525H, Pacheco, CA). For CD45, the secondary antibody was from Vector ABC R-T-U universal kit (VECTOR LABS, PK-7200, Burlingame, CA).

Briefly, after the xylene x 3 times deparrafin treatment and sequential alcohol rehydration, sections of TMA were treated with TE buffer (pH = 9.0) at 100 °C for 45 min using a steamer for antigen retrieval. After cooling in room temperature for 30 min, endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution. Then nonspecific binding was blocked with nonimmune horse serum (VECTOR LABS, S-2000, Burlingame, CA) for 20 min. The primary antibodies of anti FOXP3/CD25 cocktails and anti CD45 were incubated for 1 h at room temperature in a moisture chamber. Five times washing was performed with 1× PBS washing buffer between the primary and secondary antibodies. The Biocare Medical double secondary antibody for FOXP/CD25 or ABC R-T-U for CD45 was incubated at room temperature for 30 min. Signals were visualized after adding 3, 3′-diaminobenzidine tetrahydrochloride (DAB) and Vulcan Fast Red Chromogens. Tonsil tissue was used as the positive control, and TMA section with no primary antibody served as the negative control.

2.3. FOXP3+/CD25+, FOXP3+, and CD45+ lymphocyte evaluation

The IHC stained slides were reviewed by a board certified surgical pathologist, and 3 pictures in 3 different fields with the most active signals were taken at 400× with the surface area of each image about 0.088 mm^2. The digital images were analyzed with ImageJ software for FOXP3+/CD25+, FOXP3+, and CD45+ lymphocytes. The cell counts

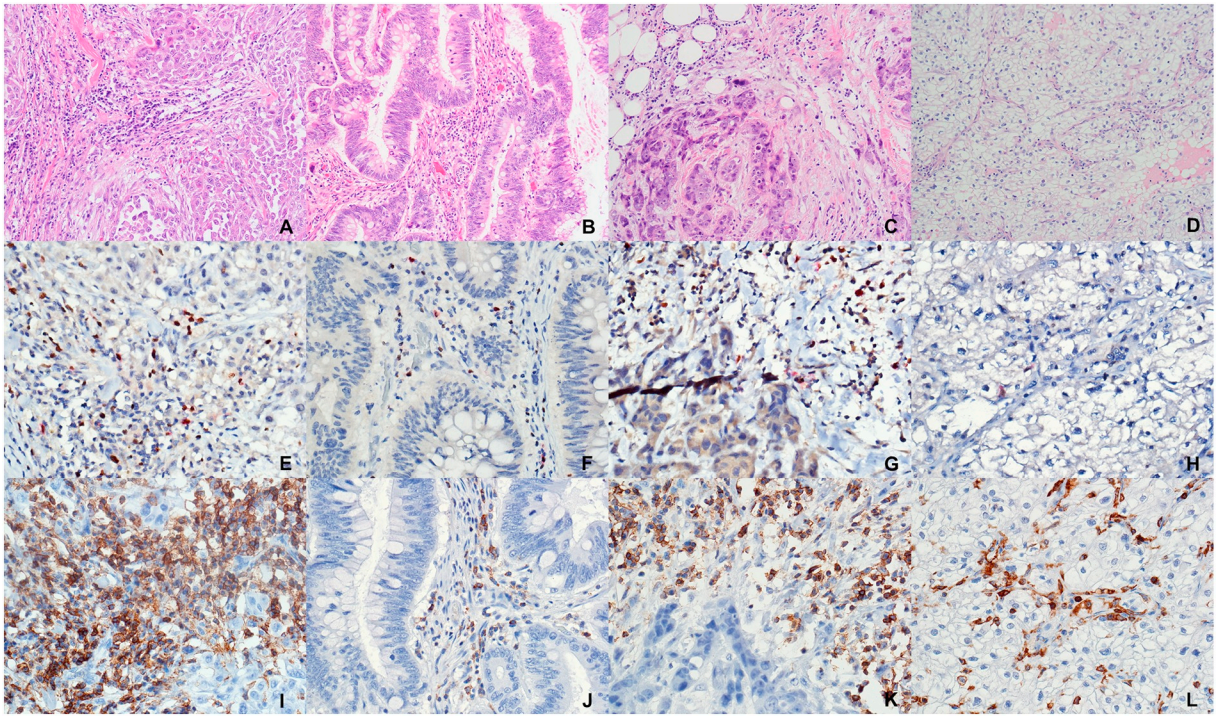

Fig. 1. H&E images (200×) of head neck squamous cell carcinoma (A), colorectal adenocarcinoma (B), breast invasive ductal carcinoma (C), and renal clear cell carcinoma (D); Images E, F, G and H the corresponding FOXP3 (brown nuclear stain) and CD25 (red cytoplasm/membrane stain) double antibody cocktail images (400×); Images I, J, K, and L the corresponding CD45 images (400×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were converted to cell density which was expressed as cell count/mm^2.

2.4. Statistical analysis

The *t*-test was used for comparing the cell density, and Pearson correlation analysis was used for studying the correlations between FOXP3+, and FOXP3+/CD25+, and CD45+ lymphocytes. The $p < 0.05$ was used as the cutoff for statistical significance.

3. Results

FOXP3/CD25 antibody cocktails were successfully performed in all the tested tissue on the TMA, and the FOXP3+/CD25+ and FOXP3+ lymphocytes were seen in both intraepithelial and tumor stroma (Fig. 1). The FOXP3+, FOXP3+/CD25+ and CD45+ lymphocytes were counted with ImageJ software. Using Pearson Correlation analysis, FOXP3+ and FOXP3+/CD25+ lymphocytes had strong positive correlation (r = 0.76), but FOXP3+ and FOXP3+/CD25+ lymphocytes showed weak positive correlation with the CD45+ lymphocytes (r = 0.26 and 0.14 respectively).

The FOXP3+ and FOXP3+/CD25+ lymphocyte count showed significant variation among different types tumor tissues (Table 2). Renal clear cell carcinoma had the lowest and lung carcinomas (both adenocarcinoma and squamous cell carcinoma) had the highest FOXP3+ and FOXP3+/CD25+ lymphocytes among all studied tumor tissues.

4. Discussion

Tregs are considered as the suppressor of antitumor immunity in cancer-bearing hosts, and infiltration of Tregs in tumor stroma reflects the host local immune condition [19,20]. The degree of Tregs infiltration is associated with the prognosis in various types of cancer [21-25]. Currently, detecting FOXP3+/CD25+ Tregs is usually performed with flow cytometry [16-18,26]; however, flow cytometry analysis can only use the fresh tissue. Most available tumor tissues are paraffin embedded, and IHC is the most valuable method for studying Tregs in formalin-fixed, paraffin-embedded tissues. In our study, we established an IHC protocol using the FOXP3/CD25 antibody cocktail to detect the FOXP3+ and FOXP3+/CD25+ Tregs in different types of tumor tissues. Immunohistochemistry with FOXP3/CD25 double staining will provide some detailed information about the different types of Tregs and distribution of Tregs in tumor tissue which may have prognostic values.

Our study showed variable FOXP3+ and FOXP3+/CD25+ Tregs counts and variable FOXP3+/CD25+ to FOXP3+ ratio in different tumor types. Head & neck squamous cell carcinoma (HNSCC), lung SCC and lung adenocarcinoma had high FOXP3+ Tregs count, and renal clear cell carcinoma (RCCC) had the lowest FOXP3+ cell count. For FOXP3+/CD25+ double positive lymphocytes, HNSCC, lung SCC, colorectal carcinoma and pancreatic ductal carcinoma were among the top 4 tumors with high count, and RCCC had the lowest count. The FOXP3+/CD25+ to FOXP3+ ratio was variable ranging from 0.05 to 0.41. Pancreatic ductal carcinoma had the highest ratio (0.41) and lung adenocarcinoma had the lowest ratio (0.05). The significant variation on Tregs cell counts and variable ratio between FOXP3+ and FOXP3+/CD25+ in different tumors may reflect the different tumor immunity. Also, the FOXP3+ and FOXP3+/CD25+ counts were only weakly correlated with the total tumor infiltrating lymphocytes (TILs, CD45+ cells), indicating the cellular composition of TILs may be important for studying tumor immunity. Even though the FOXP3+ and FOXP3+/CD25+ lymphocyte counts had strong overall positive correlation (r = 0.76) in general, the FOXP3+/CD25+ to FOXP3+ ratio showed significant variation among different types of tumors. Further analysis on FOXP3+, FOXP3+/CD25-, and FOXP3+/CD25+ Tregs counts and the distribution in tumor tissue by IHC may provide more

Table 2
FOXP3+ and FOXP3+/CD25+ lymphocytes in variety tumor tissues.

Tumor types	FOXP3+/mm2	*p* Value	FOXP3+/CD25+/mm2	*p* Value	(FOXP3+/CD25+)/FOXP3+ ratio
High grade serous carcinoma ($N = 10$)	88.8		26.2		0.29
Renal clear cell carcinoma ($N = 14$)	6.5	0.007	0.5	0.02	0.08
Colorectal adenocarcinoma ($N = 21$)	159.1	0.10, 0.0006	45.5	0.2, 0.01	0.29
Breast invasive ductal carcinoma ($N = 8$)	120.3	0.37, 0.069, 0.32	12.3	0.2, 0.068, 0.10	0.10
Uterine endometrioid carcinoma ($N = 7$)	41.7	0.15, 0.006, 0.035, 0.23	8.2	0.15, 0.0001, 0.09, 0.36	0.20
Lung adenocarcinoma ($N = 4$)	244.3	0.06, 0.001, 0.19, 0.23, 0.03	13.3	0.3, 0.005, 0.2, 0.5, 0.2	0.05
Lung squamous cell carcinoma ($N = 4$)	298.3	0.005, < 0.00001, 0.06, 0.13, 0.0003, 0.3,	61.5	0.12, 0.001, 0.33, 0.05, 0.03, 0.1	0.15
Head & Neck SCC ($N = 4$)	354.2	0.008, 0.00004, 0.03, 0.10, 0.006, 0.29, 0.36	69.1	0.10, 0.002, 0.27, 0.05, 0.04, 0.12, 0.44	0.20
Pancreatic ductal adenocarcinoma (N-3)	82.1	0.46, 0.001, 0.21, 0.41, 0.10, 0.17, 0.03, 0.08	34.1	0.38, < 0.00001, 0.39, 0.12, 0.0004, 0.06, 0.27, 0.26	0.41
Variety sarcomas (N = 8)	57.4	0.27, 0.008, 0.07, 0.3, 0.29, 0.06, 0.002, 0.01, 0.29	4.4	0.12, 0.02, 0.08, 0.26, 0.15, 0.13, 0.04, 0.04, 0.0004	0.08

information on tumor immunity and may help to guide targeted therapy on tumors.

5. Conclusions

The IHC using FOXP3/CD25 antibody cocktail can be easily performed on all formalin-fixed paraffin embedded tumor tissues for studying Tregs, and the FOXP3/CD25 double staining IHC provides more information on the Tregs distribution (intraepithelial vs stroma) and the subtypes and functions of different Tregs (such as FOXP3 + / CD25 + vs FOXP3 + /CD25-). Digital image auto-analysis software can make the IHC Tregs evaluation more objective and feasible.

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Conflict of interest disclosure

We have no conflict of interest to declare.

Disclosure

None.

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