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Immune Environment in Serrated Lesions of the Colon: Intraepithelial Lymphocyte Density, PD-1, and PD-L1 Expression Correlate with Serrated Neoplasia Pathway Progression

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Abstract

The serrated neoplasia pathway accounts for approximately 20% of colorectal carcinomas (CRCs). Sessile serrated adenomas (SSAs), the main precursor lesion of the serrated pathway, are molecularly driven by *MLH1* promoter methylation and microsatellite instability (MSI) in their progression to CRC. MSI-high (MSI-H) lesions are highly immunogenic and associated with a high density of tumor infiltrating lymphocytes. Our study's aim was to determine how the kinetics of this immune environment relates to SSAs in their progression through low-grade (SSA-LD) to high-grade dysplasia (SSA-HD), and CRC. We analyzed 74 cases (16 CRCs, 14 SSA-HD, and 44 SSA-LD). Cases of hyperplastic polyp and SSA without dysplasia were analyzed for comparison. MSI status, intraepithelial lymphocyte (IEL) density, and immune checkpoint expression were assessed by immunohistochemistry for mismatch repair proteins, CD3, and PD-1/PD-L1; respectively. Average IEL density was 12, 18.6, 21.6, and 31 for SSA, SSA-LD, SSA-HD, and CRC; respectively, as opposed to 8.1 in normal colon ($p < 0.0001$). Average PD-1/PD-L1 lymphocytic expression was 1.1/1.0, 1.2/2.9, 4.8/6.9, and 12.4/15.2 in SSA, SSA-LD, SSA-HD, and CRC; respectively, compared to 0.5/0 in normal crypts ($p < 0.0001$). IEL and PD-1/PD-L1 lymphocytic expression values of MSI-H lesions were 22.6, 27.7, 36.8; and 3/6.5, 6.2/10.6, and 18.3/17.6 in MSI-H SSA-LD, SSA-HD, and CRCs; respectively (p ranged from 0.0478 to 0.3529). PD-L1 epithelial expression was positive in 40% of SSAs, 59.1% of SSAs-LD, 100% SSAs-HD, and 60% of CRCs ($p < 0.0001$). Increased IELs and PD-1/PD-L1 expression correlate with sequential progression of SSAs, through development of cytologic dysplasia, to CRC and MSI-H status.

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Keywords

serrated neoplasia pathway; immune environment; sessile serrated adenoma; dysplasia; MSI; PD-1; PD-L1; tumor infiltrating lymphocytes

1.1 Introduction

The realization that serrated lesions progress from sessile serrated adenoma (SSA) to sessile serrated adenoma with cytologic dysplasia (SSA-D) to invasive carcinoma led to recognition of the serrated pathway of colorectal carcinogenesis. SSAs, through stepwise progression through the aforementioned dysplasia-carcinoma sequence, give rise to the vast majority of sporadic microsatellite unstable (MSI-H) colorectal adenocarcinomas (CRCs). Serrated pathway carcinomas can harbor diverse molecular alterations, but commonly share *BRAF* mutation and a CpG island methylator phenotype that can result in either microsatellite unstable or microsatellite stable (MSS) CRCs as reviewed by Jass [1] and Snover [2], *BRAF* activating mutation is an early event in the formation of an SSA from normal mucosa or possibly a microvesicular hyperplastic polyp (HP) precursor. Serrated neoplasia pathway progression from SSA to sporadic MSI-H CRC is subsequently driven by CpG island methylation of the *MLH1* promoter region, which results in epigenetic silencing and onset of DNA mismatch repair (MMR) deficiency. Once microsatellite instability (MSI) develops, lesions can rapidly accumulate additional mutations, coinciding with the development of dysplasia and progression to invasive carcinoma [1,2].

Microsatellite unstable carcinomas and carcinomas arising from the serrated pathway are frequently characterized by high numbers of tumor infiltrating lymphocytes [3–5], and express the immune checkpoint receptor PD-L1 [6,7]. However, few studies have examined the immune environment in both serrated and non-serrated precursor lesions [8,9]. Rau et al. were the first to study the immune environment in serrated precursor lesions, with an emphasis on tumor infiltrating lymphocytes (TILs) [8]; while Rubio et al. analyzed TIL density in colorectal conventional adenomas with low and high-grade dysplasia, and carcinomas [9]. However, no studies have analyzed immune checkpoint protein expression, particularly PD-1/PD-L1, in precursor serrated lesions and its relationship to the onset of MSI-H status and morphologic dysplasia. Therefore, the objective of this study was to examine the density of intraepithelial lymphocytes (IELs), expression of PD-1 in infiltrating lymphocytes, expression of PD-L1 in infiltrating lymphocytes and lesion epithelium, and MSI status, at various stages of progression through the serrated neoplasia pathway.

1.2 Materials and Methods

1.2.1 Patients and samples

Study cases were identified by retrospective search of pathology databases at Rhode Island Hospital and Miriam Hospital over a 10-year period and at Beth Israel Deaconess Medical Center over 3-years using keywords “sessile serrated adenoma” or “sessile serrated polyp” in combination with “dysplasia” or “carcinoma.” A total of 74 cases, including both biopsy and resection specimens, of SSAs associated with cytologic dysplasia or invasive carcinoma

were analyzed. This included 44 SSAs with low-grade dysplasia (SSAs-LD), 14 SSAs with high-grade dysplasia (SSAs-HD), and 16 adenocarcinomas (ACAs). Ten cases each of hyperplastic polyp (HP) and sessile serrated adenoma without dysplasia were prospectively identified during routine pathologic evaluation and included for comparison. Pathology and endoscopy reports were reviewed for patient clinical features, including age at diagnosis, gender, and anatomic location. No cases of Lynch syndrome were included. Left sided neoplasia was defined as polyps or cancer arising from the splenic flexure distally and right sided those proximal to the splenic flexure.

This study was performed under a protocol approved by the Rhode Island Hospital and Beth Israel Deaconess Medical Center Institutional Review Boards.

1.2.2 Histomorphologic subtypes

Hematoxylin and eosin-stained slides of potential study cases were retrieved and reviewed by two independent Gastrointestinal Pathologists for subtype classification, through strict assessment of cytological and architectural features. Criteria for SSAs-LD and SSAs-HD study inclusion included presence of areas with serrated architecture, characterized by luminal in-folding or “saw-tooth” appearance of epithelium, and at least one unequivocal architecturally distorted crypt with basal dilatation and/or horizontal branching (particularly if associated with inverted maturation or asymmetric proliferation zone) [10]. In addition, a transition to cytologically dysplastic areas within the same tissue fragment displaying architectural features described above was a requisite for study inclusion. Representative examples of histomorphologic classification subtypes are shown in Figure 1. Cases with separate tissue fragments with dysplasia and serrated architecture, but no single fragment displaying both, were excluded. ACAs with associated architecturally unequivocal areas of SSA with contiguous or abrupt transition to cytologic dysplasia in the vicinity of invasion met study criteria and were included as serrated neoplasia pathway ACAs.

1.2.3 Immunohistochemistry

Table 1 contains a summary of the immunohistochemical stains used. In brief, stains for each antigen were performed on 4 µm paraffin tissue sections. The panel included antibodies against CD3, PD-1, PD-L1, PMS2, and MSH6. The stains were loaded on the Ventana XT Autostainer (Roche, Switzerland) and run to completion using the Discovery DabMap Detection Kit (Roche).

1.2.3.1 Immunohistochemical assessment and scoring—IEL density, corresponding to highest density area of CD3 positive cells, and intraepithelial lymphocytic expression of PD-1 and PD-L1 per 200 epithelial cells were separately scored for areas of hyperplastic polyp, SSA, SSA-LD, SSA-HD, and ACA, and also for areas corresponding to MSI-H. Normal adjacent colonic crypts were also scored for internal control purposes. Extent and intensity of PD-L1 expression in epithelium was scored semi quantitatively in the different morphologic areas. The extent of epithelial PD-L1 membranous staining, either complete or apical, was semiquantitatively scored from 0-3 according to the percentage of staining cells (0 = 0%; 1 = less than 25%; 2 = 25-50%; 3 = more than 50%). Epithelial PD-L1 staining intensity was considered weak if there was absent or barely perceptible staining

at 10× magnification, moderate if easily identifiable at 10× magnification, and intense if easily identifiable at 4× magnification. Only cases with moderate-to-intense staining intensity were considered positive. A two-panel test for PMS2 and MSH6 expression by immunohistochemistry was utilized to evaluate MSI status in serrated precursor lesions. The sensitivity and specificity of the two panel test as a screen for MSI approached 100% when compared to the traditional four panel test (11). Carcinomas were tested for all four MMR proteins (MLH1, PMS2, MSH2, and MSH6) by IHC to assess MSI status.

1.2.4 Statistical analysis

Statistical analysis included mean values for CD3 IEL density, PD-1 and PD-L1 lymphocytic expression, and percentage positive cases for PD-L1 epithelial expression; which were measured within each histopathologic lesion subtype and according to MSI status. Results were compared using analysis of variance (ANOVA) (followed by Tukey's pairwise comparison if necessary), t-test, and chi-square test (followed by Fisher's exact test if necessary). All statistical analysis was done using JMP Pro 13.0 (Cary, NC). $P < 0.05$ was considered statistically significant.

1.3 Results

1.3.1 Clinicopathological features

The clinicopathologic features are summarized in Table 2. The mean patient age increased with lesion progression with means of 58 yr. for HPs, 57 yr. for SSAs, 65 yr. for SSAs-LD, 72 yr. for SSAs-HD, and 77 yr. for ACAs with serrated precursor lesions ($P < 0.0001$). Sixty-five of the 94 included cases were from female patients (69%); including 40% of HPs, 80% of SSAs, 66% of SSAs-LD, 71% of SSAs-HD, and 88% of ACAs. Excluding HPs, which were all located in the sigmoid or rectum, the vast majority of lesions were located in the right colon (81%).

1.3.2 Immunohistochemistry

1.3.2.1 Correlation of IEL density with serrated neoplasia pathway progression—CD3-positive intraepithelial lymphocyte density correlated with progression of serrated pathway precursor lesions, HPs to SSAs, through the dysplasia-carcinoma sequence (Figure 2, Table 2). Mean IEL density was 12.9 per 200 epithelial cells in HPs and 12 in SSAs, compared to 8.1 in normal adjacent colonic crypts. The IEL density increased to 18.6 in SSAs-LD, 21.6 in SSAs-HD, and 31 in CRCs; a statistically significant trend ($p < 0.0001$). An example of CD3-positive intraepithelial lymphocytic distribution is seen in supplementary Figure 1.

1.3.2.2 Correlation of PD-1 and PD-L1 lymphocytic expression with serrated neoplasia pathway progression—PD-1 and PD-L1 expression within intraepithelial lymphocytes, corresponding to highest IEL density area by CD3 immunostain, were also scored separately for all different histomorphologic lesion subtypes. Similar to IEL density results, PD-1 and PD-L1 expression within lymphocytes increased with stepwise progression through serrated carcinogenesis (Figures 3–4, Table 2). While none of the ten cases of HPs assessed showed PD-1 expression, mean values increased from 1.1, to 1.2, 4.8,

and 12.4 in SSA, SSA-LD, SSA-HD, and CRC; respectively, compared to mean PD-1 lymphocytic expression of 0.5 in normal colonic epithelium ($p < 0.0001$). The extent of PD-L1 expression within lymphocytes showed a similar trend within serrated pathway lesions, with values of 1.0, 2.9, 6.9, and 15.3 in SSAs, SSAs-LD, SSAs-HD, and CRC; respectively, compared to 0 in normal colonic mucosa ($p < 0.0001$)

1.3.2.3 Correlation of PD-L1 epithelial expression with serrated neoplasia pathway progression—

Lesions were scored as positive for PD-L1 expression in epithelium (at least moderate staining in 25% of cells) in 50% of HPs, 40% of SSAs, 59.1% of SSAs-LD, 100% of SSAs-HD, and 60% of ACAs; compared to 26.3% in non-lesional colonic epithelium ($p = 0.008$), refer to Figure 5 and Table 2. PD-L1 epithelial positivity showed an overall upward trend with increased lesion severity.

1.3.3 Correlation of microsatellite instability with immune microenvironment

Results of MSI status correlation with immune microenvironment are summarized in Table 3. A total of 22 of the 74 (29.7%) study cases showed immunohistochemical evidence of microsatellite instability, with all cases showing loss of expression of mismatch repair protein PMS2. Among the 22 total MSI-H cases, there were 5 SSAs-LD (11%), 7 SSAs-HD (50%), and 10 CRCs (62%). The onset of microsatellite instability had the strongest association with increased IEL density and lymphocytic PD-1/PD-L1 expression. Mean IEL densities were 22.6, 27.7, and 36.8 for MSI-H SSAs-LD, SSAs-HD, and CRCs; respectively. In comparison, microsatellite stable counterparts mean IEL values were 18, 15.6, and 21.3 ($p = 0.546$). Similarly, PD-1/PD-L1 lymphocytic expression values were 3/6.5, 6.2/10.6, and 18.3/17.6 ($p = 0.2964$ and 0.0478 , respectively) for MSI-H SSAs-LD, SSAs-HD, and CRCs; respectively. Corresponding results for MSS lesions were 1.1/2.3, 3/1.8, and 4.7/11.8 ($p = 0.022$ and < 0.0001 , respectively). Epithelial PD-L1 expression was also higher in MSI-H lesions, with 21 of the 22 (95%) MSI-H cases positive for PD-L1 expression, compared to 44 out of 52 (85%) MSS SSAs-D/CRC cases. Of note, individual study cases with areas of intact mismatch repair protein expression adjacent to areas with lost expression showed this same pattern with higher IEL density and PD-1/PD-L1 expression corresponding to MSI-H areas when compared to MSS regions within the same slide (Figure 5).

1.4 Discussion

In our study, we set out to evaluate immune features of the serrated carcinogenesis pathway and their correlation with morphologic stepwise dysplasia-carcinoma progression and MSI status. To our knowledge this is the first study to look at immune checkpoint expression patterns in serrated precursor lesions, by analyzing expression patterns of PD-1 and PD-L1 in serrated carcinogenesis and how these relate with lesion progression, dysplasia, and MSI-H status. Of note, while onset of microsatellite instability is classically associated with progression to carcinoma, a significant proportion of carcinomas that arise through the serrated pathway are MSS, which we also include in our study. Very few studies have analyzed the particulars of the immune microenvironment of these MSS serrated carcinomas.

Our results show that IEL density, lymphocytic PD-1/PD-L1, and epithelial PD-L1 expression increase as lesions sequentially progress through the dysplasia-carcinoma sequence of serrated carcinogenesis. Our findings of increased IEL density with serrated neoplasia progression are in line with those previously described by Rau et al. [8]. We also demonstrate a strong correlation of increased IEL density and immune checkpoint expression with MSI-H status, as MSI-H SSAs-D and CRCs had significantly higher IEL density values and PD-1/PD-L1 expression when compared to MSS serrated carcinomas. Interestingly, we found that increased numbers of IELs, including PD-1 and PD-L1 expressing IELs, and PD-L1 epithelial expression can be seen prior to the development of MMR loss. Thus, infiltration of lymphocytes and upregulation of PD-L1 in precursor lesions is not solely dependent on generation of immunogenic neoantigens resulting from MMR deficiency, but is likely dependent on earlier, as yet undefined, molecular mechanisms.

The serrated neoplasia pathway is one of two major morphologic stepwise colorectal carcinogenesis pathways, accounting for approximately 20% of all colorectal carcinomas (CRCs). Presently, sessile serrated adenomas (SSAs) are recognized as premalignant lesions associated with distinct molecular alterations in their progression to CRC. SSAs commonly harbor somatic *BRAF* mutations and arise within a background of global genome hypermethylation as reviewed by Jass and Snover [1,2]. Molecularly, this epigenetic instability is the main driver of serrated neoplasia pathway progression. By means of a yet unknown initiating mutation or epigenetic event, vast promoter CpG island hypermethylation leads to progressive silencing of tumor suppressor genes and a CpG island methylator phenotype (CIMP) [12]. When and if, hypermethylation of the *MLH1* gene promoter occurs, gene expression is silenced and mismatch repair deficiency ensues. The onset of mismatch repair deficiency and subsequently MSI serve as a major boost in serrated lesion carcinogenesis. The hypermutator phenotype characteristic of MSI-H lesions results in mutation burden accumulation, increasing the probability of progression to carcinoma [13]. Morphologically, this progression manifests in SSAs by development of cytologic adenomatous dysplasia, with stepwise progression from low-grade (SSA-LD) to high-grade dysplasia (SSA-HD), and ultimately carcinoma (ACA) [14,15].

SSAs with cytologic dysplasia are the intermediate lesion in the progression of SSAs to CRC through serrated carcinogenesis. SSAs-D are molecularly advanced lesions that frequently harbor MSI with loss of MLH1 expression, and are associated with significantly higher rates of invasive carcinoma than conventional adenomas [16]. A recent study evaluated MLH1 expression by immunohistochemistry in SSAs-D and found that 73% of 266 analyzed lesions had loss of MLH1, while 19% were associated with invasive carcinoma [17]. Additionally, other studies have shown retained expression of MLH1 in areas of SSA with concomitant loss within areas of dysplasia within the same lesion; suggesting that onset of MSI is an important driver in the dysplasia-carcinoma progression [18,19].

Onset of mismatch repair deficiency and MSI-H phenotype leads to accumulation of frameshift mutations within microsatellites located in coding regions of tumor cell DNA. As neoplastic cells proliferate and divide, previously unrecognized new “non-self” or neoantigens are produced, setting off a powerful immune response and acting as a homing factor that attract numerous tumor infiltrating lymphocytes (TILs) that have been primed for

an anti-tumor cytotoxic response. Indeed, studies of the immune environment in CRCs with MSI have shown that MSI-H tumors are associated with a higher density of TILs than MSS lesions [3–5] and that these TILs are often cytotoxic and activated [20,21]. Additionally, other studies have shown that overall number of frameshift mutations in CRCs with MSI correlates with tumor infiltrating lymphocyte density, specifically CD8+ lymphocyte density [22,23].

While the MSI tumor microenvironment is rich in antitumor TILs, multiple anti-immune counter-regulatory pathways (PD-1/PD-L1, IDO-1) may limit immune response and resist tumor elimination. Upregulation of immune checkpoints, which are a constellation of different intrinsic immune inhibitory pathways designed to maintain self-tolerance and prevent damage to our own healthy tissues [24], by tumor cells counter the initial host cytotoxic response and allow neoplastic cells to evade the immune system. Llosa et al. showed that the immune environment in MSI CRCs is highly active and that tumors can escape the immune response through upregulated expression of multiple different immune checkpoint ligands [25]. This was further supported by a study of medullary CRC that showed increased expression of immune checkpoint ligands IDO and PD-L1 in medullary tumor cells and greater numbers of CD8+, PD-1+, PD-L1+ and FoxP3+ TILs; highlighting the complexity and dynamism of the immune microenvironment of MSI CRCs [26].

The PD-1/PD-L1 and programmed death ligand-2 (PD-L2) is one of the most important and currently studied checkpoints. In the PD-1 pathway, interaction between the PD-1 receptor expressed on the surface of T-cells and PD-L1/PD-L2 ligands expressed on the tumor cell surface renders T-cells inactive. Studies have shown that mismatch-repair deficient tumors have significantly higher rates of PD-L1 and lymphocytic PD-1 expression when compared with mismatch-repair-proficient tumors [6]. PD-L1 expression in CRC is also associated with clinicopathologic and molecular features of the serrated pathway of colorectal carcinogenesis. Specifically, PD-L1 expression in CRCs is associated with high TIL density, BRAF mutation, medullary morphology, and microsatellite instability [7].

While many studies have analyzed the immune landscape of CRCs with MSI, only one study evaluated the immune microenvironment of serrated precursor lesions, including SSAs with dysplasia (SSA-D) [8]. Rau et al. examined IEL density in different serrated polyps and SSAs-D. The authors found that numbers of intraepithelial lymphocytes (IELs) were significantly higher in SSA-D compared to SSAs, which in turn showed significantly greater numbers of IELs when compared to HPs and conventional adenomas [8].

In conclusion, our results show that increased IEL density and PD-1/PD-L1 expression correlate with progression of SSAs to SSAs with cytologic dysplasia to CRC, and particularly with MSI-H status. Our findings support the stepwise dysplasia-carcinoma sequence of serrated carcinogenesis and the hypermutator phenotype mechanism of MSI-H lesions, which stimulates a host immune response characterized by brisk lymphocytic infiltration counterbalanced by immune checkpoint expression upregulation by neoplastic cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Study highlights

IEL density, lymphocytic PD-1/PD-L1, and epithelial PD-L1 expression increase with sequential progression of serrated neoplasia

Increased IEL density and immune checkpoint protein expression strongly correlate with MSI-H status

Increased numbers of IELs and PD-1/PD-L1 expression can be seen in serrated precursor lesions prior to onset of MSI

Figure 1. Serrated neoplasia morphologic dysplasia to carcinoma stepwise progression. A, Sessile serrated adenoma (H&E, original magnification $\times 100$). B, Sessile serrated adenoma with low-grade dysplasia (H&E, original magnification $\times 100$). C, Sessile serrated adenoma with high-grade dysplasia (H&E, original magnification $\times 100$). D, Adenocarcinoma arising from sessile serrated adenoma (H&E, original magnification $\times 20$).

Figure 2.

Intraepithelial lymphocyte density in serrated neoplasia pathway progression. A, Sessile serrated adenoma (CD3 IHC, original magnification $\times 200$). B, Sessile serrated adenoma with low-grade dysplasia (CD3 IHC, original magnification $\times 200$). C, Sessile serrated adenoma with high-grade dysplasia (CD3 IHC, original magnification $\times 200$). D, Adenocarcinoma arising from sessile serrated adenoma (CD3 IHC, original magnification $\times 200$).

Figure 3. Intraepithelial PD-1 lymphocytic expression in serrated neoplasia pathway progression. A, Sessile serrated adenoma (PD-1 IHC, original magnification $\times 200$). B, Sessile serrated adenoma with low-grade dysplasia (PD-1 IHC, original magnification $\times 200$). C, Sessile serrated adenoma with high-grade dysplasia (PD-1 IHC, original magnification $\times 200$). D, Adenocarcinoma arising from sessile serrated adenoma (PD-1 IHC, original magnification $\times 200$).

Figure 4. Epithelial and intraepithelial PD-L1 lymphocytic expression in serrated neoplasia pathway progression. A, Sessile serrated adenoma (PD-L1 IHC, original magnification $\times 200$). B, Sessile serrated adenoma with low-grade dysplasia (PD-L1 IHC, original magnification $\times 200$). C, Sessile serrated adenoma with high-grade dysplasia (PD-L1 IHC, original magnification $\times 200$). D, Adenocarcinoma arising from sessile serrated adenoma (PD-L1 IHC, original magnification $\times 200$).

Figure 5.

Immune microenvironment of serrated neoplasia pathway with onset of microsatellite instability. A, Sessile serrated adenoma with abrupt transition to low-grade dysplasia (H&E, original magnification $\times 100$). B, Sessile serrated adenoma with low-grade dysplasia showing loss of PMS2 expression in dysplasia component (PMS2 IHC, original magnification $\times 100$). C, Higher IEL density in area of SSA-LD with MSI versus adjacent area of SSA with intact mismatch repair protein expression (CD3 IHC, original magnification $\times 100$). D, Increased PD-L1 epithelial and lymphocytic PD-L1 expression in area of SSA-LD with MSI versus adjacent area of SSA with intact mismatch repair protein expression (PD-L1 IHC, original magnification $\times 100$).

Table 1:

Summary of Antibodies and Methods

Antibody	Source	Host	Antigen Retrieval	Dilution	Detection Method
CD3	Dako Carpinteria, CA	Rabbit Polyclonal	EDTA pH 9 100°C, 40 min	RTU	Ventana XT DabMap Kit
PD1	Abcam Cambridge, MA	Mouse Monoclonal, Clone NAT 105	EDTA pH 9 100°C, 40 min	1:100	Ventana XT DabMap Kit
PDL1	Abcam Cambridge, MA	Rabbit Monoclonal, Clone EPR1161	EDTA pH 9 100°C, 40 min	1:1500	Ventana XT DabMap Kit
MSH6	Dako Carpinteria, CA	Rabbit Monoclonal, Clone EP49	EDTA pH 9 100°C, 40 min	RTU	Ventana XT DabMap Kit
PMS2	Dako Carpinteria, CA	Rabbit Monoclonal, Clone EP51	EDTA pH9 100°C, 40 min	RTU	Ventana XT DabMap Kit

Table 2:

Clinical characteristics and marker expression of study lesions

	Normal (n=78)	HP (n = 10)	SSA-ND (n = 10)	SSA-LD (n = 44)	SSA-HD (n = 14)	ACA (n = 16)	p-values
Age							<0.0001
Mean	N.A.	58	57	65	72	77	
SEM		3.0	3.0	1.5	3.3	2.0	
Sex							0.1150
Female	N.A.	4 (40%)	8 (80%)	29 (65.9%)	10 (71.4%)	14 (87.5%)	
Male		6 (60 %)	2 (20%)	15 (34.1%)	4 (28.6%)	2 (12.5%)	
Location							<0.0001
Right	N.A.	0	7 (70%)	33 (75%)	13 (92.8%)	14 (87.5%)	
Left		10 (100%)	3 (30%)	11 (25%)	1 (7.2%)	2 (12.5%)	
IEL (CD3+)							<0.0001
Mean	8.1	12.9	12	18.6	21.6	31 *	
SEM	0.6	1.5	1.8	1.4	3.5	5.3	
PD-1							<0.0001 **
Mean	0.5	0	1.1	1.2	4.8	12.4	
SEM	0.1	0	0.4	0.4	1.8	4.3	
PD-L1							<0.0001 ***
Mean	0	0.2	1	2.92	6.91	15.26	
SEM	0	0.13	0.33	0.55	1.78	2.09	
PD-L1(E)							0.0008 ****
Negative	28 (73.7%)	5 (50%)	6 (60%)	9 (40.9%)	0	6 (40%)	
Positive	10 (26.3%)	5 (50%)	4 (40%)	13 (59.1%)	9(100%)	9 (60%)	
MSI status							<0.0001 ****
MSS	N.A.	N.A.	N.A.	38 (88.4%)	7 (50%)	6 (37.5%)	
MSI-H				5 (11.6%)	7 (50%)	10 (62.5%)	

Abbreviations: HP, hyperplastic polyp; SSA-ND, Sessile serrated adenoma without dysplasia; SSA-LD, sessile serrated adenoma with low-grade dysplasia; SSA-HD, sessile serrated adenoma with high-grade dysplasia; ACA, adenocarcinoma

* ANOVA followed by Tukey's pairwise comparison: ACA group has significantly more IELs than normal crypt, HP, SSA-ND, and SSA-LD. Normal crypt group has significantly less IEL than SSA-LD, SSA-HD, and ACA.

** ANOVA followed by Tukey's pairwise comparison: ACA group has significantly more PD-1 (+) IELs than all the other groups.

*** ANOVA followed by Tukey's pairwise comparison: ACA group has significantly more PD-L1(+) IELs than all the other groups; SSA-HD group has significantly more PD-L1(+) IELs than SSA, HP and NL groups SSA-LD group has significantly more PD-L1(+) IELs than SSA, HP and NL groups.

**** Fisher's exact test.

Table 3.

IEL populations in colonic neoplasm based on MSI status.

Markers	SSA-LD (44)	SSA-HD (14)	ACA (16)	p-value
PD-L1(+) IEL				
MSS	2.3±0.5	1.8±0.6	11.8±1.9	<0.0001 *
MSI-H	6.5±2.0	10.6±2.1	17.6±3.1	0.0478 **
PD-1 (+) IEL				
MSS	1.1 ±0.5	3.0±2.1	4.7±1.5	0.022 ***
MSI-H	3.0±0	6.2±2.7	18.3±8.0	0.2964
CD3(+) IEL				
MSS	18.0±1.4	15.6±1.7	21.3±7.3	0.546
MSI-H	22.6±6.2	27.7±6.2	36.8±6.8	0.3529

* Tukey's pairwise comparison: ACA group had significant more PD-L1(+) IELs than SSA-LD and SSA-HD groups. No difference was seen between SSA-LD and SSA-HD groups.

** Tukey's pairwise comparison: no significant difference between individual groups.

*** Tukey's pairwise comparison: ACA group had significant more PD-1(+) IELs than SSA-LD group.