TITLE: Lynch Syndrome – Cancer Pathways, Heterogeneity and Immune Escape

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CONFLICTS OF INTEREST

None.

WORD COUNT: 2218 (abstract 109)

ABSTRACT:

Recent work has provided evidence for genetic and molecular heterogeneity in colorectal cancers (CRCs) arising in patients with Lynch syndrome (LS), dividing these into two groups: G1 and G2. In terms of mutation and gene expression profile, G1 CRCs bear resemblance to sporadic CRCs with microsatellite instability (MSI), whereas G2 CRCs are more similar to microsatellite stable CRCs. Here we review the current state of knowledge on pathways of precursor progression to CRC in LS and how these might tie in with the new findings. Immunotherapy is an active field of research for MSI cancers and their potential use for cancer therapy for both sporadic and LS MSI cancers is discussed.

KEYWORDS:

Lynch syndrome

Colorectal cancer

DNA mismatch repair

Microsatellite instability

Immune escape

ABBREVIATIONS

APC	Adenomatous polyposis coli (protein)
CAR	Chimeric antigen receptor
CRC	Colorectal cancer
CTL	Cytotoxic T-lymphocytes

CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
FDA	Food and Drug Administration (USA)
FSP	Frame-shift peptide
LS	Lynch syndrome
LS-CRC	Lynch syndrome colorectal cancer
MMR	DNA mismatch repair
MSI	Microsatellite instability
MSS	Microsatellite stability
MHC	Major histocompatibility complex
PD-1	Programmed cell death protein-1
PD-L1	Programmed death-ligand 1
TCR	T-cell receptor

Lynch syndrome

Lynch syndrome (LS) is probably the most common hereditary cause of cancer in humans: a genetic condition with a prevalence of at least 1 in 200, that predisposes to various cancers, most frequently colorectal (CRC) and endometrial adenocarcinoma [1]. It is caused by constitutional ("germline") mutations in one of four DNA mismatch-repair (MMR) genes: *MLH1*, *MSH2*, *MSH6* and *PMS2*. Such heritable mutations, when combined with a sporadic somatic mutation of the normal allele, lead to a deficiency of DNA mismatch repair that results in the accumulation of genetic length-changing mutations at microsatellites – so-called 'microsatellite instability' (MSI). This is accompanied by an incidental 100 – 1000-fold increase in mutation rate, but more importantly a reduced susceptibility to apoptosis induced by DNA damage recognised by the MMR pathway, and it is this which confers a strongly selectable Darwinian advantage to such cells. A subset (~14%) of sporadic colon cancers which arise from right-sided serrated lesions are also MMR-deficient, and thus have MSI, because of hypermethylation of the *MLH1* gene's promoter [2]. In contrast, sporadic rectal cancers with MMR-deficiency are rare and most rectal cancers with loss of MMR are due to LS [3,4]. LS accounts for 3.3% of bowel cancers in the UK, a similar proportion to Denmark (2.8%) and the USA (3.1%) [5–7].

The number of mutations reported in MMR genes varies [8]. This is a consequence of mutations in *MLH1* and *MSH2* conferring the highest risks, *MSH6* mutations less so, and *PMS2* mutations least of all, so cases of LS identified on the basis of family history are mostly found to have mutations in *MSH2* or *MLH1*. However, this may change as case finding by systematic testing of incident cancers becomes the norm [e.g. 3]. So far, gene, gender, age, and previous cancer have all been identified as variables in LS cancer risk [10], as well as various environmental and lifestyle factors [1].

Molecular heterogeneity of Lynch syndrome tumours

Whilst research in sporadic CRC has focussed on delineating molecular heterogeneity, little work has been done on such heterogeneity in LS-CRC. Previously, it was assumed that CRCs occurring in LS patients were all part of the same disease process. However, recent studies have identified at least two precursors of LS-CRC. Firstly, via adenomatous polyp formation in which *APC* mutations (and other genetic or epigenetic changes) which occur early, and probably sporadically, and drive conventional polypoid adenoma formation. Subsequent loss of MMR in LS patients leads to invasive adenocarcinoma via more genetic mutations. Secondly, a separate pathway in which MMR deficiency occurs within single non-dysplastic crypt foci, that subsequently acquire more genetic changes to become flat intra-mucosal neoplastic lesions, that are more difficult to diagnose endoscopically [11]. Intriguingly, evidence is coming forward of a third pathway, related to the first two, comprising a subset of lesions that initially proceed along the second pathway, but which on acquisition of somatic *APC* mutations, secondary to the MMR deficiency, become dysplastic and polypoid [12]. It is thought that approximately half of the CRCs in LS may evolve from these flat, non-polypoid, neoplasms to form sub-mucosal “immediately invasive” cancers. These are often associated with *CTNNB1* mutational activation, instead of *APC* inactivation, although mutations of *ASTE1/HT001, AIM2* and *BAX* may also contribute to the progression of MMR-deficient precursors into MMR-deficient cancers. Moreover, these must arise at a slow rate, given the 10,000 or so MMR-deficient crypts which have an inactivated second MMR allele in a LS large bowel, and yet LS patients develop only zero, one or perhaps two CRCs in a lifetime [13,14]. This is in contradistinction to the original school of thought that LS-CRCs must derive from very rapidly growing adenomas – an understandable logical consequence of assuming all CRCs derive from polypoid adenomas.

In turn, this starts to explain recent findings that surveillance colonoscopy in LS patients only reduces CRC-related mortality in LS by half, mostly by downstaging, whilst making no

discernible impact on the rate at which LS-CRCs occur – in complete contrast to the situation in the general population undergoing screening, where adenoma removal very significantly impacts on future CRC incidence [15,16]. Significantly, individuals who have inherited mutations in both copies of the DNA repair genes *MSH2, MLH1, MSH6, PMS2, MSH3* and *MUTYH* can develop multiple colorectal adenomas, but patients with a mutation in only one copy of these genes do not.

Further new evidence now supports the contention that, in addition to different precursor lesions, distinct molecular and cellular subtypes of LS-CRC exist. Writing in this edition of *The Journal of Pathology*, Binder and colleagues utilise CRC resection specimens from LS patients to demonstrate at least two distinct genetic subtypes of LS-CRC, termed G1 and G2 [17]. The DNA sequencing data shows that G1 tumours have a different mutation spectrum compared to G2 tumours, with higher mutation numbers and greater MSI. Interestingly, G1 cancers share these characteristics with sporadic MSI CRC, whereas the mutational profile of G2 cancers tends more to resemble that of microsatellite stable (MSS) sporadic CRC. Additionally, a higher proportion of G1 cancers arise on a background of germline *MLH1* mutation compared with G2 cancers. These findings suggest that, even within LS, varying degrees of mutation frequency and MSI exist, consistent with multiple pathways to carcinogenesis in LS.

The authors also perform gene expression analysis on these two LS tumour groups. The G1 reference mucosa has upregulated transcription of genes associated with inflammatory pathways, whereas G1 tumours have strong upregulation of cell cycle and proliferation-associated genes. In contrast, G2 reference mucosa and tumours show a more heterogeneous expression signature with either a stromal or mucosal transcriptional programme. The inflammatory signature in G1 reference mucosa is characterised by overexpression of various T- and B-lymphocyte markers (e.g. *CD3*, *CD4*, *CD8* and *CD19*), and this is backed up by strong CD4+ staining demonstrated by immunohistochemistry.

Moreover, G1 reference mucosa shows higher expression of genes related to MHC antigen presentation and of chemokine receptor-ligand pairs compared with G1 tumours. These data suggest that G1 tumours undergo transcriptional reprogramming to dysregulate the immune response and hence evade immune destruction. The differences between G1 and G2 LS-CRCs are summarised and compared to sporadic CRC in Table 1.

Role of the Immune System in Tumour Immunosurveillance and Immune Escape

The link between the immune system and cancer was probably first recognised in the nineteenth century, when Rudolf Virchow noticed an infiltrate of leucocytes within tumours [18]. However, the exact role the immune system plays is complex and our understanding of this is continually evolving. Somewhat paradoxically, both 'avoiding immune destruction' and 'tumour-promoting inflammation' are now regarded as hallmarks of cancer [19], suggesting that the immune system has dual roles in protecting from and promoting carcinogenesis. The mechanisms by which cancer cells avoid immune destruction can be conceptualised as a continual process of 'immunoediting' and split into three phases: elimination, equilibrium and escape [20].

MSI cancers with high mutation rates producing many mutant proteins tend to be particularly immunogenic and are typically associated with a strong lymphocytic infiltrate [21]. Moreover, they tend to show overexpression of immune-checkpoint proteins (e.g. PD-1 and CTLA-4) [22], and early-stage clinical trials show promising results for anti-tumour efficacy of immune-checkpoint inhibitors in the treatment of advanced MSI CRC [23,24]. It is hypothesised that this immunogenicity is due to the generation of a multitude of neo-antigens that are recognised as 'foreign' and trigger a strong immune response [25]. With deficiency of MMR, cells develop a characteristic mutator phenotype, and accumulate indel mutations within microsatellites and other repetitive sequences. If this occurs within coding

DNA, it can lead to a frameshift within the translational reading frame and hence synthesis of novel frameshift peptides (FSP) that can act as tumour-specific antigens. This hypothesis is backed up by previous work that shows tumour-infiltrating T-lymphocytes exhibiting reactivity against predicted FSPs are present in sporadic MSI, but not MSS, CRCs [26]. Interestingly, peripheral FSP-reactive T-cells are also identified in LS patients without CRC, but not individuals without LS, or CRC patients with cancers that do not have MMR deficiency. This shows that patients with LS are 'auto-immunised' against FSP neo-antigens prior to cancer formation, and may explain the better survival [27] and reduced rate of metastasis [21] seen in LS-CRCs compared to sporadic CRCs.

In the work by Binder et al. [17], G1 reference mucosa is shown to be more immunogenic than that of G2 with a greater CD4+ infiltrate and greater expression of immune response-associated genes. Furthermore, even within the G1 group, expression of MHC class II genes and the predicted number of FSP neo-antigens (from sequencing data) rises as a function of mutational load. This supports a conceptual model whereby MMR deficiency leads to hypermutation within coding repetitive sequences, which in turn leads to generation of FSPs and consequently a strong anti-tumour immune response. Such a strong immune response was not seen in G2 reference mucosa, supporting the contention that there is heterogeneity concerning the mutation frequencies and degree of MSI in LS.

G1 tumours, in contrast with matched reference mucosa, show reduced expression of MHC class I and II genes as well as reduced infiltration with CD4+ cells [17], suggesting that they have undergone immune escape through reduced presentation of FSP neo-antigens. Such immune escape in MSI CRC has previously been linked to mutation in genes that regulate MHC class I (e.g. *B2M*) and class II (e.g. *CIITA, RFX5*) function [28,29]. Additionally, in this work, the authors describe recurrent mutations in *AIM2* (an upstream regulator of MHC class II function [30]) as a mechanism for reduced FSP presentation. Interestingly, G1 tumours show reduced expression of the T-cell immune checkpoint

proteins, PD-1 and CTLA-4, compared to reference mucosa, consistent with a reduced infiltration with lymphocytes. Conversely, sporadic MSI CRCs tend to have a strong lymphocytic infiltrate and show increased expression of immune-checkpoints [21,22]. Overall, these findings are consistent with marked immunoediting of G1 CRCs that arise in LS. In the early stages of carcinogenesis, the immune system is able to keep small foci of neoplastic cells in check (elimination phase). However, the inevitable accumulation of somatic mutations in MMR-deficient cells leads to mechanisms to avoid immune destruction and the progression to CRC (equilibrium and escape phases) [see Figure 1].

Implications for cancer therapy and clinical practice

Clinically, MMR-deficient cancers tend to be immunogenic and therefore have the potential to be targeted with specific immunotherapy. MSS CRCs, on the other hand, are not as immunogenic and would be less likely to respond to immunotherapy. Although LS-CRCs have MMR deficiency, their response to immunotherapy may be more difficult to predict. G2 CRCs are less immunogenic, bearing resemblance to sporadic MSS CRCs, and therefore might be less likely to respond. G1 CRCs arise in a highly immunogenic environment, suggesting that they would be good candidates for treatment with immunotherapy. However, these tumours have undergone immune escape with reduced antigen presentation and reduced lymphocytic infiltrate, meaning that some immunotherapies may not be as effective as compared with sporadic MSI CRCs.

Clinical trials are already underway for the use of immune-checkpoint inhibitors such as pembrolizumab and nivolumab for MSI CRC [23,24]. Another approach, based on the idea of ‘auto-immunisation’ in patients with LS, is to develop a vaccine against common FSP neo-antigens expressed by MSI CRCs. Such a vaccine has completed phase I/IIa clinical trials with long-term results awaited [31]. Different approaches for vaccine delivery have

also been tried, including peptide loading of patient's monocyte-derived dendritic cells *ex vivo* and administration to the patient as a cellular vaccine [32]. In addition, as adoptive transfer of 'CAR T-cells' (cytotoxic T-lymphocytes engineered to express a chimeric antigen receptor recognising a particular antigen [33]) has recently gained FDA approval for the management of various haematological malignancies [34,35], then such an approach has the potential to be used in MSI CRC by creating engineered T-cells, either with an engineered T-cell receptor (TCR) or CAR, against FSP neo-antigens. Adoptive transfer of cytotoxic T-cells, with an engineered TCR against a common MSI FSP, has demonstrated efficacy in a xenograft mouse model [36], but a trial in humans is yet to be conducted. Although not yet experimentally tested, CAR T-cells against membrane-associated FSP neo-antigens could represent an ideal way to target G1 LS-CRCs and sporadic MSI CRCs that have undergone immune escape, as there is no requirement for FSP neo-antigens to be presented by MHC class I (since the immunoglobulin domain of a CAR can directly target FSPs).

The implications of these findings of LS tumour heterogeneity include the need for greater use of MMR analysis in all LS and sporadic MSI cancers, both biopsies and resections, including CRCs, endometrial cancers and other LS-related cancers. This should include combined MMR immunohistochemistry and tumour MSI testing for LS screening, prognosis, prediction of treatment responsiveness (both conventional chemotherapy and immunotherapies), and pre-operative planning of appropriate surgical operations. Binder et al's work [17] also argues for further work to establish whether this should also now involve further tumour testing for G1 versus G2 MSI subtypes, along with quantitative analysis and characterisation of tumour infiltrating lymphocytes for more sophisticated prediction of responsiveness to the emerging range of immunotherapies. Moving forward into the era of digital pathology, more advanced image-analysis techniques will facilitate such a quantitative description of tumour infiltrating lymphocytes, and prognostic parameters such

as the ‘Immunoscore’ [37] are likely to become an integral part of the pathology report in CRC.

AUTHOR CONTRIBUTIONS

Sidhant SETH: wrote the initial draft

All: planned the manuscript, commented on and amended the draft text and figure

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Table 1: Comparison of properties of G1 and G2 LS-CRC (as defined by Binder et al. [17]) with sporadic CRC, both MSI and MSS. Data all from [17].

	Colorectal cancers			
	Lynch Syndrome		**Sporadic**	
	MSI (G1)	**MSI (G2)**	**MSI**	**MSS**
Relative Mutation Numbers	High	Low	High	Low
Fraction of microsatellites exhibiting deletions	High	Low	High	Low
Gene expression profile	DOWN-regulation of immune system and inflammatory genes. UP-regulation of cell cycle and proliferation genes	UP-regulation of mucosal and stromal genes	UP-regulation of cell cycle and proliferation genes	UP-regulation of cell cycle and proliferation genes

Figure 1: Mechanisms of Immunoediting in MMR-deficient CRC. Both G1 CRCs in LS and sporadic MSI CRCs elicit a strong immune response and undergo significant immunoediting. Cytotoxic T-lymphocytes (CTL) are able to recognise immunogenic frameshift peptides (FSPs) that are synthesised in MMR-deficient cells as a result of MSI. However, tumour cells may evolve mechanisms to prevent immune destruction including inhibition of CTLs by upregulating expression of immune-checkpoints (e.g. PD-L1) or through various genetic mutations leading to loss FSP antigen presentation.