

BRIEF REPORT

Clear cell ovarian cancers with microsatellite instability: a unique subset of ovarian cancers with increased tumor infiltrating lymphocytes and PD-1/PD-L1 expression

Brooke E. Howitt^{1*}, Kyle C. Strickland^{1*}, Lynette M. Sholl¹, Scott Rodig¹, Lauren L. Ritterhouse¹, Dipanjan Chowdhury², Alan D. D'Andrea², Ursula A. Matulonis³, Panagiotis A. Konstantinopoulos^{3**}

¹ Department of Pathology, Brigham and Women's Hospital, Harvard Medical School

² Division of Genomic Stability and DNA Repair, Dana Farber Cancer Institute, Harvard Medical School

³ Medical Gynecologic Oncology Program, Dana Farber Cancer Institute, Harvard Medical School

*Brooke Howitt and Kyle Strickland contributed equally to this work.

**Address for correspondence:

Panagiotis A. Konstantinopoulos, M.D., Ph.D.

Dana Farber Cancer Institute, YC-1424, 450 Brookline Ave, Boston, MA 02215

Email: panagiotis_konstantinopoulos@dfci.harvard.edu

Phone: 617-632-5269

Fax: 617-632-3479

ABSTRACT

Clear cell ovarian carcinoma (CCOC) represents a distinct histologic subtype of ovarian cancer associated with significantly worse prognosis across all stages and no effective therapeutic options. Here, we report a rare but clinically important cohort of CCOCs with MSI (MSI-CCOCs), which are highly immunogenic and may thus be very responsive to immune checkpoint blockade. CCOCs with MSI exhibit significantly higher number of CD8+ TILs, higher CD8/CD4 ratio and higher PD-1+TILs compared to microsatellite stable (MSS) CCOCs and compared to high grade serous ovarian cancers, which are the most common histologic subtype of ovarian cancer. Of note, PD-L1 expression in tumor cells or immune cells was noted in all cases of CCOCs with MSI. These observations open an alternative therapeutic avenue for a fraction of patients with CCOC and argue for routine testing of CCOCs for MSI, a test that is not currently routinely performed.

KEYWORDS

Clear Cell Ovarian Cancer, Microsatellite Instability, Tumor infiltrating lymphocytes, Immunotherapy, PD-1, PD-L1

INTRODUCTION

Clear cell carcinoma is a distinct histologic subtype of epithelial ovarian cancer (EOC) which accounts for approximately 10% of all EOCs. With the exception of very early stage disease, clear cell ovarian cancers (CCOC) are associated with worse prognosis compared with other EOC histologic subtypes across all stages[1]. CCOCs pose a significant clinical challenge as they are relatively resistant to the conventional platinum based chemotherapy used traditionally in EOC, and there are no effective alternative therapeutic strategies.

Interestingly, while immune checkpoint inhibitors have demonstrated only modest responses collectively in patients with EOC [2, 3], isolated responses have been observed in patients with CCOCs. Specifically, in a study of nivolumab in platinum resistant ovarian cancer, one of two patients with clear cell histology exhibited complete response, and in a study of avelumab in recurrent/refractory OC, both patients with clear cell tumors exhibited partial response [2, 3]. Identification of CCOCs that respond well to immune checkpoint blockade may provide patients with a powerful therapeutic option against this otherwise devastating disease.

Response to PD-1/PD-L1 blockade in multiple tumor types has been associated with presence of tumor infiltrating lymphocytes (TILs) and elevated PD-L1 expression in tumor and/or immune cells[4]. In this study, we evaluated whether certain subsets of CCOCs exhibit a higher number of TILs and/or increased expression of PD-1/PD-L1

and may therefore be more responsive to PD-1/PD-L1 blockade. As control, we compared the pattern of TILs and expression of PD-1/PD-L1 in CCOCs to high grade serous ovarian cancers (HGSOCs), the most common histologic subtype of EOC which accounts for 70% of all cases. We evaluated CCOCs for presence of microsatellite instability (MSI) as well as specific subsets of CCOCs that are associated with endometriosis and ARID1A/BAF250A loss.

Here, we report for the first time that CCOCs with MSI may represent a unique subset of CCOCs with enhanced immunogenicity and increased expression of PD-1/PD-L1 compared to other CCOCs and HGSOCs, and may therefore be excellent candidates for therapy with immune checkpoint inhibitors.

RESULTS

Association of TILs with presence of MSI, endometriosis and ARID1A/BAF250A loss in CCOCs

Of the 30 CCOCs, 3 (10%) exhibited MMR protein expression loss by IHC and were confirmed MSI-H by PCR, 8 (26.6%) exhibited loss of ARID1A/BAF250A by IHC (Supplemental Figure 1), and 22 (73.3%) were associated with personal history and/or histologic evidence of endometriosis.

We did not observe any difference between CCOCs (n=30) with presence versus absence of endometriosis, or between CCOCs (n=30) with retention versus loss of ARID1A/BAF250A expression in terms of CD3+, CD8+, CD4+ and PD-1+ TILs (Figures 1A and 1B). Conversely, CCOCs with MSI had a statistically significantly higher CD8+ TILs (53 vs 20.4, $p=0.016$), higher CD8/CD4 ratio (4.74 vs 1.5, $p<0.001$) and higher PD1+TILs (22 vs. 3.56, $p<0.001$) compared to the remaining microsatellite stable (MSS) CCOCs (Figure 1C) while there was a trend for higher CD3+ TILs (mean 52.33 vs 33, $p=0.3$). Figure 2 shows two representative MSS-CCOC and MSI-CCOC cases with staining results for CD3+, CD8+ and PD-1+ TILs.

Presence of TILs in CCOCs versus HGSOCs

We compared 30 CCOC cases, of which 3 exhibited MSI, with 53 previously published HGSOCs [5]. Overall, there was no statistically significant difference in the number of CD3+ TILs (34.9 vs 36.2, $p=0.83$), CD8+ TILs (23.7 vs 28.7, $p=0.33$), CD4+ TILs (15.9

vs 13.4, $p=0.34$) and PD-1+ TILs (5.4 vs 3.5, $p=0.19$) between CCOCs (all cases) and HGSOCs (all cases), (Figure 3A). However, when we compared specifically CCOCs with MSI to HGSOCs, we noted that CCOCs with MSI had significantly higher number of PD-1+TILs (22 vs. 3.45, $p<0.001$) compared to HGSOCs (Figure 3B), and a trend for higher CD3+ TILs (52.33 vs 36.2, $p=0.25$) and CD8+ TILs (53 vs 28.7, $p=0.072$).

PD-L1 expression in CCOCs

Evaluation of PD-L1 expression in tumor cells and in intraepithelial and peritumoral immune cells in CCOCs showed that all 3 CCOCs with MSI expressed PD-L1 either in the tumor cells (in one out of 3 CCOCs with MSI) or the intraepithelial or peritumoral immune cells (in 3 out of 3 CCOCs with MSI). Conversely, only 44.4% of the CCOCs with MSS expressed PD-L1 either in the tumor cells or the intraepithelial or peritumoral immune cells. Figure 2 shows two representative MSS-CCOC and MSI-CCOC cases with staining results for PD-L1. We did not observe any difference between CCOCs with presence versus absence of endometriosis, or between CCOCs with retention versus loss of ARID1A/BAF250A expression in terms of PD-L1 expression in tumor cells or the intraepithelial or peritumoral immune cells.

DISCUSSION

CCOC, especially when diagnosed at an advanced stage, is associated with poor prognosis due to the lack of effective therapeutic options. Strikingly, our study highlights a unique subset of CCOCs with MSI (MSI-CCOCs) that are associated with enhanced immunogenicity and may therefore be susceptible to immunotherapy. CCOCs with MSI exhibited significantly higher number of TILs and PD-1+ TILs compared to the remaining CCOCs (MSS-CCOCs) and compared to HGSOEs, and uniformly expressed PD-L1 in the tumor cells and/or the intraepithelial or peritumoral immune cells. Our study suggests that MSI-CCOCs may be excellent candidates for PD-1/PD-L1 blockade and supports routine evaluation of CCOCs for MSI, a test that is not performed routinely for these patients. Our study extends previous observations that, regardless of tissue of origin, tumors with DNA repair defects such as tumors with MSI, *POLE* mutations, or homologous recombination deficiency are associated with higher number of TILs which is counterbalanced by overexpression of immune checkpoints such as PD-1/PD-L1[5-8], and further supports the notion that these tumors may respond well to immune checkpoint blockade.

Unlike MSI status, we did not observe any difference in immunogenicity between CCOCs with presence versus absence of endometriosis, or between CCOCs with retention versus loss of ARID1A/BAF250A expression in this study. However, a non-statistical trend for lower CD3+ and CD8+ TILs was noted for tumors with ARID1A loss (Figure 1B) which may be worth to be explored in a larger study.

We acknowledge a limitation of our study which is the small number of MSI-CCOC cases. However, to put things into perspective, CCOC is an uncommon subtype of ovarian cancer, representing only 10% of all EOCs. We were able to identify and examine in our institution a total 30 cases of CCOCs and found 3 CCOCs cases (i.e. 10% of CCOC cases) that harbor MSI. Based on this information, we estimate that MSI-CCOCs represent a very rare cohort of EOCs, i.e. only approximately 1% of all EOCs. Although the small number of MSI-CCOC limits the power of detecting statistically significant differences, we were still able to detect statistically significant differences in the number of TILs and PD-1+ TILs between MSI-CCOCs and the remaining CCOCs as well as HGSOCs which are the more common type of EOCs. Furthermore we observed a consistent pattern of high number of TILs (including PD-1+ TILs) in all MSI-CCOC cases, with expression of PD-L1 in the immune cells and/or the tumor cells in all MSI-CCOC cases. It is important to underscore that the pattern of TILs and PD-1/PD-L1 expression in the MSI-CCOC cases was clearly distinct compared to the MSS-CCOC, and was entirely consistent with previous reports of increased presence of TILs and enhanced PD-1/PD-L1 expression in other MSI positive tumors of non-ovarian origin, such as MSI positive colorectal and endometrial cancers [6, 7]. Of note, MSI positive colorectal cancers have been shown to respond well to PD-1/PD-L1 blockade[9, 10], and several trials of PD-1/PD-L1 blockade in non-colorectal cancers with MSI are currently ongoing.

In conclusion, our study highlights a clinically important cohort of CCOCs with MSI (MSI-CCOCs), which are highly immunogenic and may thus be very responsive to immune checkpoint blockade. These findings open an alternative therapeutic avenue

for a fraction of patients with CCOC, a disease that is resistant to standard chemotherapies and for which there are essentially no effective therapeutic options. Importantly, our study argues for routine testing of CCOCs for MSI, a test that is not routinely performed.

MATERIALS AND METHODS

Tumor samples

This study evaluated 30 cases of CCOC and a previously reported cohort of 53 cases of HGSOc[5]. All cases were retrieved from the Pathology archives of Brigham and Women's Hospital under institutional review board approved protocol.

Determination of MSI status and ARID1A/BAF250A loss

MSI status was determined using immunohistochemistry for the mismatch repair (MMR) proteins MSH2, MSH6, PMS2 and MLH1 (Supplemental Table 1). For cases with loss of expression of one or more MMR proteins, MSI was confirmed by a PCR-based assay using fluorescently labeled primers to five DNA microsatellites (BAT 25, BAT 26, BAT 40, BAT 34c4, and D18S55). Alterations in the length of the repetitive sequences were detected by sizing the PCR products by capillary electrophoresis. The normal and tumor allele patterns were compared for each marker. MSI-high (MSI-H) was defined as instability in two or more markers. Loss of ARID1A/BAF250A was assessed by immunohistochemistry using an antibody (Sigma, HPA005456) directed against the human BAF250a protein (Supplemental Table 1).

Immunohistochemistry and evaluation of tumor infiltrating lymphocytes (TILs)

Immunohistochemistry (IHC) was performed for CD3, CD4, CD8, PD-1 and PD-L1 on formalin fixed paraffin embedded (FFPE) tissue samples using standard protocols as previously reported (see supplemental Table 1) [5, 6]. For evaluation of TILs, we

focused only on intraepithelial lymphocytes, i.e. lymphocytes located within the tumor epithelium, rather than in the peritumoral stroma. The number of intraepithelial PD-1 positive lymphocytes was determined as the average count from three 40X high power fields (HPFs). Positive tumor expression of PD-L1 was defined as greater than or equal to 5% of tumor cells with PD-L1 positivity. Positive PD-L1 expression in intraepithelial and peritumoral immune cells was defined as any PD-L1 positivity within intraepithelial and peritumoral immune cells.

Statistical analyses

Staining results were compared among CCOCs with MSI, endometriosis, or ARID1A loss, and between CCOCs and HGSOCs using Student's t-test as well as Fisher's exact test. All reported p values are two-sided.

FIGURE LEGENDS

Figure 1: Comparison of CD3, CD8, CD8/CD4 ratio and PD-1+ lymphocytes between CCOCs with endometriosis vs no endometriosis (1A), ARID1A/BAF250a loss vs retention (1B) and between MSI-CCOCs and MSS-CCOCs (1C).

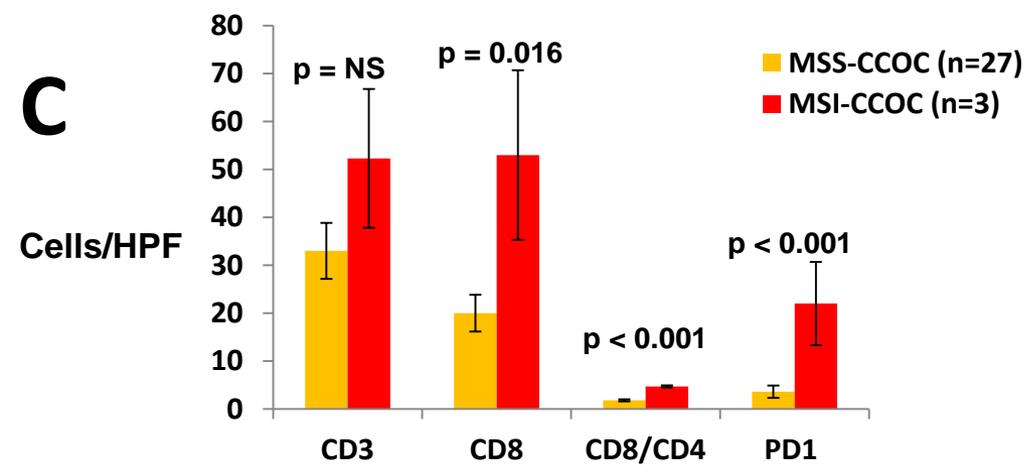
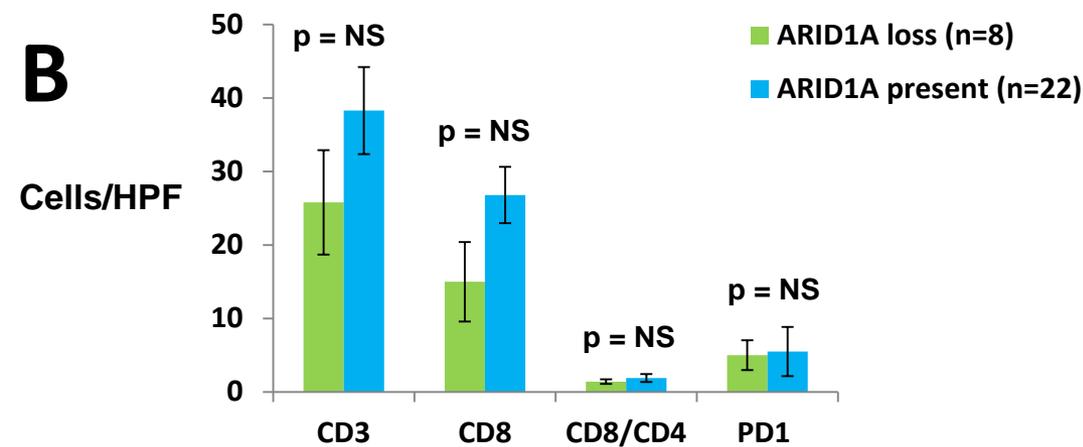
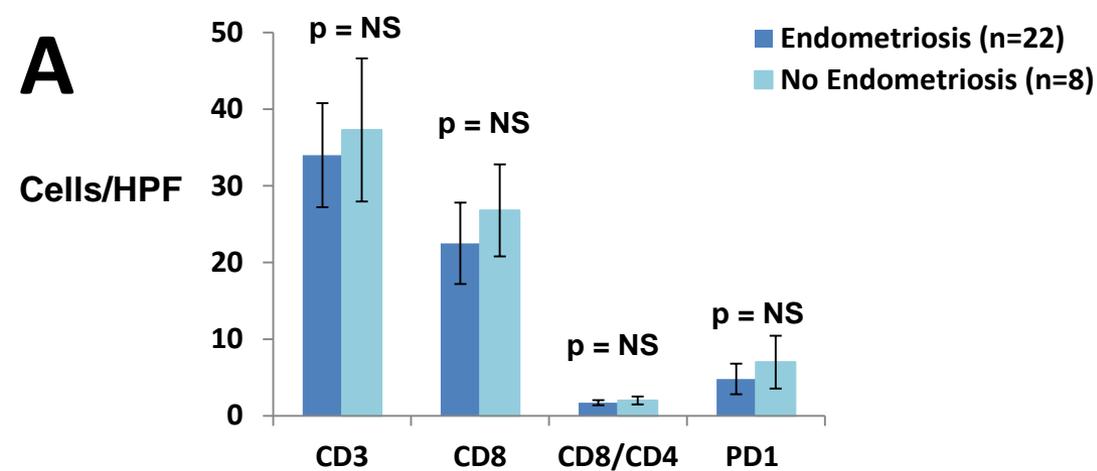
Figure 2: Two representative MSS-CCOC and MSI-CCOC cases with staining results for CD3, CD8, PD-1 and PD-L1 (PD-L1 staining in tumor cells).

Figure 3: Comparison of CD3, CD8, CD8/CD4 ratio and PD-1+ lymphocytes between all CCOCs and all HGSOCs (1A) and between MSI-CCOCs (n=3) and all HGSOCs (1B).

REFERENCES

1. Crotzer DR, Sun CC, Coleman RL, Wolf JK, Levenback CF and Gershenson DM. Lack of effective systemic therapy for recurrent clear cell carcinoma of the ovary. *Gynecol Oncol.* 2007; 105(2):404-408.
2. Hamanishi J, Mandai M, Ikeda T, Minami M, Kawaguchi A, Murayama T, Kanai M, Mori Y, Matsumoto S, Chikuma S, Matsumura N, Abiko K, Baba T, Yamaguchi K, Ueda A, Hosoe Y, et al. Safety and Antitumor Activity of Anti-PD-1 Antibody, Nivolumab, in Patients With Platinum-Resistant Ovarian Cancer. *J Clin Oncol.* 2015; 33(34):4015-4022.
3. Disis ML, Patel MR, Pant S, Infante JR, Lockhart AC, Kelly K, Beck JT, Gordon MS, Weiss GJ, Ejadi S, Taylor MH, von Heydebreck A, Chin KM, Cuillerot J and Gulley JL. (2015). Avelumab (MSB0010718C), an anti-PD-L1 antibody, in patients with previously treated, recurrent or refractory ovarian cancer: A phase Ib, open-label expansion trial. American Society of Clinical Oncology. (Chicago, IL.
4. Meng X, Huang Z, Teng F, Xing L and Yu J. Predictive biomarkers in PD-1/PD-L1 checkpoint blockade immunotherapy. *Cancer Treat Rev.* 2015; 41(10):868-876.
5. Strickland KC, Howitt BE, Shukla SA, Rodig S, Ritterhouse LL, Liu JF, Garber JE, Chowdhury D, Wu CJ, D'Andrea AD, Matulonis UA and Konstantinopoulos PA. Association and prognostic significance of BRCA1/2-mutation status with neoantigen load, number of tumor-infiltrating lymphocytes and expression of PD-1/PD-L1 in high grade serous ovarian cancer. *Oncotarget.* 2016; 7(12):13587-13598.

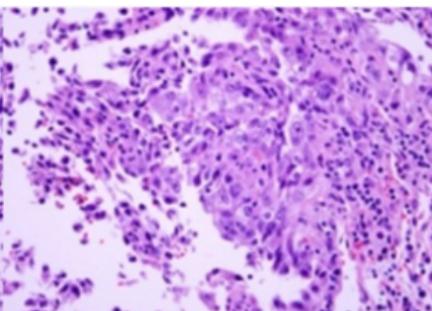
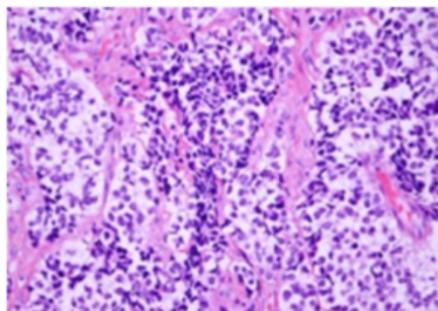
6. Howitt BE, Shukla SA, Sholl LM, Ritterhouse LL, Watkins JC, Rodig S, Stover E, Strickland KC, D'Andrea AD, Wu CJ, Matulonis UA and Konstantinopoulos PA. Association of Polymerase e-Mutated and Microsatellite-Instable Endometrial Cancers With Neoantigen Load, Number of Tumor-Infiltrating Lymphocytes, and Expression of PD-1 and PD-L1. *JAMA Oncol.* 2015; 1(9):1319-1323.
7. Llosa NJ, Cruise M, Tam A, Wicks EC, Hechenbleikner EM, Taube JM, Blosser RL, Fan H, Wang H, Lubber BS, Zhang M, Papadopoulos N, Kinzler KW, Vogelstein B, Sears CL, Anders RA, et al. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. *Cancer Discov.* 2014; 5(1):43-51.
8. van Gool IC, Eggink FA, Freeman-Mills L, Stelloo E, Marchi E, de Bruyn M, Palles C, Nout RA, de Kroon CD, Osse EM, Klenerman P, Creutzberg CL, Tomlinson IP, Smit VT, Nijman HW, Bosse T, et al. POLE Proofreading Mutations Elicit an Antitumor Immune Response in Endometrial Cancer. *Clin Cancer Res.* 2015; 21(14):3347-3355.
9. Fader AN, Diaz LA, Armstrong DK, Tanner EJ, Uram J, Eyring A, Wang H, Fisher G, Greten T and Le D. (2016). Preliminary results of a phase II study: PD-1 blockade in mismatch repair-deficient, recurrent or persistent endometrial cancer. 47th Annual Meeting of the Society of Gynecologic Oncology. (San Diego, CA.
10. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Lubber BS, Azad NS, Laheru D, Biedrzycki B, Donehower RC, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med.* 2015; 372(26):2509-2520.



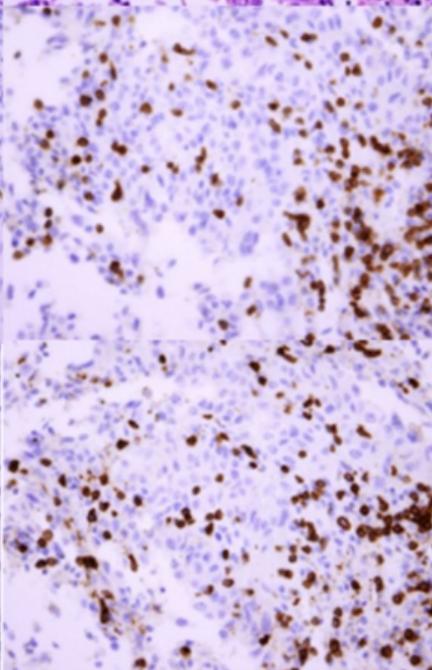
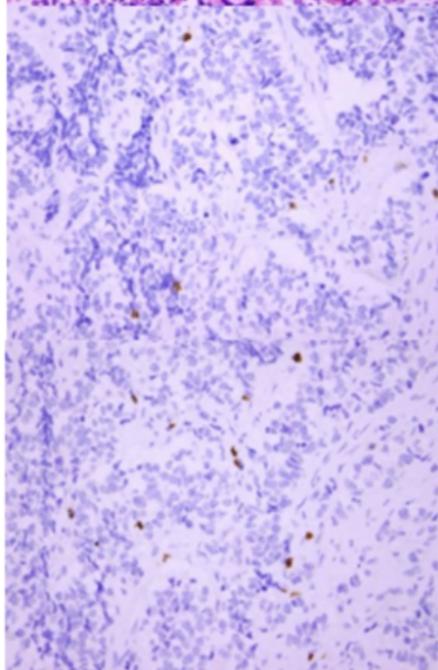
MSS-CCOC

MSI-CCOC

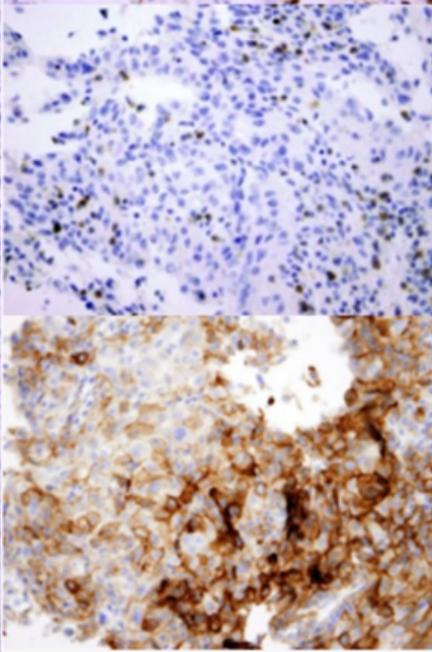
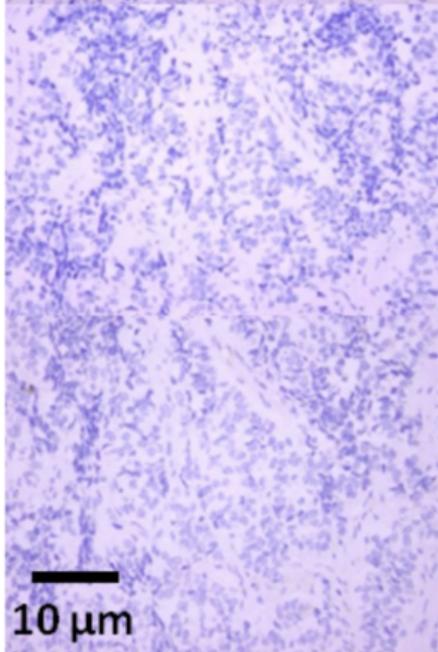
H&E



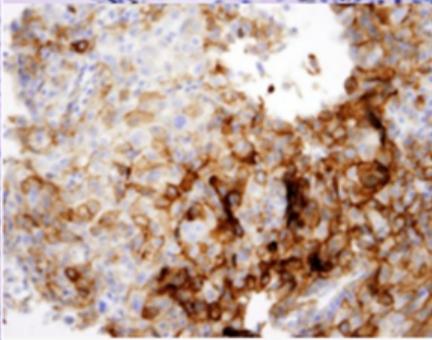
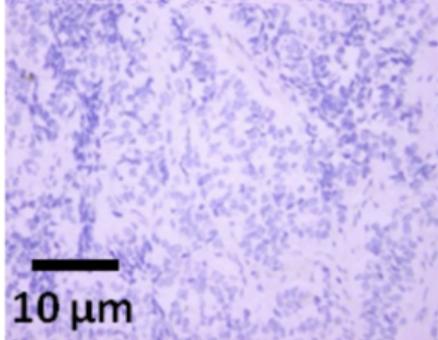
CD3



CD8



PD-1



PD-L1

10 μ m

