

# A Multiplex Immunohistochemistry Evaluation of Immune Checkpoint Receptors and Mismatch Repair Proteins in Colorectal Carcinoma

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## Introduction

Colorectal carcinoma (CRC) is the third most common cancer type in men and women in the United States and represents a significant burden of disease. A recent, small cohort study demonstrated that refractory, metastatic CRC patients with mismatch repair deficient (dMMR)/microsatellite instability-high (MSI-H) CRC are favorable candidates for checkpoint immunotherapy. A 40% objective response rate was achieved for MSI-H CRC compared with 0% for microsatellite stable (MSS) CRC following the treatment of the immunotherapy, pembrolizumab.<sup>1</sup> This initial success along with five multi-cohort clinical trials has led to the accelerated FDA approval of pembrolizumab as a first-line immunotherapy for refractory and advanced CRC.<sup>2</sup> With this achievement in the field of immunotherapy, several combination therapy trials have begun. Key immune inhibitory ligands, receptors and metabolic enzymes such as LAG3, IDO1, CTLA4, and GITR are being investigated in clinical trials in combination with PD-1 or PD-L1 inhibitors.<sup>3,4</sup> Drugs such as CD137 (4-1BB) that have been found to be direct immune stimulators are currently in active clinical testing in combination with PD-1 or PD-L1 inhibition.<sup>4,5</sup> In this study, we have evaluated a large panel of immune checkpoint receptors (ICRs) in the tumor microenvironment (TME) on FFPE tissue of CRCs by multiplex immunohistochemistry (IHC).

## Design

Tissue sections were initially reviewed by H&E to confirm the presence of CRC with adequate pathological features including histology, grade, and abundance of tumor-infiltrating lymphocytes (TILs). An MMR protein panel (MLH1, MSH2, MSH6, and PMS2) was tested by IHC to determine dMMR/MSI-H status through loss of MLH1/PMS2 or MSH2/MSH6. Multiple ICRs including CD4, CD8, CD137, CTLA4, FOXP3, GITR, IDO1, LAG3, PD-1 and PD-L1 (Table 1) were tested by multiplex IHC on 18 CRC cases consisting of MSI-H CRCs (n=8) and MSS CRCs (n=10) using four chromogenic end-points. Expression of ICRs in the TME was determined at four sites: within-tumor, tumor invasive margin, peri-tumor and tertiary lymphoid structure (TLS). ICR expression levels were determined by counting and averaging positive tumor-infiltrating lymphocytes in three high-power fields (HPF, 400X) and categorized in the following manner: 0 (negative, no expression), 1 (weak, 1-25 cells), 2 (moderate, 26-50 cells), and 3 (high, >50 cells).

## Results

Each of the multiplexed immune checkpoint receptors exhibited clear and concise staining throughout all MSI-H and MSS colorectal carcinoma cases (Figures 1-9). PD-L1 expression was found in 10-30% of tumor cells in 50% of MSS cases (5/10) and in 25% of MSI-H cases (2/8). For the remaining cases, tumors did not express PD-L1; however, the tumor-infiltrating immune cells expressed PD-L1. All cases of MSI-H and MSS demonstrated high counts (>50/HPF) of CD8+ T cells within-tumor and in the invasive margin, peri-tumor and TLS. 50% of MSI-H cases (5/10) showed moderate to high PD-1 expression and 30% (3/10) were weak for PD-1 expression (Figures 6-8). All MSS cases showed weak to moderate PD-1 expression. There were high levels of PD-1+ immune cells in the invasive margin and within-tumor; however, expression of the PD-1 immune cells was scarce in peri-tumor and TLS. CD4, FOXP3, CTLA4, IDO1, LAG3 and CD137 had weak to moderate expression in all CRC cases (Figures 1-9). CD4+, FOXP3+, CTLA4+, LAG3+ and CD137+ immune cells were found mostly in the invasive margin, within-tumor, and peri-tumoral areas. IDO1 was expressed in tumor cells, dendritic cells and macrophages with the majority present within-tumor, invasive margin, and peri-tumor (Figures 5 and 9). GITR was expressed in TILs that were present within-tumor, invasive margin, peri-tumor and TLS (Figures 2-5 and 9). Within the TLS, the predominant cellular components were CD8+, CD4+ and PD-1+ T cells in all cases. Co-expression of GITR and LAG3, GITR and FOXP3, CD137 and FOXP3, CTLA4 and FOXP3, and CTLA4 and PD-1 were observed (Figures 1-9).

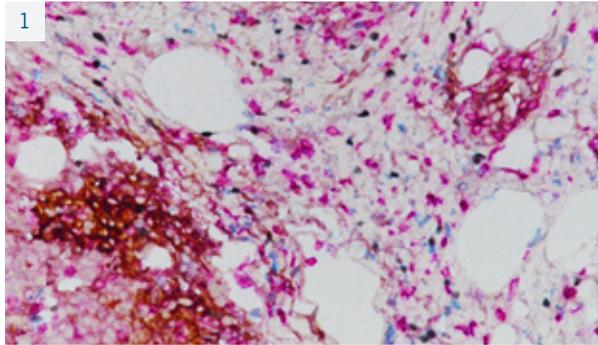
## Conclusion

In the present study, a greater number of MSS CRCs expressed PD-L1 than MSI-H CRCs in tumor cells and tumor-infiltrating lymphocytes (TIL). PD-1 was positive in all CRC cases with variable expression between MSI-H and MSS CRCs. There appears to be no significant difference in TIL expression of the additional immune checkpoint receptors tested in microsatellite instability-high colorectal carcinomas and microsatellite stable colorectal carcinomas.

Multiplex technology appears to allow for superior evaluation of the synergistic relationship between immune checkpoint receptors as well as the interconnection between inhibitory barriers to CD8+ effector T cells and regulatory T cells developed by immune checkpoint receptors within each of the tumoral regions. Further study of expression patterns of immune checkpoint receptors may provide useful information for clinical management of patients with colorectal carcinoma.

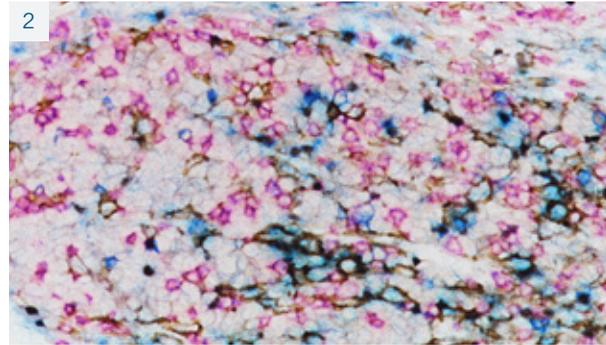
# Figures 1-9: CRC Cases

Figure 1: Invasive margin and within-tumor



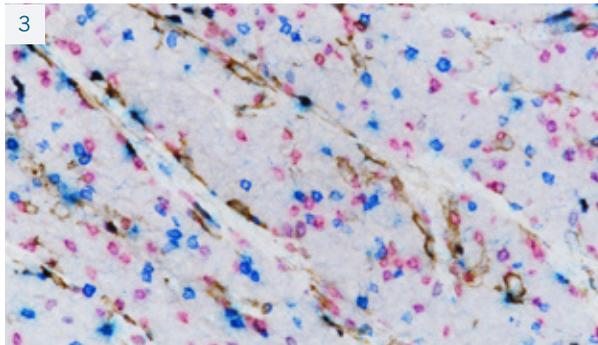
**CD8 (Red), PD-L1 (Brown), FOXP3 (Black) and CD4 (Blue)**  
 CD8+ T cells, CD4+ T cells, and FOXP3+ T regulatory cells are intermixed. PD-L1 labels tumor cells and tumor-infiltrating immune cells.

Figure 2: Invasive margin and within-tumor



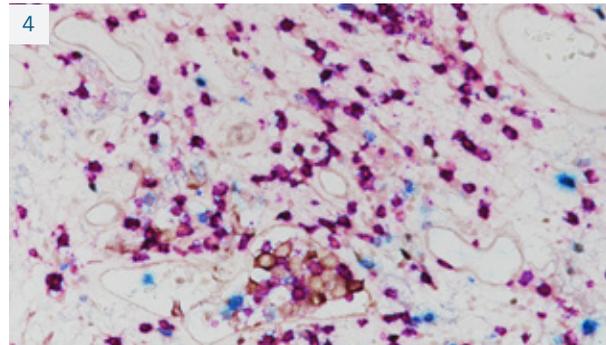
**CD8 (Red), PD-L1 (Brown), FOXP3 (Black) and GITR (Blue)**  
 CD8+ T cells (dominant), GITR+ T cells, and FOXP3+ T regulatory cells are intermixed. PD-L1 marks tumor cells and tumor-infiltrating immune cells.

Figure 3: Within-tumor



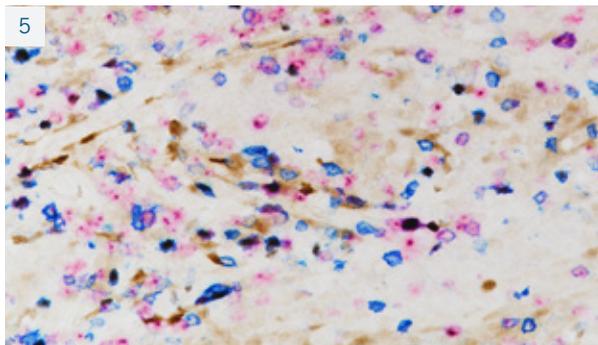
**CD8 (Red), PD-L1 (Brown), FOXP3 (Black) and GITR (Blue)**  
 CD8+ T cells, GITR+ T cells, and FOXP3+ T regulatory cells are intermixed. Many FOXP3+ T regulatory cells co-express GITR. PD-L1 decorates tumor cells and immune cells.

Figure 4: Peri-tumoral



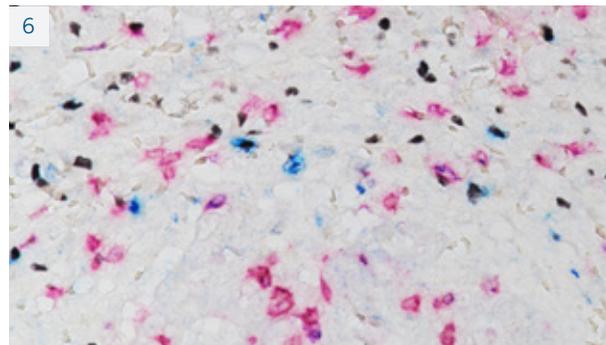
**CD8 (Red), PD-L1 (Brown), FOXP3 (Black), and GITR (Blue)**  
 CD8+ T cells (dominant), GITR+ T cells, and FOXP3+ T regulatory cells are intermixed. PD-L1 labels tumor-associated macrophages and immune cells.

Figure 5: Invasive margin and within-tumor



**LAG3 (Red), IDO1 (Brown), FOXP3 (Black) and GITR (Blue)**  
 LAG3+ T cells, GITR+ T cells, and FOXP3+ T regulatory cells are intermixed. LAG3 & GITR are co-expressed in some T cells. GITR and FOXP3 are co-expressed in many T cells. IDO1 labels tumor cells, tumor-associated macrophages, & dendritic cells.

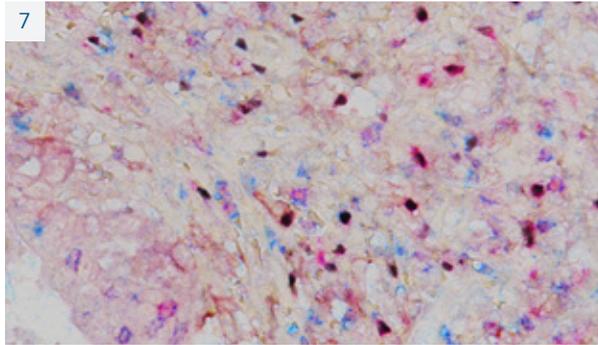
Figure 6: Invasive margin and within-tumor



**CD8 (Red), PD-1 (Brown), FOXP3 (Black) and CD137 (Blue)**  
 CD8+ T cells, CD137+ T cells, and FOXP3+ T regulatory cells are intermixed. Occasionally FOXP3 and CD137 are co-expressed on T cells.

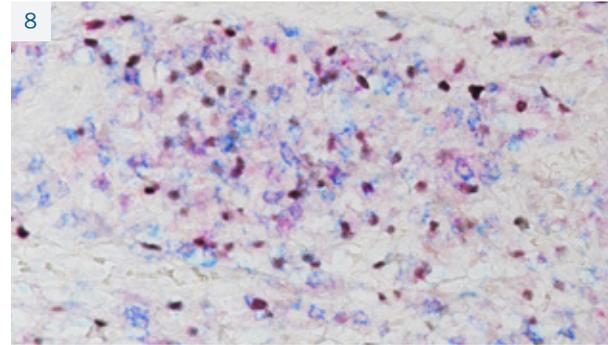
## Figures 1-9: CRC Cases

Figure 7: Invasive margin and within-tumor



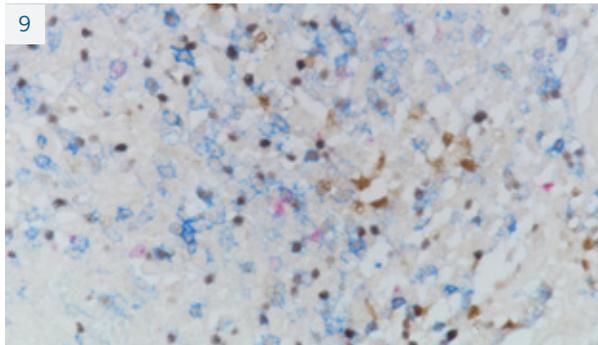
**CTLA-4 (Red), PD-L1 (Brown), FOXP3 (Black) and PD-1 (Blue)**  
 CTLA-4+ T cells, PD-1+ T cells, and FOXP3+ T regulatory cells are intermixed. CTLA-4 and FOXP3 are co-expressed in certain T cells. Some T cells show co-expression of CTLA-4 and PD-1.

Figure 8: Tertiary lymphoid structure



**CTLA4 (Red), PD-L1 (Brown), FOXP3 (Black) and PD-1 (Blue)**  
 CTLA4+ T cells, PD-1+ T cells, and FOXP3+ T regulatory cells are present. Some CTLA-4+ T cells co-express PD-1. Co-expression of CTLA-4 and FOXP3 can be seen on certain immune cells.

Figure 9: Peri-tumoral



**LAG3 (Red), IDO1 (Brown), FOXP3 (Black) and GITR (Blue)**  
 LAG3+ T cells, GITR+ T cells, & FOXP3+ T regulatory cells are present. Some LAG3+ T cells co-express GITR. Co-expression of GITR and FOXP3 can be seen on some immune cells. IDO1 labels tumor cells, tumor-associated macrophages, and dendritic cells.

Table 1

Antibody	Target/Gene	Immune Category
PD-L1	Programmed cell death 1 ligand	Immune checkpoint receptor
PD-1	Programmed cell death 1	Immune checkpoint receptor
FOXP3	Forkhead box protein P3	T-regulatory cells
CD8	CD8	Cytotoxic T cells
CD4	CD4	T helper cells
GITR	Glucocorticoid-induced TNFR-related protein	Co-stimulatory receptor
LAG3	Lymphocyte-activation gene 3	Co-inhibitory receptor
IDO1	Indoleamine 2,3-dioxygenase 1	Metabolic immune checkpoint
CTLA4	Cytotoxic T lymphocyte antigen 4	Co-inhibitory receptor
CD137	4-1BB	Co-stimulatory receptor

## References

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