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Immunohistochemical multiplex staining strategies with CD8, CD103, PD-1, FOXP3 and pan melanoma cocktail in tumor infiltrating lymphocytes in melanoma

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Introduction

Melanoma accounts for approximately 1% of skin cancer cases, but causes a large majority of skin cancer deaths.[1] About 76,380 new melanomas will be diagnosed (about 46,870 in men and 29,510 in women). Advanced melanoma has historically been associated with a poor prognosis, with a median overall survival of 8–10 months and a 5-year survival rate of only 10%.[2]

Immunotherapy is the use of targeted therapy to stimulate patient's own immune system to recognize and destroy cancer cells more effectively. Recently, former President Jimmy Carter, who had metastatic melanoma to the brain and liver, was treated with the drug pembrolizumab, a type of drug known as an immune checkpoint inhibitor, which blocks a protein called PD-1. The complete remission of Jimmy Carter's melanoma shows the great potential of immunotherapy for melanoma.[3] This new type of immunotherapy has harnessed and released the body's own biological weapons to create mass tumor destruction.

In melanoma, tumor-associated immune suppression can lead to defective T-cell mediated antitumor immunity. CD8 cytotoxic T-cells play a critical role in host defense against cancers; however, the presence of antigen-specific CD8 T-cells does not always imply that cancers and/or pathogens are efficiently eliminated in the body.[4] In tumor infiltrating lymphocytes (TILs), markers including CD8, CD103, PD-1 and FOXP3 are broadly expressed and have shown a wide range of immunoregulatory and important roles in T-cell activation and in T-cell regulatory and in programmed cell-death checkpoints.[5-9] Studies have also shown that the high ratios and/or the co-expression of PD-1$^+$ and CD8$^+$ in tumor cells identified poor prognosis; and conversely, the co-expression of CD8$^+$ and CD103$^+$ identified a favorable prognosis.[5-7] In another study, the authors reported that the majority of TILs, including MART-1 melanoma antigen-specific CD8 T-cells, predominantly expressed PD-1.[8] Anichini *et al*, identified tumor-reactive CD8$^+$ (early effector T-cells) at tumor sites in primary and metastatic melanoma; and concluded the CD8$^+$ FOXP3$^+$ "early effector" subset may be an invaluable tool for monitoring immunity at tumor sites.[9] Therefore, a multiplex immunohistochemical stain utilizing a melanoma marker as a staining mask, could separate the malignant tumor cells from stromal cells and may be a good strategy to facilitate better interpretation and cell counting for prognostication.

Design

Cases of formalin-fixed paraffin embedded (FFPE) melanoma were 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.

Two-color double stain assays

Pan Melanoma Cocktail-2 (PMC-2) is compose of MART-1 + Tyrosinase (MM) antibodies and was cocktailed with CD8 (RM), or CD103 (RM), or FOXP3* (RM) or PD-1*(RM) antibodies (Biocare Medical, Concord, CA, *Epitomics, Burlingame, CA) (Table 1). The antibody cocktails were applied on tissue sections for 30 minutes, followed by a secondary polymer mixture of goat anti-mouse alkaline phosphatase (AP) and goat anti-rabbit horseradish peroxidase (HRP). Visualization was achieved with Warp Red or Deep Space Black chromogens

Three-color triple stain assays

Triple Stain #1

PD-1 (MM) + CD8 (RM) cocktail was applied on tissue sections for 30 minutes, followed by a secondary polymer mixture of goat anti-mouse HRP and goat anti-rabbit AP detection. Visualization was achieved with an application of Deep Space Black (PD-1) and Warp Red (CD8) chromogens. For third color application, tissue sections were then sequentially stained by first applying a denaturing (elution) step for 20 minutes and applying the PMC-2 for 30 minutes. A secondary goat anti-mouse HRP-polymer was applied for 30 minutes and visualized with Vina Green chromogen. Tumors were evaluated in the growing and non-growing zones of melanoma.

Triple Stain #2

FOXP3 (MM) + CD8 (RM) cocktail was applied on tissue sections for 30 minutes, followed by a secondary polymer mixture of goat anti-mouse HRP and goat anti-rabbit AP and visualized with Deep Space Black (FOXP3) and AP Ferangi Blue (CD8) chromogens. For third color application, the tissue sections were sequentially stained by first applying a denaturing step for 20 minutes and applying PMC-2 for 30 minutes. A secondary anti-mouse AP-polymer was applied for 30 minutes and visualized with Warp Red chromogen.

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Statistical Analysis Results

Comparative analysis of staining results with CD8⁺ with and without denaturing solution for triple stains was achieved using a two-tailed Student's t-Test (GraphPad Prism 6.0, GraphPad) Software on an Applied Spectral Imaging System.

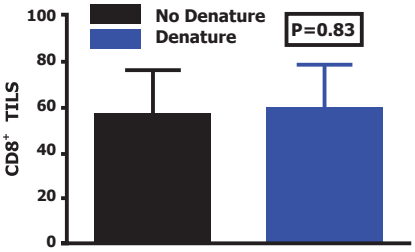
Table 1

Antibody	Source	Clone
CD8	Mouse	CD8/144B
CD8	Rabbit	SP16
CD103	Rabbit	EP206
FOXP3	Mouse	236A/E7
FOXP3	Rabbit	EPR 15038-69
PD-1	Mouse	NAT105
PD-1	Rabbit	EP239
PMC-2	Mouse	M2-7C10 + M2-9E3 + T311

Results

In corresponding melanoma spots on adjacent tissue sections, CD8⁺ TIL counts were similar before and after denaturing solution was applied to tissues (P=0.83, n=6) (Figure 1).

Figure 1



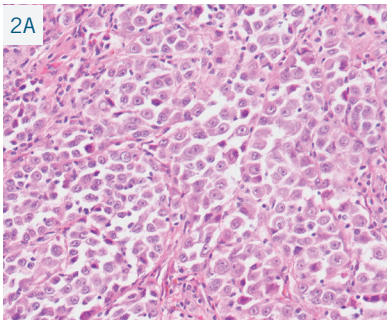
Double Stains

Simultaneous double stains were easily obtained and red and black chromogens produce the highest contrast (Figures 2A-F).

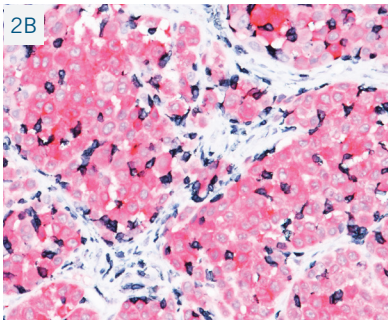
Triple Stains

Both triple stains used 3 different antibodies and 3 different chromogens in the same tissue sections (Figure 2F, 3A, B, and 4). PMC-2 was sequentially stained and was used as a staining mask in red or green chromogens (Figure 2F, 3A, B, and 4). Visualizing co-expression of PD-1⁺ and CD8⁺ cells was best achieved by using red and black chromogens (Figure 2F, 3B), and sequential staining with PMC-2 using the green chromogen.

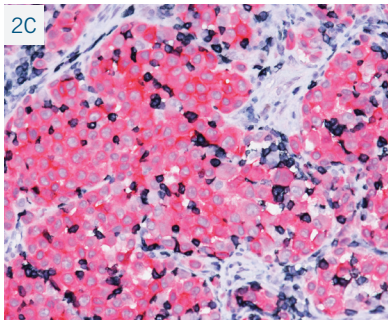
Figure 2



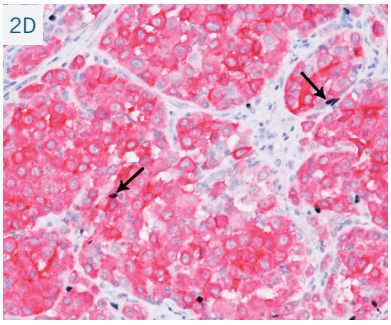
Melanoma stained with Hematoxylin and Eosin, 20X



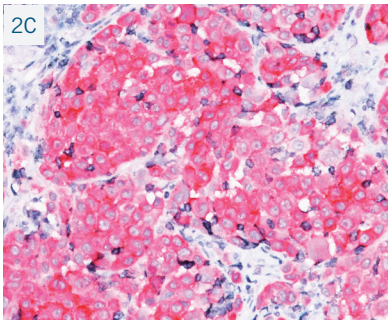
Melanoma stained with CD8⁺ (black) and PMC-2 (red mask), 20X



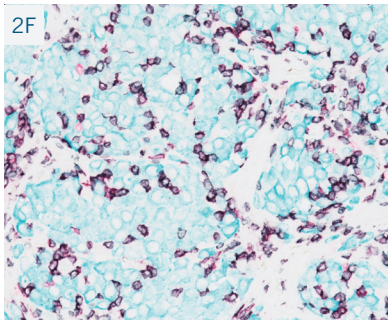
Melanoma stained with CD103⁺ (black) and PMC-2 (red mask) 20X



Melanoma stained with FOXP3⁺ (black) and PMC-2 (red mask), 20X

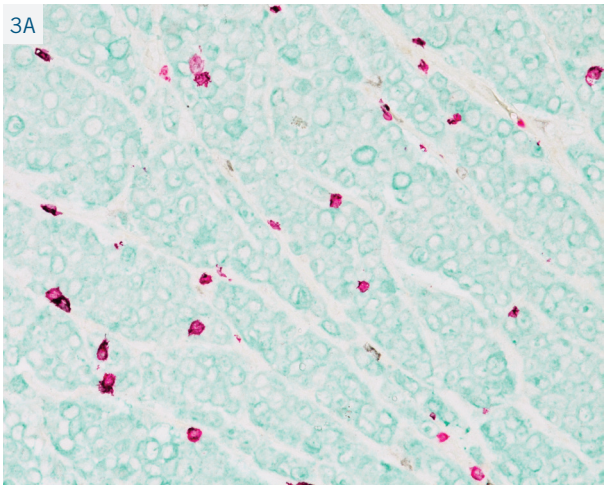


Melanoma stained with PD-1⁺ (black) and PMC-2 (red mask), 20X Note: PD-1⁺ cells are co-expressing with CD8⁺ cells (black/red)

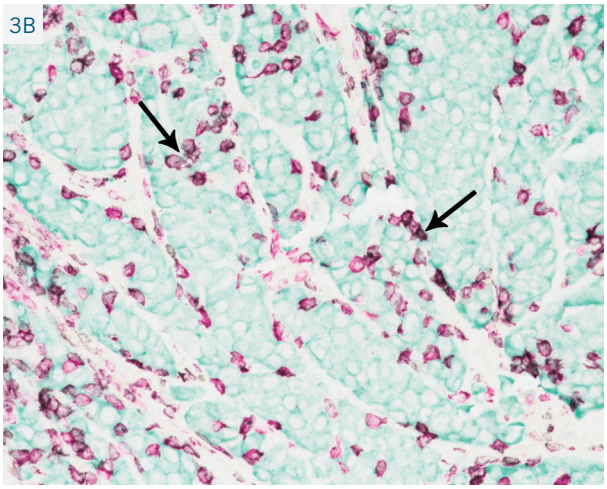


Melanoma stained with PD-1⁺ (black), CD8⁺ (red) and PMC-2 (green mask) 20X

Figure 3

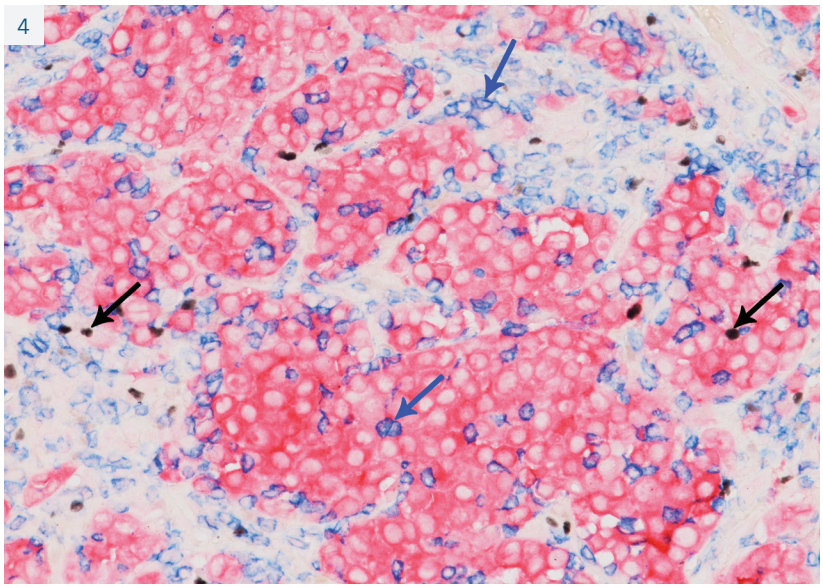


PD-1⁺ (black), CD8 (red) and PMC-2 (green mask) positive cells in the non-growing zone in melanoma, 20X



PD-1⁺ (black), CD8⁺ (red) and PMC-2 (green mask) positive cells in the growing zone in melanoma, 20X Note: PD-1⁺ and CD8⁺ TILs are co-expressing in the same cell (black arrows)

Figure 4



Triple Stain #2; Melanoma TILs stained with FOXP3⁺ (black, black arrows), CD8⁺ (blue, blue arrows) and PMC-2 (red mask), 20X

Conclusion

This study represents one of first fully automated Multiplex IHC assays to simultaneously label and identify different subtypes of TILs; and then sequentially applying PMC-2 as a staining mask (red or green) to identify tumor and non-tumor cells in a single section. Visualizing the co-expression of 2 antibodies was best achieved with black and red chromogens. Different subtypes of TILs were easily identified and could be 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.

References

1. www.cancer.org
2. Redman JM, *et al*. Advances in immunotherapy for melanoma. BMC Med. 2016 Feb 6;14(1):20.
3. http://blog.dana-farber.org
4. Eikawa S, *et al*. Monitoring multifunctionality of immune-exhausted CD8 T cells in cancer patients. Methods Mol Biol. 2014;1142:11-7.
5. 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 Oct;117(1):165-70. 6
6. Webb JR, *et al*. 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.
7. Djenidi F *et al*. $CD8^+$ $CD103^+$ tumor-infiltrating lymphocytes are tumor-specific tissue-resident memory T cells and a prognostic factor for survival in lung cancer patients. J Immunol. 2015 Apr 1;194(7):3475-86.
8. Ahmadzadeh M, *et al*. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. Blood 2009 Aug 20;114(8):1537-44
9. Anichini A, *et al*. Tumor-reactive $CD8^+$ early effector T-cells identified at tumor site in primary and metastatic melanoma. Cancer Res. 2010 Nov 1;70(21):8378-87.

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