Immune Profiling of Premalignant Lesions in Patients With Lynch Syndrome

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IMPORTANCE Colorectal carcinomas in patients with Lynch syndrome (LS) arise in a background of mismatch repair (MMR) deficiency, display a unique immune profile with upregulation of immune checkpoints, and response to immunotherapy. However, there is still a gap in understanding the pathogenesis of MMR-deficient colorectal premalignant lesions, which is essential for the development of novel preventive strategies for LS.

OBJECTIVE To characterize the immune profile of premalignant lesions from a cohort of patients with LS.

DESIGN, SETTING, AND PARTICIPANTS Whole-genome transcriptomic analysis using next-generation sequencing was performed in colorectal polyps and carcinomas of patients with LS. As comparator and model of MMR-proficient colorectal carcinogenesis, we used samples from patients with familial adenomatous polyposis (FAP). In addition, a total of 47 colorectal carcinomas (6 hypermutants and 41 nonhypermutants) were obtained from The Cancer Genome Atlas (TCGA) for comparisons. Samples were obtained from the University of Texas MD Anderson Cancer Center and “Regina Elena” National Cancer Institute, Rome, Italy. All diagnoses were confirmed by genetic testing. Polyps were collected at the time of endoscopic surveillance and tumors were collected at the time of surgical resection. The data were analyzed from October 2016 to November 2017.

MAIN OUTCOMES AND MEASURES Assessment of the immune profile, mutational signature, mutational and neoantigen rate, and pathway enrichment analysis of neoantigens in LS premalignant lesions and their comparison with FAP premalignant lesions, LS carcinoma, and sporadic colorectal cancers from TCGA.

RESULTS The analysis was performed in a total of 28 polyps (26 tubular adenomas and 2 hyperplastic polyps) and 3 early-stage LS colorectal tumors from 24 patients (15 [62%] female; mean [SD] age, 48.12 [15.38] years) diagnosed with FAP (n = 10) and LS (n = 14). Overall, LS polyps presented with low mutational and neoantigen rates but displayed a striking immune activation profile characterized by CD4 T cells, proinflammatory (tumor necrosis factor, interleukin 12) and checkpoint molecules (LAG3 [lymphocyte activation gene 3] and PD-L1 [programmed cell death 1 ligand 1]). This immune profile was independent of mutational rate, neoantigen formation, and MMR status. In addition, we identified a small subset of LS polyps with high mutational and neoantigen rates that were comparable to hypermutant tumors and displayed additional checkpoint (CTLA4 [cytotoxic T-lymphocyte–associated protein 4]) and neoantigens involved in DNA damage response (ATM and BRCA1 signaling).

CONCLUSIONS AND RELEVANCE These findings challenge the canonical model, based on the observations made in carcinomas, that emphasizes a dependency of immune activation on the acquisition of high levels of mutations and neoantigens, thus opening the door to the implementation of immune checkpoint inhibitors and vaccines for cancer prevention in LS.

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Lynch syndrome (LS) is the most common hereditary colorectal cancer (CRC) syndrome and constitutes an attractive model to elucidate carcinogenesis in the setting of DNA mismatch repair (MMR) deficiency, which is the basis of approximately 15% of sporadic CRC due to somatic epigenetic inactivation.1 Lynch syndrome results from the presence of germ-line mutations in 1 of the DNA MMR genes (MLH1, MSH2, MSH6, PMS2, and EPCAM), which are involved in repairing base-to-base mismatches and insertion-deletion (indels) loops, and predisposes patients not only to development of CRC, but also endometrial, ovarian, small intestine, and urinary tract tumors.2 Lynch syndrome has an autosomal-dominant transmission and causes an estimated lifetime risk for CRC as high as 80%, compared with 5% in the general population.3 The estimated prevalence of LS is 1:2,800, affecting a total of 1.1 million people in the United States.4 Although screening with annual colonoscopy has been demonstrated to decrease cancer incidence in patients with LS,5 many patients continue developing CRC at a young age due to poor adherence to screening recommendations or the rapid development of interval cancers owing to biologic reasons that are not yet well defined.6 Therefore, there is an urgent need for a better understanding of the pathogenesis of colorectal premalignant lesions, instrumental for the development of novel preventive strategies for LS.7

Carcinomas arise in patients with LS secondary to the acquisition of a somatic hit in the alternative wild-type allele of the same MMR gene that carries the germline alteration, thus inducing deficiency in the DNA-repairing machinery and consequently resulting in an excessive accumulation of frame-shift mutations (ie, hypermutated tumors) that generate neoantigens. As a consequence, LS carcinomas are infiltrated by abundant tumor-infiltrating lymphocytes.8 It has been reasoned that these neoantigens also upregulate inhibitory molecules such as PD-1 (programmed cell death 1), PD-L1 (programmed cell death 1 ligand 1), lymphocyte activation gene 3 (LAG3), and other checkpoints to counterbalance the infiltrating immune cells,9 thus suggesting that these tumors are particularly susceptible to immune-based destruction. In fact, treatment of MMR-deficient tumors with the checkpoint inhibitor pembrolizumab or nivolumab has demonstrated clinical benefit in terms of prolongation of progression-free and overall survival.10-12 Therefore, the potential T-cell infiltration and expression of immune checkpoints in LS premalignant lesions would represent an opportunity for immunoprevention in patients with LS.

In this study, our goal was to characterize the immune profile of premalignant lesions (polyps) from a cohort of patients with LS. We hypothesized that mutational load and neoantigen formation are molecular features that arise late in MMR-deficient carcinogenesis (either advanced premalignant lesions or carcinomas) and are independent of the immune profile displayed by premalignant lesions.

Methods

Participants and Samples

RNA sequencing was performed in a total of 28 colorectal polyps (26 tubular adenomas and 2 hyperplastic polyps) from 21 patients with a diagnosis of familial adenomatous polyposis (FAP) (n = 10) or LS (n = 11) at the University of Texas MD Anderson Cancer Center (UTMDACC) and 3 early-stage colorectal tumors (1 stage I tumor and 2 stage II tumors) from 3 patients with a diagnosis of LS obtained by Nouscom SRL from “Regina Elena” National Cancer Institute, Rome, Italy (eTables 1 and 2 in the Supplement). We chose as the comparator group samples from patients with FAP because they represent the premalignant model for colorectal carcinogenesis developing in a background of chromosomal instability and proficiency of the MMR system. Written informed consent was obtained from all individuals, and the UTMDACC Institutional Review Board approved this study. In addition, data from 47 CRCs (6 hypermutants and 41 nonhypermutants) were downloaded from The Genomics Data Commons13 for comparative analysis with carcinomas.

Illumina Sequencing, Whole Transcriptomic Analysis, and Mutational Analysis

Sample preparation, library construction, and sequencing were performed at the UTMDACC Sequencing Core Facility and the Center for Genomics and Transcriptomics (Tübingen, Germany). In brief, normalized counts were transformed into log2 counts per million (CPM) with limma_3.30.9 for statistical analysis. Genes linked to the immune microenvironment of CRC and MMR deficiency were grouped by lineage and/or function (helper T cell 1/cytotoxic T cell 1 [Th1/Tc1], cytotoxic T lymphocyte, Th17, regulatory T cells, proinflammatory, and metabolism) as previously reported9 to distinguish which genes were differentially expressed on the basis of LS and FAP status. Messenger RNA CPM were plotted in GraphPad Prism, version 6.0 (GraphPad Software). Mutation rates were calculated by dividing the number of somatic mutations by the number of callable bases (defined as >10x in polyp and matched normal mucosa sample). Somatic allelic imbalances were detected using HapLOHseq with event prevalence of 0.001 and with matched normal mucosa as reference.10 Somatic allelic imbalance events in APC and MMR genes were found by overlapping their genomic coordinates (hgdownload.cse.ucsc.edu/goldenpath/hg19/database/refGene.txt.gz) with allelic imbalance regions. Mutation signatures were detected and plotted with
Neoantigen Discovery
We used seq2HLA, version 2.2,\textsuperscript{17} with default settings to generate 4-digit typing for major histocompatibility complex (MHC) class I and II on FAP and LS normal mucosa. Then, we ran pvac-seq, version 4.0.8,\textsuperscript{18} to generate neoantigen MHC class I and II predictions on each sample.

Results
Immune Profiling of LS Premalignant Lesions
RNA sequencing was performed in a total of 28 colorectal polyps (eTables 1 and 2 in the Supplement). All of the polyps analyzed from patients with FAP (n = 17) were confirmed to be early tubular adenomas, smaller than 1 cm in diameter, and without signs of high-grade dysplasia. All LS polyps (n = 11) were early adenomas of 1 cm in diameter, with the exception of 2 that were hyperplastic polyps. A total of 4 LS polyps displayed MMR deficiency by loss of staining in MSH2 and/or MSH6, and the rest were MMR proficient (eTable 2 in the Supplement). Overall, LS polyps showed a significantly higher expression of CD4, IFNG, LAG3, and CD274/PDL1 (checkpoints), IL12A, and TNF (proinflammatory) compared with FAP polyps and displayed a consistent trend among the genes integrated in these pathways regardless of their MMR status (Figure 1 and eTable 3 in the Supplement). Interestingly, LAG3 was observed to be the most significantly upregulated. Then, we analyzed the evolution of immune activation in MMR carcinogenesis by comparing LS polyps with carcinomas and observed additional consistent activation among genes in the proinflammatory and metabolism pathways that were absent in premalignant lesions (eFigure 1A and eTable 4 in the Supplement). Of note, LS premalignant lesions showed activation of both PD-L1 and LAG3 and carcinomas showed deregulation of additional checkpoints such as CTLA4. This expression pattern displayed by LS polyps and carcinomas is consistent with a strong enrichment for additional immune-related gene sets such as immune activation, immune response, PD-1 activation, and T-cell reaction (eFigure 1B and eTable 5 in the Supplement).\textsuperscript{19} These results suggest the existence of a robust immune microenvironment in LS premalignant lesions secondary to T-cell infiltration.\textsuperscript{9}

Mutational Rate and Mutation Signature in LS Premalignant Lesions
On the basis of the previous findings made in carcinomas, we hypothesized that one possible explanation for the immune deregulation observed in LS premalignant lesions is the

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Figure 1. Immune Profile of Lynch Syndrome (LS) Premalignant Lesions

The graphs display values for each premalignant lesion (circles), the mean for each group (horizontal bar), and statistically significant differences between FAP and LS using the Welch t test and multiple comparisons by Benjamini-Hochberg method.

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Messenger RNA expression levels of immune-related genes involved in CD4, helper T cell 1 (cytotoxic T cell 1) (TH1/TC1), cytotoxic T lymphocyte (CTL), checkpoint response, T\textsubscript{H}17, regulatory T cells (Treg), proinflammation, and metabolism comparing LS and familial adenomatous polyposis (FAP) polyps.
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Figure 2. Mutational Rate and Mutation Signature Distribution in Lynch Syndrome (LS) Premalignant Lesions

A. Comparisons of mutation rate among familial adenomatous polyposis (FAP), hypermutated and nonhypermutated LS polyps, hypermutated LS, and The Cancer Genome Atlas (TCGA) colorectal cancer (CRC) tumors. The graphs display values for each tumor (symbols), the mean for each group (horizontal bar), and statistically significant differences between groups. B, Hierarchical clustering of mutation spectrum of FAP and LS polyps, LS tumors, and sporadic TCGA CRC stage I and II colorectal tumors with known microsatellite instability and hypermutation status. A total of 3 hypermutated LS polyps, 1 LS tumor, and 4 hypermutated TCGA tumors display mutational signature 6, which is caused by defective DNA mismatch repair (MMR).

acquisition of high levels of somatic mutations (hypermutation). To assess the mutation rate in our samples, we called mutations from RNA-sequencing data and compared the results with hypermutant and nonhypermutant sporadic carcinomas from TCGA and our LS carcinomas. To this end, we first demonstrated the feasibility of using RNA sequencing to estimate somatic mutation rates by observing a statistically significant correlation between mutation rates called from whole-exome sequencing data and RNA sequencing in 47 TCGA samples with matched data (R² = 0.339; P < .001) (eFigure 2 in the Supplement). Overall, polyps displayed low mutation rates compared with carcinomas; however, among LS polyps, 3 were found to be hypermutated (Figure 2A and eTable 6 in the Supplement) and exhibited a mutation signature with distinct C>T changes that are associated with deficiency in the DNA MMR system (signature 6 described by Alexandrov et al²⁰ and eFigure 3 in the Supplement). These 3 hypermutant LS polyps clustered with sporadic hypermutant CRCs from TCGA and LS carcinomas based on mutation signature 6 (Figure 2B), and displayed loss of staining of MMR proteins (ie, MMR deficiency) (eTable 2 in the Supplement). At the same time, FAP polyps and nonhypermutated LS polyps shared a similar mutation spectrum lacking the distinct MMR-deficient pattern. Furthermore, a comparative analysis of the immune profile of hypermutant and nonhypermutant LS polyps was only significant for the regulatory T-cell–related gene FOXP3 and the immune checkpoint CTLA4 (eTable 7 in the Supplement). We confirmed with immunohistochemical analysis the promi- nent infiltration by FOXP3-positive T cells of hypermutant LS polyps (eFigure 4 in the Supplement). This fact suggests that the immune activation program that is displayed by all LS polyps of this cohort (both hypermutant and nonhypermutant) is independent of the mutation rate.

Neoantigens and Their Relation to Mutation Rates and Immune Profiling

We postulated that the immune profile observed in all LS polyps could be secondary to an increase in neoantigen rate and independently from the global mutational rate. To determine this, we performed MHC class I and II typing (eTable 8 in the Supplement) and detected tumor-specific neoantigens using bioinformatics methods (eTable 9 in the Supplement). The 3 hypermutated LS polyps displayed a neoantigen burden that was similar to that for LS carcinomas and higher than for the nonhypermutant LS and FAP polyps (Figure 3). This difference was statistically significant for both high- and low-binding affinity neoantigens binding MHC class I and II (eFigure 5A and B in the Supplement) and secondary to the
accumulation of indels (eFigures 5C and D in the Supplement). Overall, the total number of neoantigens in FAP samples did not vary as a function of the mutational rate ($R^2 = 0.02$), but it did correlate well in LS polyps ($R^2 = 0.8; P < .001$) (eFigures 5E and F in the Supplement). These analyses confirm that a higher neoantigen load is secondary to an increase in mutational rate in hypermutant LS polyps but this is not responsible for the overall immune profile displayed by LS premalignant lesions.

**Neoantigen Pathway Enrichment Analysis in LS and FAP Premalignant Lesions**

We proceeded to discover gene pathways affected by emerging neoantigens unique to LS premalignant lesions using impartiality pathway analysis. Among the most important pathways that were significantly enriched by both class I and II neoantigens, we identified alterations in DNA repair mechanisms that could contribute to additional accumulation of somatic mutations in advanced LS polyps and carcinomas such as the role of BRCA1 in DNA damage response and ATM signaling (Figure 4 and eTable 10 in the Supplement). On the other side, FAP polyps acquired neoantigens in the Wnt pathway (Figure 4) and also accumulated somatic genomic events in APC (eTable II in the Supplement). As has been recently suggested, the activation of β-catenin ($CTNNB1$) secondary to deregulation of the Wnt pathway is responsible for immune exclusion in carcinomas$^{19}$; therefore, we decided to assess the expression levels of $CTNNB1$ in our samples and found that all FAP polyps presented with Wnt/β-catenin activation compared with normal adjacent mucosa. In contrast, LS polyps did not display any significant activation of $CTNNB1$, thus supporting the contribution of Wnt/β-catenin to immune exclusion in FAP premalignant lesions (eFigure 5D in the Supplement). However, in the absence of high neoantigen rates and MMR deficiency, the mechanism responsible for the immune activation in LS premalignant lesions remains elusive.

**Discussion**

Our results show a distinct immune profile in LS polyps, independent of the DNA mutation rate, the emergence of neoantigens that is secondary to frameshift mutations, and the MMR status. Among the immune checkpoints upregulated in polyps stands LAG3, which constitutes a promising target for immune interception in this patient population. Therefore, the emergence of high mutation burdens and neoantigens cannot simply be applied as a biomarker to guide implementation and development of immunoprevention strategies. In addition, we observed that neoantigen formation correlates with a high mutational rate present in the subgroup of LS polyps that are hypermutants. The acquisition of additional MHC class I and II neoantigens by hypermutated LS polyps was associated with the introduction of alterations in DNA damage repair mechanisms and LAG3 expression.
Figure 4. Pathway Analysis of Neoantigens Present in Familial Adenomatous Polyposis (FAP), Lynch Syndrome (LS) Polyps, and LS Tumors

Figure 5. Schematic Model of the Immune Activation in Lynch Syndrome (LS) Carcinogenesis

Follow-up Study reported a correlation between neoantigen load and density of FOXP3-positive T-cell infiltrates. In addition, neoantigens accumulated along with the acquisition of additional indels that generate new open reading frames in hypermutant polyphs are more immunogenic than single-nucleotide variants. Overall, these observations are consistent with the results from pan-TCGA analysis that indicated that indel load is more closely associated with overall immunogenicity and response to checkpoint inhibition. Therefore, our results challenge the concept that immune activation in LS is a consequence of the excessive accumulation of somatic variation secondary to MMR deficiency because all polyphs analyzed presented a consistent immune profile regardless of the mutation rate or abundance of high-affinity binding neoantigens. This canonical concept could be the case at later stages of premalignant lesions (advanced polyphs) and progression into carcinoma. However, immune deregulation could precede the accumulation of genomic aberrations and neoantigen formation in initial steps of carcinogenesis (Figure 5). Finally, this observation will advocate for the development of vaccine strategies to prevent the progression of carcinogenesis by priming T cells to antigens displayed by early lesions that will be cleared at the premalignant stage. Furthermore, combinations of immune checkpoint inhibitors and vaccines could be exploiting both components displayed by MMR-deficient premalignant lesions.

An additional interesting finding of this study is the presence of deregulation of additional DNA repair pathways that manifest as neoantigens, which may stimulate additional genomic deregulation beyond MMR deficiency. Accumulation of...
mutations in target genes involved in immune surveillance contribute in later stages of carcinogenesis to promote immune escape, and further progression such as mutations in the β2-microglobulin gene (B2M) that causes the loss of MHC class I antigen presentation. Moreover, we have identified somatic mutations in genes regulating MHC class II (CIITA, RFX5, and RFXAP) that have been reported previously as microsatellite instability targets in the LS hypermutant polyps and 1 of the LS carcinomas (eTable II in the Supplement). In contrast, FAP polyps accumulated neoantigens that were enriched for the Wnt pathway. This accumulation of genomic events in the Wnt pathway led to activation of β-catenin in T cells, which potentially justifies the immune exclusion in FAP premalignant lesions.

We believe that the results of this study open the field of immunoprevention in LS to checkpoint inhibitors as an immune intervention strategy. This class of agents have shown a high level of clinical activity in the treatment of stage IV MMR-deficient CRC. Although toxicity has to be carefully considered in the setting of prevention in healthy carriers and cancer survivors, it is ubiquitously accepted that cancer risks associated with LS outweigh potential toxic effects cataloged to date. Moreover, the clinical experience gained on the management of the adverse effects of these drugs has rapidly improved in recent years. Our data are particularly compelling for the use of LAG3 and dual LAG3/PD1 inhibitors in the prevention space as demonstrated by the upregulation of both molecules in LS polyps. LAG3 is a molecule found on the cell surface that plays a role in the negative regulation of T cells and binds MHC II molecules with high affinity. Currently, LAG3 inhibitors are being developed in several clinical trials (NCT01968109, NCT02061761). In the first-in-human phase 1 trials, IMP321 showed no dose-limiting toxicity and the adverse effects were minimal. Therefore, in light of the current efforts to develop cancer prevention vaccines based on the presence of frame-shift peptides that have been detected across different types of LS-associated tumors such as colorectal, endometrial, and gastric using computational tools and existing genomic data from TCGA, combinations of vaccine approaches and single/dual checkpoint blockade are logical next steps in immunoprevention development in this hereditary disease.

Limitations
We acknowledge that our study has several limitations. First, we used RNA-sequencing data as the only source to estimate mutational rate in polyps. It would have been ideal to assess the mutational rate by both whole-exome sequencing and RNA sequencing; however, all the lesions analyzed were smaller than 10 mm and required pathology interpretation to rule out the presence of high-grade dysplasia and adenocarcinoma, which limited our access to only 1 endoscopic biopsy. However, we have proved using TCGA pairs of RNA-sequencing and whole-exome sequencing data in early-stage CRC that a high degree of concordance between mutational rates estimated from both data sources exists. In addition, we have implemented a rigorous analytic protocol for best interpretation of mutational calls from transcriptomic data. A second limitation is the limited access to additional RNA to validate our expression data using alternative techniques. However, next-generation sequencing has proved to be a robust tool to define expression profiles. A third limitation is the relatively limited number of polyps analyzed in the LS cohort. A simple explanation for this fact is that only 25% of patients with LS undergoing screening colonoscopies present with a premalignant lesion. Therefore, a subsequent validation study using technologies that are capable of rendering robust results in archival tissues is warranted.

Conclusions
We have shown that LS polyps exhibit a unique immune profile with upregulation of checkpoints that is independent of mutational rates and neoantigen formation, suggesting that the development of an immune environment is present in early steps of MMR-deficient carcinogenesis. These findings have an important implication in the development of immunotherapies as checkpoint inhibitors and vaccines for cancer prevention in patients with LS.

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Additional Information: Data sets: RNA-sequencing files have been deposited in GEO. The following links can be used to access the data for reanalysis: GSE88945 (3 FAP polypos) https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE88945; GSE106500 (13 FAP polypos and 11 LS polypos) https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE106500.

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