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Immunohistochemical Double and Triple Stain Strategies with CD8, CD103, FOXP3, PD-1 and CK8/18 in Colon Adenocarcinoma: Quantitation, Prognosis and Immunotherapy

Wei Yuan, DMV, PhD, Jillian Tyrrell, PhD and David Altree-Tacha, PhD, HTL (ASCP); Biocare Medical, Concord, CA

Background

It is estimated that 1 in every 20 people will develop colorectal cancer. In 2015, it is projected that there will be 93,090 new cases of colon cancer and 39,610 new cases of rectal cancer in the U.S. Approximately 23,600 deaths are expected in the U.S. and 700,000 deaths worldwide.[1]

Currently, immunotherapies for colorectal cancer fall into broad categories: monoclonal antibodies, checkpoint inhibitors, vaccines and adjuvant immunotherapies. Immunotherapies for colorectal cancer are still in early-phase clinical trial (phase I and II), but their successful use in other types of cancers suggests that they may ultimately prove useful for colorectal cancer.[2]

Tumor-associated immune suppression can lead to defective T-cell mediated anti-tumor immunity. $CD8^+$ T-cells play a critical role in host defense against cancer; however, the presence of antigen-specific $CD8^+$ T-cells does not always imply that cancers are efficiently eliminated or indicate a good prognosis.[3] Biomarkers that identify tumor infiltrating lymphocytes (TILs) include CD8, CD103, FOXP3 and PD-1; all of which play important roles in T-cell cytotoxicity, activation, regulatory and programmed cell death checkpoints, respectively.[4-8] Understanding the distribution of multiple subsets of immune cells in tumors is important in cancer. Quantification and ratios of these distributions may have prognostic value and other implications, which may lead to strategies for immunotherapy in colorectal cancers.[9-12]

Existing methods for biomarker detection, such as immunohistochemistry (IHC) and flow cytometry, can either deliver immunohistochemical information on single biomarkers in fixed tissues or give phenotypic information on homogenous liquid samples; however, it would be advantageous to simultaneously visualize the distribution of multiple biomarkers in TILs to discriminate stromal tissue vs. tumor tissue. This can be achieved using IHC with double and triple staining (i.e. multiplex staining). Multiplex IHC may be an important strategy for improving our understanding of the distributions of biomarkers in cancer and the implications they may have.

In this study, we demonstrate multiplex staining of TILs using IHC, and show its use in combination with CK8/18 rabbit/mouse monoclonal antibodies as a marker (staining mask) for colon adenocarcinoma. This technique enables visualization of simultaneous single or double staining in TILs, and additionally allows for visualization of tumors cells stained with CK8/18 to easily discriminate between TILs in stroma and colon adenocarcinoma.

Design

Cases of formalin-fixed paraffin embedded (FFPE) colon adenocarcinoma were randomly selected and processed for immunohistochemistry. All tissue sections were deparaffinized and hydrated to water. Slides were placed in a modified citrate buffer and heated in a pressure cooker at 110° C for 15 minutes.

Mouse monoclonal (MM) antibodies to CD8 [C8/144B], FOXP3 [236A/E7], PD-1 [NAT105] and CK8/18 [5D3], and rabbit monoclonal (RM) antibodies to CD8 [SP16], CD103 [EP206] and CK8/18 [EP17/EP30] (Biocare Medical) were tittered for optimum dilutions and incubated for 30 minutes on tissue sections, followed by a secondary polymer of anti-mouse conjugated with horseradish-peroxidase (MACH2 HRP Polymer, Biocare Medical) or of an anti-rabbit conjugated with alkaline-phosphatase (AP) (MACH2 AP Polymer, Biocare Medical). Visualization was accomplished with 3,3'-diaminobenzidine (DAB) or fast red chromogens. Slides were counterstained in hematoxylin.

Double stain cocktails were developed for PD-1 (MM) + CD8 (RM) and CD8 (RM) + FOXP3 (MM). Antibodies CD8 (MM), CD103 (RM), FOXP3 (MM), PD-1 (MM) were cocktailed with CK8/18 (RM/MM). Single stained slides were compared to adjacent double stained slides for comparative analysis. Antibody cocktails were applied on tissues sections for 30 minutes, followed by a secondary polymer mixture of anti-mouse HRP and anti-rabbit AP. Visualization was achieved with DAB and fast red chromogens.

A triple stain was also developed with an antibody cocktail of FOXP3 (MM) + CD8 (RM). The cocktail was applied on tissues for 30 minutes, followed by a secondary polymer mixture of mouse and rabbit HRP/AP detection. HRP (Black) and AP (Blue) chromogens were used for visualization. For the third color application, tissue sections were stained sequentially by first applying a denaturing (elution) step. CK8/18 (RM) antibody was then applied for 30 minutes, followed by a secondary anti-rabbit AP-polymer, and fast red chromogen for visualization.

Statistical Analysis

Comparative analysis of antibodies with single/double/triple stains was achieved using a two-tailed Student's t-Test (GraphPad Prism 6.0, GraphPad Software). P-values of <0.05 were considered statistically significant.

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Figures

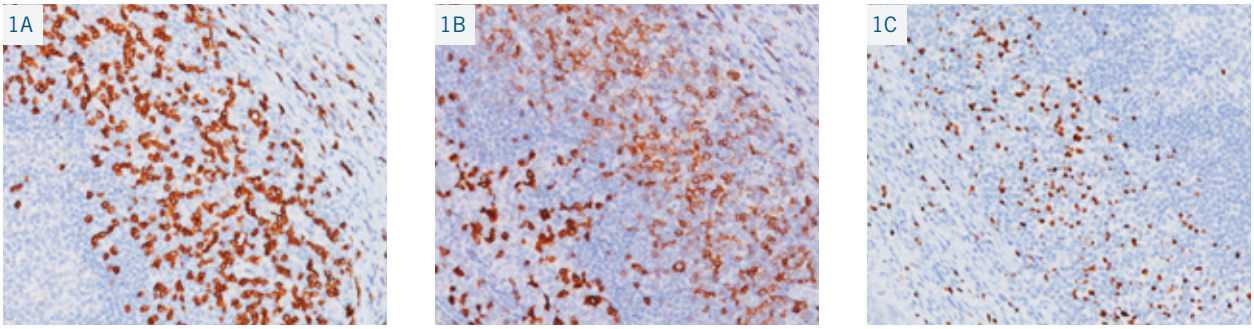

Figure 1: CD8 and PD-1 showed a cellular membrane staining pattern in Peyer's patches (1A and 1B); and FOXP3 showed nuclear staining (1C). Note the staining patterns/location and the number of stained cells for each antibody. (100X)

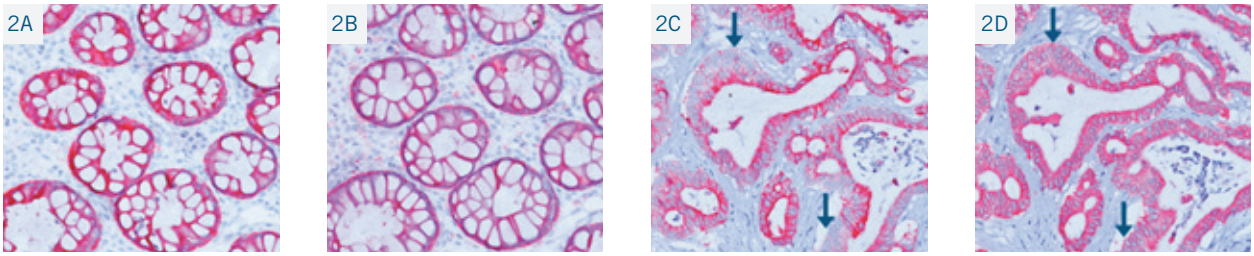

Figure 2: Normal colon epithelia stained with CK8/18 (MM) (Figure 2A) and normal colon epithelia stained with CK8/18 (RM) (Figure 2B). Colon adenocarcinoma stained with CK8/18 (MM) (2C) and CK8/18 (RM) (2D). Arrows indicate the difference of staining in tumor cells by mouse monoclonal vs. rabbit monoclonal anti-CK8/18 (Figures 2C and 2D)

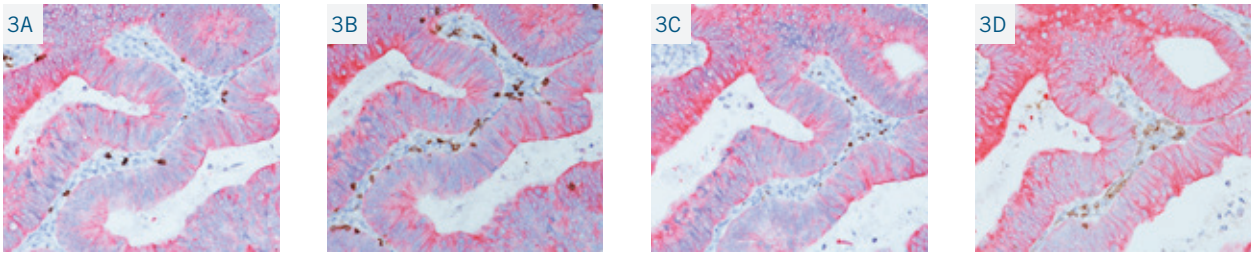

Figure 3: Double staining showed that well differentiated colon adenocarcinoma was masked with CK8/18 (red) and CD8 (3A), CD103 (3B), FOXP3 (3C) and PD-1 (3D) were mostly expressed in stromal tissues (brown), rather within tumor.

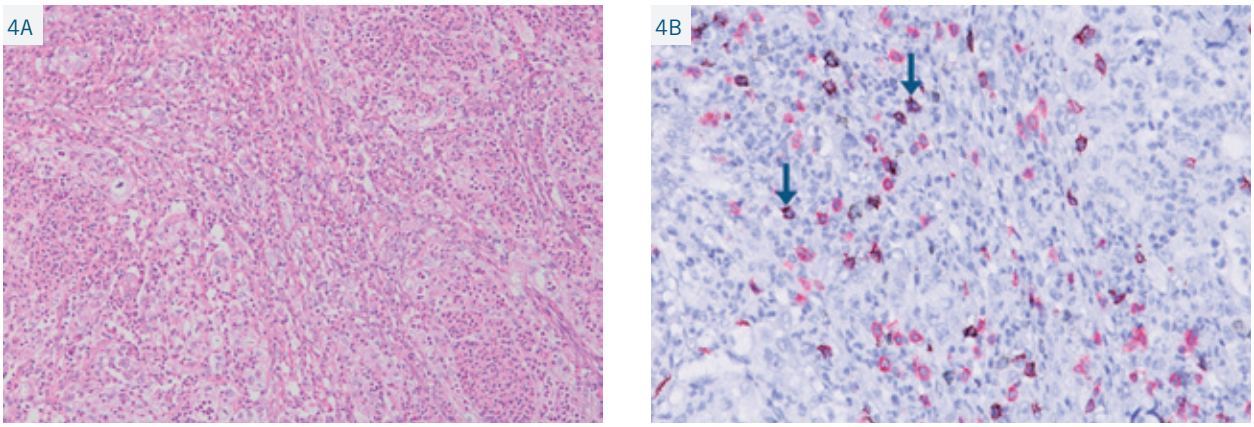

Figure 4: Poorly differentiated colon adenocarcinoma, H&E stain (4A) and TILs with co-expression of PD-1^{+} and CD8^{+} are seen within the tumor (4B, arrows).

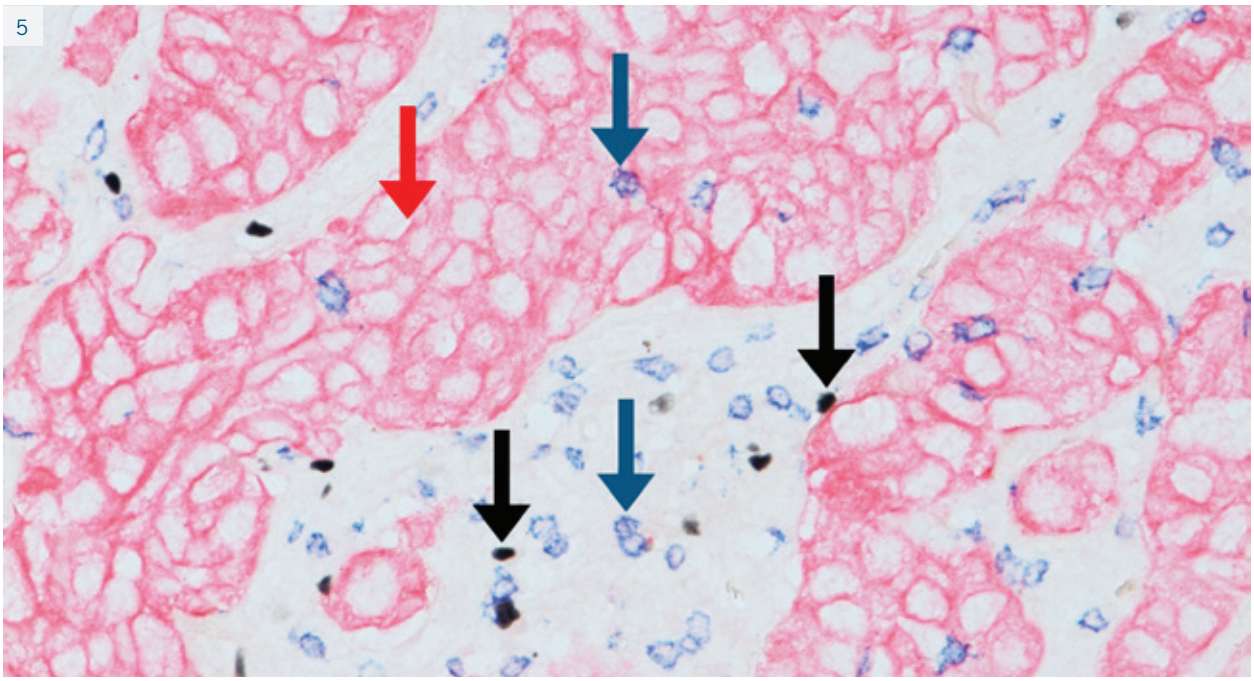

Figure 5: Triple staining of poorly differentiated colon adenocarcinoma showing $CD8^+$ (blue arrows), $FOXP3^+$ (black arrows) and $CK8/18^+$ colon adenocarcinoma (red arrow).

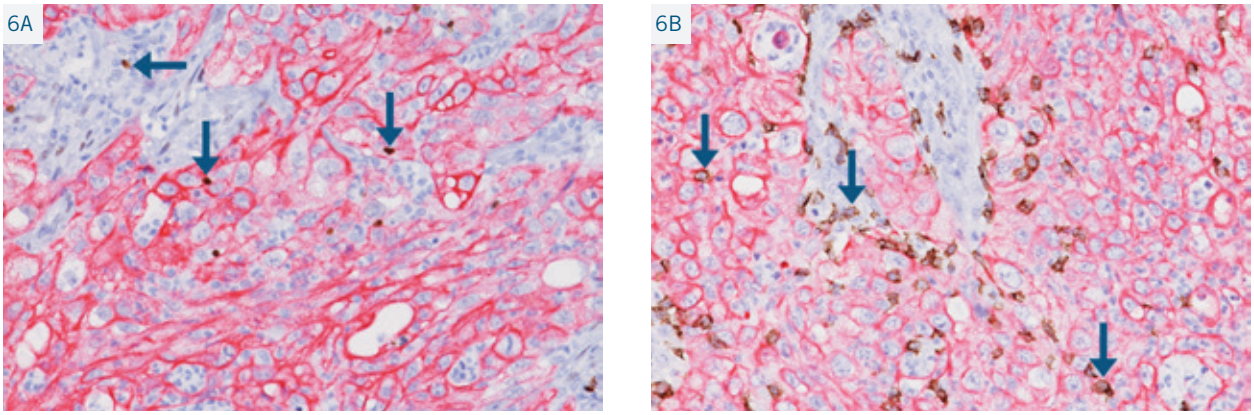

Figure 6: Double staining of TILs in colon adenocarcinoma. 6A: Demonstrates FOXP3 within the stroma and tumor cells. 6B: Demonstrates CD8 in and stroma and tumor cells. In figure 6A and B CK8/18 (Red) only stains tumor cells.

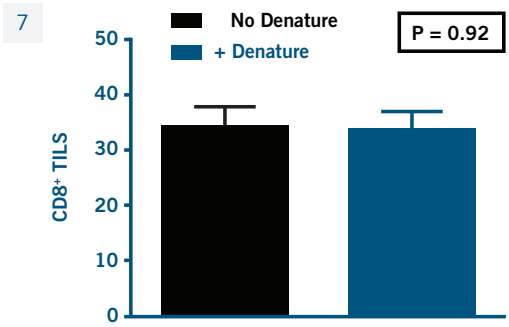

Figure 7: $CD8^+$ TILs in colon adenocarcinoma before and after denaturing solution was applied. The denaturing process had no effect on $CD8^+$ TIL counts (n=6).

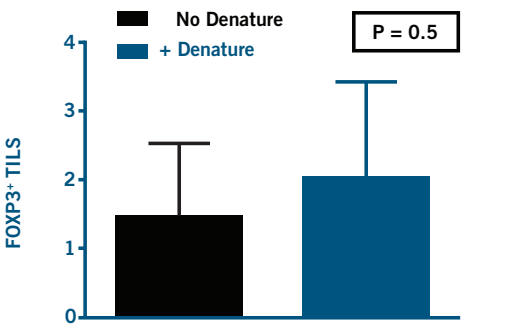

Figure 8: $FOXP3^+$ TILs in colon adenocarcinoma before and after denaturing solution was applied. The denaturing process had no significant effect on $FOXP3^+$ TIL counts (n=6).

Results

Single Stains

In this study, CD8 and PD-1 displayed cellular membrane staining in lymphoid cells (Peyer's patch) adjacent to colon adenocarcinoma (Figures 1A and 1B). FOXP3, a transcriptional T-cell regulator, was localized in the nucleus in stroma and tumor cells and in normal lymphoid cells (Figure 1C).

Double Stains

CK8/18 (MM) and CK8/18 (RM) showed clear and similar cytoplasmic staining in normal colon (Figures 2A, B). However, in colon adenocarcinoma, CK8/18 (MM) staining (red) displayed less uniformity (Figure 2C, arrow) compared to CK8/18 (RM) (Figure 2D, arrow). Therefore, the CK8/18 (RM) staining mask was slightly superior in colon adenocarcinoma compared to CK8/18 (MM). In cases of well-differentiated colon adenocarcinoma, the CK8/18 staining mask (red), clearly showed that CD8, CD103, FOPX3 and PD-1 were predominantly expressed in stromal tissue, but absent within the tumor (Figure 3A-D) In a case of poorly differentiated colon adenocarcinoma (Figure 4A, H&E), a double stain clearly showed co-expression of PD-1 (brown) and CD8 (red) in TILs within the tumor (Figure 4B).

Triple Stains

In the triple stain procedure, colon adenocarcinoma was stained with CD8 (blue), FOXP3 (black) and CK8/18 (red), (Figure 5). In a poorly differentiated case of colon adenocarcinoma with numerous TILs, the CK8/18 staining mask clearly made the localization of FOXP3 (Figure 6A, brown) and CD8 (Figure 6B, brown) easy to identify within the stroma and tumor.

Statistical Analysis

In corresponding carcinoma spots on adjacent tissue sections, $CD8^+$ TILS counts were similar before and after denaturing solution was applied to tissues (Figure 7, P=0.92, n=6). The number of $FOXP3^+$ TILS was also similar before and after denaturing solution was applied (Figure 8, P=0.50, n=6).

Conclusion

This study represents the first Multiplex IHC assay to simultaneously label and identify different subtypes of TILs, and apply CK8/18 as a staining mask to identify tumor and non-tumor cells in a single section. Different subtypes of TILs were easily identified and quantified both manually and with an image analysis system. This technique may help facilitate acquisition of important prognostic information and could help support new strategies in immunotherapy and other therapeutic treatments.

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References

1. American Cancer Society: Colorectal Cancer - Facts and Figures; www.cancer.org
2. Cancer Research Institute: www.cancerresearch.org/cancer-immunotherapy/impacting-all-cancers/colorectal-cancer.
3. Eikawa S, Mizukami S, Udono H. Monitoring multifunctionality of immune-exhausted CD8 T cells in cancer patients. Methods Mol Biol. 2014;1142:11-7.
4. Lim YJ, *et al.* High ratio of programmed cell death protein 1 (PD-1)(+)/CD8(+) tumor-infiltrating lymphocytes identifies a poor prognostic subset of extrahepatic bile duct cancer undergoing surgery plus adjuvant chemoradiotherapy. Radiother Oncol. 2015;117:165-70.
5. Webb JR, Milne K, Nelson BH. PD-1 and CD103 Are Widely Coexpressed on Prognostically Favorable Intraepithelial CD8 T Cells in Human Ovarian Cancer. Cancer Immunol Res. 2015;3:926-35.
6. Wu X, *et al.* PD-1(+) CD8(+) T cells are exhausted in tumours and functional in draining lymph nodes of colorectal cancer patients. Br J Cancer. 2014;111:1391-9.
7. Droeser RA, *et al.* Clinical impact of programmed cell death ligand 1 expression in colorectal cancer. Eur J Cancer. 2013;49:2233-42.
8. Yoon HH, *et al.* Prognostic impact of $FoxP3^+$ regulatory T cells in relation to $CD8^+$ T lymphocyte density in human colon carcinomas. PLoS One. 2012;7(8)
9. Correale P, *et al.* Regulatory ($FoxP3^+$) T-cell tumor infiltration is a favorable prognostic factor in advanced colon cancer patients undergoing chemo or chemoimmunotherapy. J Immunother. 2010;33:435-41.
10. Turksma AW, *et al.* Extent and Location of Tumor-Infiltrating Lymphocytes in Microsatellite-Stable Colon Cancer Predict Outcome to Adjuvant Active Specific Immunotherapy. Clin Cancer Res. 2016;22:346-56.
11. Gulbake A, Jain A, Jain SK. Insight to drug delivery aspects for colorectal cancer. World J Gastroenterol. 2016;22:582-99.
12. Shaza L, *et al.* Colorectal cancer: ten years of illusion of progress but advances are on the horizon. Rev Med Brux. 2015;36:263-6.

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4040 Pike Lane
Concord CA 94520
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