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

Jason A. Willis1, Laura Reyes-Uribe2, Kyle Chang2,3, Steven M. Lipkin4, and Eduardo Vilar2,3

ABSTRACT

Mismatch repair (MMR)–deficient colorectal cancers (dMMR colorectal cancer) are characterized by the expression of highly immunogenic neoantigen peptides, which stimulate lymphocytic infiltration as well as upregulation 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 colorectal cancer. Importantly, preclinical studies also suggest that this correlation between potent tumor neoantigens and the immune microenvironment is present in early (premalignant) 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 coevolve throughout the dMMR adenoma-to-carcinoma pathway. We further highlight how this preclinical evidence forms the rational basis for developing novel immunotherapy-based colorectal cancer prevention strategies for patients with Lynch syndrome.

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 (1). Approximately 10% to 15% of colorectal cancers 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 as microsatellites (2, 3). MMR-deficient tumors often exhibit a high mutation burden and may express neoantigens generated by frame-shift mutations in coding microsatellites, such as the 10 adenine mononucleotide repeat in the TGFBR2 gene (4).

As part of the standard molecular workup for colorectal cancer, 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 monallelic pathogenic germline mutations in MMR pathway genes (MLH1, MSH2, MSH6, PMS2, and EPCAM; ref. 6). More frequently, MMR deficiency occurs as a sporadic (nonhereditary) process characterized by a distinctive hyperproliferative, 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 colorectal cancer represents a unique molecular subtype 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 MHC class I alleles by the adaptive immune system (Fig. 1, later stages). This model not only helps explain the favorable prognostic implications of MMR deficiency in colorectal cancer, 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 colorectal cancer (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 colorectal cancer (19, 20). Similar benefit was reported by the phase II study Keynote-164 (NCT02460198) in patients treated with single-agent pembrolizumab (anti–PD-1; ref. 19). These breakthrough results have amplified interest in the potential applications of novel immunotherapy agents not only in the adjuvant therapy setting for dMMR colorectal cancer, 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 of MMR-deficient histologically normal appearing colon crypts as the earliest definable abnormality in preneoplastic colorectal epithelium in Lynch syndrome (13). With respect to existing immunomodulatory agents, nonsteroidal anti-inflammatory drugs (NSAID) inhibit cyclooxygenase 2 (COX-2) and the downstream production of protumorigenic 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 colorectal cancer (and perhaps other sites) after a continuous exposure of at least 2 years of duration. Recent preclinical work has highlighted that naproxen sodium may have greater chemopreventive efficacy than aspirin (24), although the mechanism is not yet well delineated.

1Hematology and Oncology Fellowship Program, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas. 2Department of Clinical Cancer Prevention, The University of Texas MD Anderson Cancer Center, Houston, Texas. 3MD Anderson Cancer Center UT Health Graduate School of Biomedical Sciences, Houston, Texas. 4Department of Medicine, Weill-Cornell Medical College, Cornell University, New York, New York. Corresponding Author: Eduardo Vilar, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Unit 1360, Houston, TX 77030. Phone: 713-745-4929; Fax: 713-794-4403; E-mail: EVilar@mdanderson.org

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Toward 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 preneoplastic lesions (25). Such strategies would include novel immunomodulatory agents, tumor vaccines (26), and even low-dose immune checkpoint inhibitors. Importantly, given the unique challenges of drug development in the prevention setting, each strategy needs focused reexamination of the risks and benefits. For example, although 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, although 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 precancer state in order to shed light on possible rationales for the development of novel immunoprevention strategies.

**Functional implications of MMR deficiency in colorectal cancer 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 reannealing) 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 colorectal cancer, 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 United States and serves as a disease model in which to understand the relevance of MMR deficiency across many cancer types (6). Detrimental 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).
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 coevolve during tumorigenesis. At the earliest stage, comprehensive work by Kloor and colleagues (13) and Shia and colleagues (67) showed that MMR deficiency is present among a large proportion of nonneoplastic 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+ intraepithelial 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; refs. 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 suggest that MMR deficiency can also appear at a later step in tumorigenesis (72). Addressing this question has important implications for colorectal cancer prevention in Lynch syndrome. In particular, the prevalence of precancers (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 with carcinomas (80, 81) and yet infiltrating T cells directed against microsatellite-derived neoantigens are detectable at this stage as well (Fig. 1, precancer stage; refs. 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 precancers. 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 (Fig. 1, advanced precancer stage). In addition, there was upregulation of both proinflammatory cytokines (IL12A) and checkpoint blockade (IFNG, CD274/PD1, and LG3).

These findings correlate well with the known biology and clinical significance of immune activation in carcinomas. A high density of CD3+ cells in colorectal cancer 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 colorectal cancer portends a poorer prognosis (85).

Perhaps the best correlation may be found in the setting of advanced dMMR colorectal cancer, where treatment with 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 require thorough and specific evaluation. Toward this end, a phase II single-arm study was recently opened in which adults with Lynch syndrome with MSI-H tumors will receive nivolumab infusion every 3 months for up to 8 doses (clinicaltrials.gov ID NCT03631641; ref. 84). As a secondary prevention study, its primary objective is to determine the incidence of secondary adenomas and colorectal cancer development, and a history of partial colectomy due to advanced adenomas or colorectal cancer will receive nivolumab every 3 months for up to 8 cycles.

### 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 colorectal cancer are currently under investigation (Table 1). First, the implication of adaptive immune resistance (PD-1, LAG3, and 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 colorectal cancer, the PD-1/PD-L1 axis is an especially promising 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 require thorough and specific evaluation. Toward this end, a phase II single-arm study was recently opened in which adults with Lynch syndrome with MSI-H tumors will receive nivolumab infusion every 3 months for up to 8 doses (clinicaltrials.gov ID NCT03631641; ref. 84). As a secondary prevention study, its primary objective is to determine the incidence of secondary adenomas and colorectal cancer in Lynch syndrome patients treated with anti-PD-1.

Second, antitumor 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 noncoding microsatellites yields a robust signature of tumor/tissue-specific neoantigens that may be targeted by predesigned 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

### Table 1. Immunoprevention studies for Lynch syndrome–related and/or sporadic dMMR CRC.

<table>
<thead>
<tr>
<th>Study ID</th>
<th>Study type/ design</th>
<th>Location of study</th>
<th>Population</th>
<th>Intervention</th>
<th>Primary endpoint(s)</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT01461148</td>
<td>Phase I/II</td>
<td>Germany</td>
<td>Adults (18 years and older) Surgery resected stage III and IV colorectal cancers with MSI-H</td>
<td>Biological FSP peptides TAF18 (-1), HT001(-1), and AIM2(-1) weekly for 4 consecutive weeks and repeated every 4 weeks up to a total of 3 cycles.</td>
<td>Safety immunogenicity</td>
<td>Completed</td>
</tr>
<tr>
<td>NCT03631641</td>
<td>Phase II open-label, single arm</td>
<td>Ohio, United States Multisite</td>
<td>Adults (18 years or older) Lynch syndrome secondary to germline MLH1 or MSH2 mutation; History of hemicolectomy for advanced adenoma or CRC</td>
<td>Checkpoint blockade; nivolumab every 3 months for up to 8 cycles.</td>
<td>Incidence rate of adenomas, high-risk adenomas, CRC, and non-CRC at 3 years</td>
<td>Suspended</td>
</tr>
<tr>
<td>NCT04041310</td>
<td>Phase I</td>
<td>Rome, Italy Multisite</td>
<td>Adults (18 years and older) Stage IV MSI-H tumors</td>
<td>Checkpoint blockade combined with vaccine of virally encoded library of 209 neoantigen peptides shared in MSI tumors.</td>
<td>Safety immunogenicity</td>
<td>Active (not yet recruiting)</td>
</tr>
<tr>
<td>NCT01885702</td>
<td>Phase I/II open-label, multisite</td>
<td>Nijmegen, Gelderland, Netherlands</td>
<td>Adults (18 years or older) with Lynch syndrome and no history of CRC, or adults with a history of MSI CRC</td>
<td>Vaccine: monocyte-derived peptide-loaded dendritic cells targeting MSI-specific neoantigens and tumor-associated antigen carcinoembryonic antigen (CEA)</td>
<td>Safety</td>
<td>Active (not recruiting)</td>
</tr>
<tr>
<td>OncoPeptIV4C</td>
<td>Preclinical</td>
<td>India</td>
<td>Adults with Lynch syndrome secondary to germline MLH1 mutation</td>
<td>In silico prediction of tumor-derived neoantigen peptides.</td>
<td>Immunogenicity</td>
<td>Completed</td>
</tr>
</tbody>
</table>
the access to a wealth of genomic information from tumors (93–96). An example is the recent report from Scarselli and colleagues at NousCom 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 upregulation of immune checkpoints in Lynch syndrome-associated precancers 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 colorectal cancers. 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 colorectal cancer recurrence and increasing cancer-specific survival.

Disclosure of Potential Conflicts of Interest

E. Vilar is a consultant/advisory board member for Janssen Research and Development. No potential conflicts of interest were disclosed by the other authors.

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