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Immune Activation in Mismatch Repair Deficient Carcinogenesis: More Than Just Mutational Rate

Jason A. Willis[1], **Laura Reyes-Uribe**[2], **Kyle Chang**[2,3], **Steven M. Lipkin**[4], **Eduardo Vilar**[*,2,3]

[1]Hematology and Oncology Fellowship Program, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX

[2]Department of Clinical Cancer Prevention, The University of Texas MD Anderson Cancer Center, Houston, TX

[3]MD Anderson Cancer Center UT Health Graduate School of Biomedical Sciences, Houston, TX

[4]Department of Medicine, Weill-Cornell Medical College, Cornell University, New York, NY

Abstract

MMR-deficient colorectal cancers (dMMR CRC) are characterized by the expression of highly-immunogenic neoantigen peptides, which stimulate lymphocytic infiltration as well as up-regulation of inflammatory cytokines. These features are key to understanding why immunotherapy (specifically PD-1 and/or CTLA-4 checkpoint blockade) has proved to be highly effective for the treatment of patients with advanced dMMR CRC. Importantly, pre-clinical studies also suggest that this correlation between potent tumor neoantigens and the immune microenvironment is present in early (pre-malignant) stages of dMMR colorectal tumorigenesis as well, even in the absence of a high somatic mutation burden. Here, we discuss recent efforts to characterize how neoantigens and the tumor immune microenvironment co-evolve throughout the dMMR adenoma-to-carcinoma pathway. We further highlight how this pre-clinical evidence forms the rational basis for developing novel immunotherapy-based CRC prevention strategies for patients with Lynch syndrome.

Keywords

Lynch syndrome; Mismatch Repair Deficiency; Neoantigens; Mutational rate; Checkpoint inhibitors; Immunoprevention; Microsatellite Instability; Colorectal cancer

[*]**Corresponding Author:** Eduardo Vilar, MD, PhD; Department of Clinical Cancer Prevention – Unit 1360, The University of Texas MD Anderson Cancer Center, PO Box 301439, Houston, TX 77230-1439; P: (713) 745-4929; F: (713) 794-4403; EVilar@mdanderson.org.

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Introduction

Over the past decade, DNA mismatch repair (MMR) deficiency has emerged as a critically-important biomarker with implications for the management of both early- and advanced-stage colorectal cancer (CRC) (1). Approximately 10–15% of CRC exhibit MMR deficiency, which is characterized by a propensity for accumulating single-nucleotide mutations and insertion-deletion loops (indels) in the somatic genome, particularly within short repetitive sequences such microsatellites (2,3). MMR-deficient tumors often exhibit a high mutation burden and may express neoantigens generated by frameshift mutations in coding microsatellites, such as the 10-adenine mononucleotide repeat in the *TGFBR2* gene (4).

As part of the standard molecular workup for CRC, MMR deficiency can be assessed on the basis of microsatellite instability (MSI) and/or loss-of-expression of MMR proteins in bulk tumor tissue specimens (5). MMR-deficiency in the tumor is often secondary to Lynch syndrome, an autosomal dominant hereditary cancer syndrome caused by monoallelic pathogenic germline mutations in MMR pathway genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*, *EPCAM*) (6). More frequently, MMR deficiency occurs as a sporadic (non-hereditary) process characterized by a distinctive hyper-proliferative, serrated morphology, DNA methylation abnormalities including *MLH1* epigenetic silencing (CpG Island Methylator Phenotype, CIMP), and elevated frequency of activating *BRAF* mutations (7–9).

Altogether, dMMR CRC represents a unique molecular sub-type of this disease with distinctive histopathologic features and clinical outcomes. One of the most prominent features is the enrichment of tumor stroma with infiltrating lymphocytes, and overexpression of prostaglandins and inflammatory cytokines in dMMR tumors (10–16). This inflammatory microenvironment is thought to be driven by recognition of the high burden of tumor neoantigens on Major Histocompatibility Complex (MHC) class I alleles by the adaptive immune system (Figure 1, later stages). This model not only helps explain the favorable prognostic implications of MMR-deficiency in CRC, but also supports the rationale for immunotherapy-based treatment strategies such as with checkpoint inhibition. In this regard, pivotal examples can be found in the setting of metastatic CRC (17,18). In particular, the phase II study CheckMate-142 (clinicaltrials.gov ID NCT02060188) recently demonstrated the safety and durable efficacy of nivolumab (anti-programmed cell death protein 1 [PD-1]) given with or without low-dose ipilimumab (cytotoxic T lymphocyte associated antigen 4 [CTLA-4]) as second-line therapy for patients with advanced dMMR CRC (19,20). Similar benefit was reported by the phase II study Keynote-164 (NCT02460198) in patients treated with single-agent pembrolizumab (anti-PD-1) (19). These breakthrough results have amplified interest in the potential applications of novel immunotherapy agents not only in the adjuvant therapy setting for dMMR CRC, but also in primary prevention for patients with Lynch syndrome.

A rationale for immunotherapy-based prevention (hereafter referred to as immunoprevention) strategies in Lynch syndrome is supported by multiple lines of evidence, including the identification MMR-deficient histologically normal appearing colon crypts as the earliest definable abnormality in pre-neoplastic colorectal epithelium in Lynch syndrome (13). With respect to existing immunomodulatory agents, non-steroidal anti-inflammatory

drugs (NSAIDs) inhibit cyclo-oxygenase 2 (COX-2) and the downstream production of pro-tumorigenic prostaglandins that promote local inflammation. Prior work has shown that NSAIDs (21), more specifically aspirin (22,23), are associated with a modest but reliable chemopreventive benefit to reduce the risk of Lynch syndrome-related CRC (and perhaps other sites) after a continuous exposure of at least two years of duration. Recent pre-clinical work has highlighted that naproxen sodium may have greater chemopreventive efficacy than aspirin (24), although the mechanism is not yet well delineated.

Towards the goal of further improving Lynch syndrome-related cancer mortality, we propose that novel prevention strategies can be developed by elucidating the sequence of events that relate acquisition of MMR deficiency to accumulation of somatic mutations, generation of neoantigens, tumorigenesis and immune recognition, and characterizing the immune cells in the microenvironment of pre-neoplastic lesions (25). Such strategies would include novel immunomodulatory agents, tumor vaccines (26–29), and even low-dose immune checkpoint inhibitors. Importantly, given the unique challenges of drug development in the prevention setting, each strategy needs focused re-examination of the risks and benefits. For example, while anti-PD-1/PD-L1 antibodies may increase immune surveillance, they are also associated with significant rates of severe adverse events. These include immune-related lung, hepatic, skin, neurologic, gastrointestinal, and endocrine toxicities, some of which are fatal (30–44). Thus, while the risk:benefit ratio of PD-1/PD-L1 blockade is acceptable for patients with metastatic tumors and poor prognosis, it is almost certainly not acceptable in the setting of healthy asymptomatic Lynch syndrome patients for cancer prevention, where the tolerance for side effects is very low. PD-1/PD-L1 inhibitors also do not have clear dose response, which makes giving lower doses of these drugs for cancer prevention problematic (30–44).

Here, we will briefly review the molecular basis of neoantigen generation and immune activation as it pertains to MMR-deficient colorectal tumorigenesis. We focus particularly on the pre-cancer state in order to shed light on possible rationales for the development of novel immunoprevention strategies.

Functional implications of MMR Deficiency in CRC carcinogenesis

The highly conserved MMR system facilitates repair of two important types of errors that arise during DNA replication: base pair mismatches and indels (45). Base pair mismatches occur when incorrect nucleotides are inserted into the newly synthesized strand and escape the proofreading function of DNA polymerases. Indel loops usually arise in the context of microsatellites, which are highly polymorphic short repetitive DNA sequences found throughout both prokaryotic and eukaryotic genomes (46–48). At microsatellites, the template and primer strands are prone to slippage (i.e. dissociation and re-annealing) during replication. This generates a loop structure and, most importantly, a discordant number of repeated units between the template and newly synthesized strand (49). In humans, the repair process begins with binding of the MSH2/MSH6 heterodimer to the DNA defect. This is followed by recruitment of the MLH1/PMS2 heterodimer, formation of a sliding clamp structure, and then activation of exonuclease 1 (EXO1) to remove the error-laden DNA segment. The resulting gap is filled in by DNA polymerases, PCNA and ligases (45). Failure

to repair base mismatches or indels leads to propagation of single-nucleotide mutations or MSI, respectively.

The mutagenic process described above is observable not only at the population level, but also within specific individuals, particularly in the context of acquired MMR deficiency. In the case of CRC, MMR-deficiency may occur as an entirely sporadic process due to aberrant hypermethylation of *MLH1* in the tumor, commonly associated with the *BRAF* V600E mutation. However, the hereditary counterpart of this process is Lynch syndrome, which affects more than 1.1 million people in the Unites States, serves as a disease model in which to understand the relevance of MMR deficiency across many cancer types (6). Deleterious germline mutations in *MLH1*, *MSH2*, *MSH6*, *PMS2*, or *EPCAM* render affected individuals with only one functional allele of the respective gene. This is accompanied by somatic inactivation of the second allele (e.g. through mutation or deletion) and thus a predisposition to developing MMR-deficient neoplasms. In general, Lynch syndrome is associated with higher life-time risks of not only colorectal cancer, but also endometrial, ovarian, gastric, small bowel, and urothelial cancers (6).

To place the MMR deficiency phenotype in context, it is helpful to consider it within the mutational 'signature' framework (50,51). Recent mutational profiling efforts have revealed at least three different signatures among pre-cancer lesions (adenomas) and carcinomas of the colorectum (52). The most common of these is *'Signature 1b'*, which is characterized by C>T transitions that are produced after spontaneous deamination of 5-methyl-cytosine. This signature is thought to represent a cumulative and age-related process, as it is also observed in histologically "normal" colonic mucosa that juxtaposes carcinomas with an intact MMR system (52). By contrast, sporadic and hereditary MMR deficiency are associated with *'Signature 6'*, in which tumors accumulate C>T transitions preferentially at NpCpG loci and indels at microsatellites. Colorectal tumors and pre-cancers with this signature are enriched for mutations in *BRAF* V600E (and under-enriched for alterations in *APC*, *KRAS*, and *TP53)* (53). *'Signature 6'* is often referred to by the synonymous term "hypermutator", which denotes a high tumor mutation burden (TMB) that is conventionally defined as more than 10 mutations per megabase (Mb) when measured by whole exome sequencing (54,55). In a related way, *'Signature 10'* is observed in CRC tumors that harbor error-prone DNA polymerase activity due to somatic inactivation of POLE or germline defects in *POLE* or *POLD1* (56). Signature 10 is often termed the 'ultramutator' phenotype, with a mutation rate on the order of 150 mutations per Mb (56).

We and others propose that the biological and clinical implications of MMR deficiency for immunoprevention are best understood not only with respect to overall mutation burden, but perhaps more importantly with respect to the specific functional impacts of MSI. A comprehensive analysis by Hause et al. demonstrated that MSI can be detected in the exomes of multiple different tumor types, albeit with varying frequency and affected loci (57). The study identified a common set of microsatellites, such as those in the coding regions of *NIPBL*, *TCF4,* and *PTEN*, that showed instability across many different tumor types (57). Yet, other microsatellite loci showed instability in only a specific tumor type, thus forming an instability signature. This work builds on prior efforts over the past twenty years to catalogue instable microsatellites that occur in colorectal adenomas and established

carcinomas. For example, MSI has been detected in the coding regions of known oncogenes and tumor suppressor genes such as *TGFβRII, BAX, MSH3, MSH6, IGFIIR and MRE11A* (58–63), as well as others associated with immune surveillance and *B2M* (which is part of the neoantigen presentation machinery) in particular (64). A large set of intergenic (non-coding) microsatellite targets has also been identified (65).

MMR-deficiency not only promotes the development and progression of CRC, but also contributes to the generation of tumor-specific neoantigens. Specifically, neoantigens are created when indels arise within coding microsatellites, leading to an erroneous reading frame (15). Upon translation, the frame-shifted peptide is often truncated and non-functional. In addition to altering downstream functions of the protein, the frameshift creates a new and foreign-appearing amino acid sequence that serves as a substrate for antigen processing and presentation via MHC class I and class II (27,29,66).

Taken together, these findings have contributed to the rational basis for novel primary and/or secondary immunoprevention strategies for dMMR CRC. In particular, while colorectal adenomas may lack the high TMB typically found at later stages of MMR-deficient tumorigenesis, the presence of robust and tissue-specific neoantigens indicates an opportunity to leverage the immune microenvironment to block progression into carcinomas.

Neoantigen-mediated Immune Activation in MMR-deficient Colorectal Tissue

Lynch syndrome serves as a disease model in which to understand how MMR deficiency and the immune microenvironment co-evolve during tumorigenesis. At the earliest stage, comprehensive work by Kloor et al (13) and Shia et al (67) showed that MMR-deficiency is present amongst a large proportion of non-neoplastic intestinal crypts in patients with Lynch syndrome. This observation, which is based on loss of MMR protein staining, may be explained by clonal expansion of histologically-normal appearing crypts that acquired inactivating mutations in the remaining MMR gene allele. Furthermore, CD8+ intra-epithelial lymphocytes were more abundant in these affected crypts, suggesting recognition of microsatellite-derived neoantigens in the normal crypt cells (68). Although such a hypothesis has not been definitively tested in experimental models, striking evidence comes from the observation that neoantigen-specific T cells and antibodies can be detected in the peripheral blood of Lynch syndrome without malignancy (which is more pronounced in patients with advanced MMR-deficient tumors that have higher TMB) (69,70).

Whether MMR-deficient intestinal crypts give rise to some, or all, MMR-deficient adenomas and carcinomas remains a subject of debate (15,67,68,71), as some data suggests that MMR-deficiency can also appear at a later step in tumorigenesis (72). Addressing this question has important implications for CRC prevention in Lynch syndrome. In particular, the prevalence of pre-cancers (particularly adenomas) in Lynch syndrome is age- and gene mutation-dependent and ranges from 10.6% to 33% (73,74), but only around 50% of these adenomas display MMR-deficiency (75,76). By contrast, histologically-normal crypts with MMR-deficiency are relatively abundant in the mucosa of healthy Lynch syndrome patients. This discrepancy raises the possibilities that either MMR-deficient adenomas develop from a

different precursor lesion, or that a significant number of MMR-deficient crypts undergo "immunoediting" prior to transforming into adenomas.

Immunoediting is the process by which aberrant cell growth is halted and regressed by T-cell mediated immunity (77–79). In cases where the lesion is not fully eradicated, immunoediting is followed by equilibrium and ultimately immune escape phases, where the remaining cells are able to evade detection by the immune system. It is therefore important to understand which intrinsic or extrinsic factors permit the formation of MMR-deficient adenomas despite early immune engagement. Notably, MMR-deficient adenomas tend to harbor significantly fewer mutations compared to carcinomas (80,81) and yet infiltrating T-cells directed against microsatellite-derived neoantigens are detectable at this stage as well (Figure 1, pre-cancer stage) (70,82). These observations suggested that neither having a low mutation burden nor a relatively low abundance of neoantigens fully explains immune evasion in colorectal pre-cancers. Indeed, recent work by our group provided evidence of a robust immune activation signature in Lynch syndrome adenomas regardless of their mutation burden (81). By further characterizing the immune signature of Lynch syndrome adenomas, we also revealed global enrichment for $CD4^+$ T cells and enrichment for $FOXP3^+$ regulatory T cells in the subset with high mutation burdens (Figure 1, advanced pre-cancer stage). Additionally, there was up-regulation of both pro-inflammatory cytokines (*IL12A*) and checkpoint blockade (*IFNG*, *CD274/PD1* and, *LAG3*).

These findings correlate well with the known biology and clinical significance of immune activation in carcinomas. A high density of CD3+ cells in CRC is associated with longer cancer-specific survival (12,83,84). Similarly, the presence of CD45RO, CD8+ and CD4+ cells is associated with lower rates of metastasis, vascular or perineural invasion respectively (85). On the contrary, the presence of FOXP3-positive regulatory T cells in normal mucosa of patients with CRC portends a poorer prognosis (85).

Perhaps the best correlation may be found in the setting of advanced dMMR CRC, where treatment with single or dual-checkpoint blockade now plays a pivotal role. Recent work by Turajlic et al. showed up-regulation of multiple checkpoints in CRC tumors, including PD-1, CTLA-4, and lymphocyte activation gene 3 (LAG-3) (86–89). As noted above, checkpoint blockade is effective for a plurality of patients with advanced dMMR CRC (response rates 30–40%) and leads to durable disease control (19,20).

Opportunities and Challenges for Novel Immunoprevention Strategies

Based on the evidence outlined above, at least two novel strategies for the prevention of Lynch syndrome-related CRC are currently under investigation (Table 1). First, the implication of adaptive immune resistance (*PD-1*, *LAG3*, *CTLA-4*) in MMR-deficient colorectal adenomas (81) raises a key question of whether checkpoint blockade could halt the progression of such adenomas into carcinomas. The complete spectrum of factors that regulate the adaptive immune response in adenomas is yet unknown. However, given the availability of inhibitors already on the drug market and known efficacy for patients with advanced MMR-deficient CRC, the *PD-1*/*PD-L1* axis is an especially completing target. Across multiple disease settings and cancer types, the safety profile of single- or dual-

checkpoint blockade is relatively well established, as are common practice guidelines for management of immune-mediated toxicities (90). Nonetheless, clearly the safety and efficacy of such agents in the preventative setting requires thorough and specific evaluation. Towards this end, a phase II single-arm study was recently opened in which adults with Lynch syndrome with *MLH1* and *MSH2* germline mutations (and therefore with maximum life-time risks for colorectal cancer development) and a history of partial colectomy due to advanced adenomas or CRC will receive nivolumab infusion every 3 months for up to 8 doses (clinicaltrials.gov ID NCT03631641) (84). As a secondary prevention study, its primary objective is to determine the incidence of secondary adenomas and CRC among Lynch syndrome patients treated with anti-PD-1.

Second, anti-cancer vaccines hold significant promise not only in hereditary colorectal cancer, but in other solid tumors as well (91). For patients with Lynch syndrome, instability within coding and non-coding microsatellites yields a robust signature of tumor/tissue-specific neoantigens that may be targeted by pre-designed vaccine libraries. In fact, this concept started to be explored in early 2000 triggered by meticulous efforts to catalogue the presence of instability in coding microsatellites using computational approaches coupled with labor intensive validation via PCR-based methods (92) that led to early-phase clinical trials using peptides identified as immunogenic. This approach has now recovered interest thanks to the development of improved pipelines for neoantigen identification that also incorporates immunogenicity predictions for both HLA-I and HLA-II presentation and the access to a wealth of genomic information from tumors (93–96). An example is the recent report from Scarselli and colleagues at NousCom (Rome, Italy) on the identification of 209 frameshift peptide neoantigens shared across colorectal, gastric, and endometrial MSI tumors. Using a viral vector-based delivery system, the investigators observed strong immunogenicity of vaccine in mouse models (26,97). These efforts are resulting in upcoming phase I clinical trials that are awaiting implementation and development in the following months (26,77,98,99). The results of these investigations will be critical for defining the technical feasibility and safety of preventative vaccines for patients with Lynch syndrome.

Conclusion

The up-regulation of immune checkpoints in Lynch syndrome-associated pre-cancers despite a relatively low mutation burden suggests that neoantigen peptides are potent targets. Checkpoint blockade in the adjuvant setting may prove to be highly effective for secondary prevention in patients with Lynch syndrome or sporadic MMR deficient CRCs. However, the benefit-to-risk ratio will need to be clarified given the adverse events associated with PD-1 blockade. Neoantigen vaccination is another approach that is being used for advanced melanoma, glioblastoma and other cancers, and repurposing this approach for primary prevention of MMR deficient cancers in Lynch syndrome patients may be promising. We propose that there is also a rationale for combining vaccine therapy and checkpoint blockade under the hypothesis that a more specific and durable response could be generated to prevent malignant transformation of adenomas, thereby reducing risk of CRC recurrence and increasing cancer-specific survival.

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Abbreviations

CRC	Colorectal cancer
MMR	Mismatch Repair
indels	insertion-deletion loops
NGS	Next-Generation Sequencing
MCH	Major histocompatibility complex
CTL4	T lymphocyte associated antigen 4
PD1	Programmed Cell Death molecule 1
LAG3	Lymphocyte Activation Gene 3

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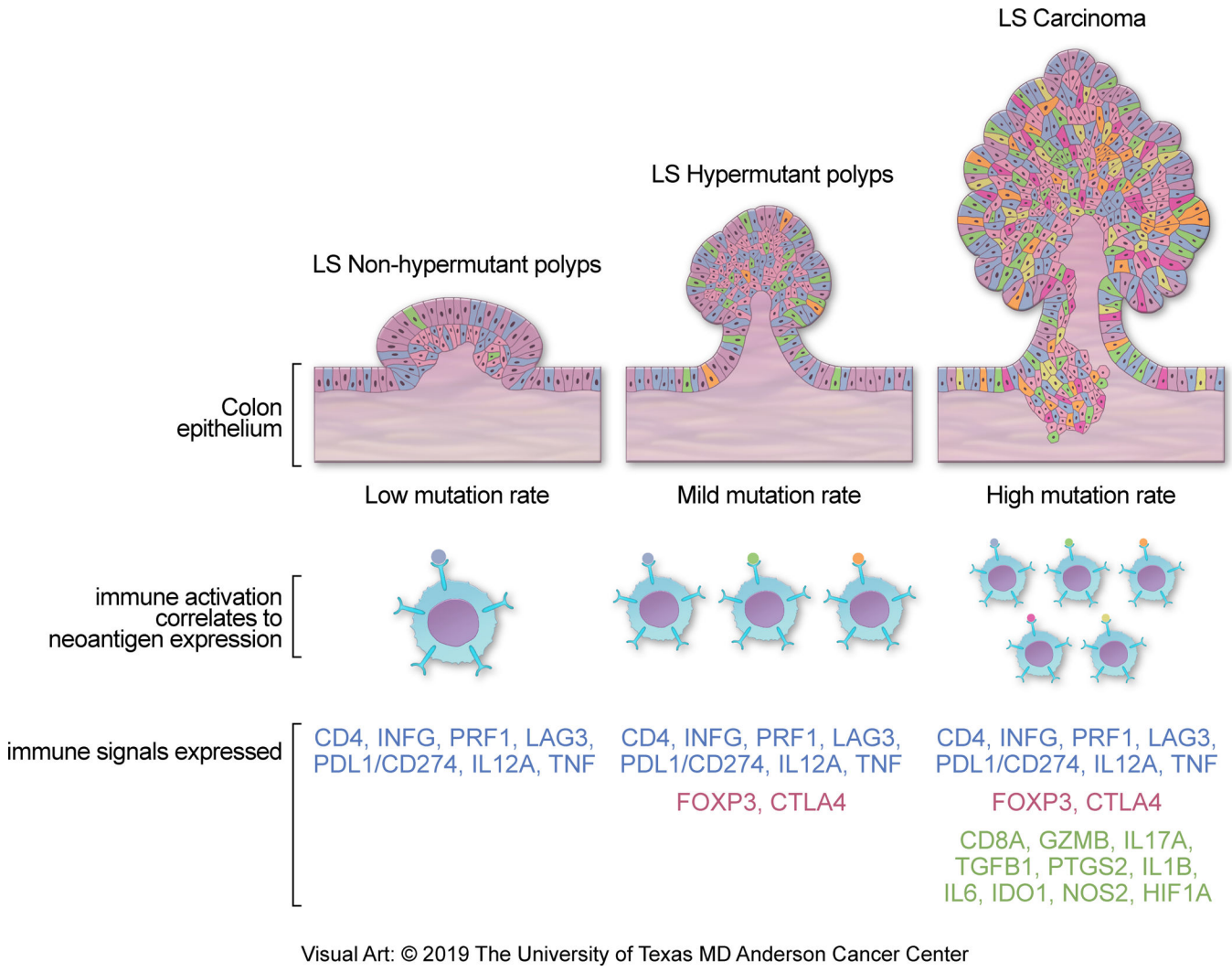

Fig. 1.

Correlation between immune activation (lower track) and neoantigen burden (middle track) along the adenoma-to-carcinoma pathway (top track) in Lynch syndrome-related CRC. The colonic epithelium is shown as confluent cells with an admixture of different colors to represent intralesional mutation and/or neoantigen diversity. Early colorectal adenomas (left column) display markers of immunoreactivity even in the absence of high somatic mutation or neoantigen burden. As the lesions progress to advanced adenomas (middle column) and carcinomas (right column), there is a corresponding rise in mutation/neoantigen burden and markers of immune tolerance. LS, Lynch syndrome. Redrawn with permission; copyright The University of Texas MD Anderson Cancer Center.

Table 1.

Immunoprevention studies for Lynch syndrome-related and/or sporadic dMMR CRC.

Study ID	Study type / design	Location of study	Population	Intervention	Primary endpoint(s)	Status
NCT01461148	Phase I/II	Germany	Adults (18 year and older) Surgically resected stage III and IV colorectal cancers with MSI-H	Biological FSP peptides TAF1B(−1), HT001(−1) and AIM2(−1) weekly for 4 consecutive weeks and repeated every four weeks up to a total of 3 cycles.	Safety Immunogenicity	Completed
NCT0363164	Phase II open-label, single-arm	Ohio, United States Multi-site	Adults (18 years or older) Lynch syndrome secondary to germline *MLH1* or *MSH2* mutation History of hemicolectomy for advanced adenoma or CRC	Checkpoint blockade: nivolumab every 3 months for up to 8 cycles	Incidence rate of adenomas, high-risk adenomas, CRC, and non-CRC at 3 years	Active (recruiting)
NOUS-209	Phase I	Rome, Italy Multi-site	Adults (18 years and older) Stage IV MSI-H tumors	Checkpoint blockade combined with Vaccine of virally-encoded library of 209 neoantigen peptides shared in MSI tumors	Safety Immunogenicity	Not yet active (planned 2019–2020)
NCT01885702	Phase I/II open-label, multi-arm	Nijmegen, Gelderland, Netherlands	Adults (18 years or older) with Lynch syndrome and no history of CRC; or adults with a history of MSI CRC	Vaccine: monocyte-derived peptide-loaded dendritic cells targeting MSI-specific neoantigens and tumor-associated antigen carcinoembryonic antigen (CEA)	Safety	Active (not recruiting)
OncoPept *VAC*	Pre-clinical	India	Adults with Lynch syndrome secondary to germline *MLH1* mutation	In silico prediction of tumor-derived neoantigen peptides	Immunogenicity	Completed