Role of the microenvironment in the tumourigenesis of microsatellite unstable and MUTYH-associated polyposis colorectal cancers

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Chapter 6

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ABSTRACT

Two forms of genomic instability can be distinguished in colorectal cancer (CRC) tumourigenesis. One is characterised by pronounced chromosomal instability (CIN), while the other relates to alterations produced at the nucleotide level that preferentially target microsatellite sequences. Tumours developing under the latter form of genomic instability possess a microsatellite instability-high (MSI-H) phenotype due to inactivation of the DNA mismatch repair system. The most recently described CRC syndrome, MUTYH-associated polyposis (MAP), shares characteristics with both MSI-H and CIN cancers. MAP carcinomas develop from the impairment of the base excision repair system, where MUTYH is involved, but also present a peculiar form of CIN. Several clinicopathological characteristics of MSI-H and MAP CRCs overlap such as tumour location, clinical prognosis and histological features. We propose that MSI-H and MAP CRCs are particularly prone to interact with their tumour microenvironment. A great deal of this interaction is probably stimulated by the immunogenic character of those tumours, known to possess a high mutagenic potential. The accumulation of mutations in coding regions of the genome of MSI-H and MAP carcinomas is likely to translate into a surplus of neo-antigens that trigger an anti-tumour immune response. The immune system constitutes thus an important vector of selective pressure that favours the outgrowth of tumour clones with immune-evasive phenotypes. In this review, we summarise the evidence for the influence of the tumour microenvironment in MSI-H and MAP tumourigenesis. Furthermore, we discuss how particular features of MSI-H and MAP CRCs can be exploited for the development of therapeutic strategies for affected patients.

INTRODUCTION

Genetic and epigenetic instability drives colorectal tumourigenesis (1). Such instability derives from the interaction between the colorectal epithelium and various mutagens like oxidising molecules and methylating agents and exposure to inflammatory and replicative stress during lifetime (2–5). Additionally, the age-related decreased functionality of caretaker and gatekeeper DNA repair systems diminishes the fidelity of DNA replication and indulges the accumulation of genetic aberrations in cells (6). Genetic and epigenetic alterations target, fortuitously, cellular tumour suppressor mechanisms and are responsible for the generation of tumour clonal diversity (7,8). This diversity is essential for the selection of the fittest tumour cell clones during tumourigenesis (9,10). Tumour cells compete against each other for growth factors, oxygen and even space. Clones that display higher proliferation rates and efficient silencing of apoptotic mechanisms will be favoured by selection (11). Additionally, genetic alterations that translate into different metabolic capacities will define which tumour clones thrive in a specific microenvironment (12). Tumour cells must also compete and coexist with adjacent non-malignant tissue. Intercellular communication comprises an efficient form of cellular control that aims to preserve the context of multicellularity in the organism and impede uncontrolled proliferation of altered clones (13). Tumour cells adulterate these communication channels by abrogating the expression of proteins involved in the negative regulation of growth and by expressing others that promote survival (14). During tumourigenesis, malignant cells might even acquire the capacity of partially modulating the stimuli provided by non-malignant tissues (15). Finally, and despite the fact that the immune system did not evolve to deal with cancer, certain tumours

evoke immune responses and introduce an additional vector of selective pressure that favours the outgrowth of clones possessing immune-evasive phenotypes (16,17).

Genetic instability in colorectal tumourigenesis mainly occurs in two distinct forms. The majority of colorectal cancers (CRCs) display pronounced chromosomal instability (CIN), including gains and losses of large portions of genetic material that translate into an aneuploid DNA content in tumour cells (18). Approximately 15% of all CRCs present a microsatellite instability-high (MSI-H) phenotype, which is characterised by the accumulation of mutations in microsatellite sequences, a consequence of the inactivation of the DNA mismatch repair (MMR) system (19). Chromosomal aberrations are relatively rare in MSI-H carcinomas and their DNA content often remains peri-diploid (20–22). In addition to the type of genetic instability affecting CIN and MSI-H colorectal tumours, certain clinicopathological characteristics further define these cancers as two different entities (23,24). Another CRC variant shares characteristics of both CIN and MSI-H tumours. MUTYH-associated polyposis (MAP) is an autosomal recessive disease caused by inactivation of MUTYH, a core protein from the base excision repair (BER) machinery (25–27). Despite the fact that MAP carcinomas arise in the context of a deficiency affecting nucleotide repair, MAP tumours display a peculiar type of CIN characterised by widespread loss of heterozygosity without chromosomal copy number alterations (28). It is estimated that MAP carcinomas might add up to 1% of all CRCs (29).

This review summarises aspects of MSI-H and MAP colorectal tumourigenesis that support a major role of the microenvironment, and particularly the immune system, in the development of these tumours. Furthermore, we discuss how this interaction can influence the management of MSI-H and MAP CRC patients.

Aetiology of MSI-H and MAP CRCs

MSI-H CRCs may arise in a sporadic or hereditary setting. Sporadic MSI-H tumours (12% of all CRCs) develop due to silencing of the *MLH1* gene by means of promoter hypermethylation (30,31). The hereditary counterpart of MSI-H CRCs is caused by germ line inactivation of a single copy of an MMR gene where *MLH1*, *MSH2*, *MSH6* and *PMS2* are most commonly affected. This syndrome, identified as Lynch syndrome, manifests in an autosomal dominant manner with inactivation of the second allele occurring somatically during life (32). Some Lynch syndrome cases have also been explained by germ line hypermethylation of MMR genes and deletions of the 3' region of the *EPCAM* gene, located upstream of *MSH2*, leading to transcriptional read-through of the latter (33,34). Lynch syndrome accounts for approximately 3% of all CRCs and further predisposes for a variety of extracolonic tumours (35). The MMR caretaker system is responsible for dealing with nucleotide mismatches, small insertions and deletions. MSH2 and MSH6 form a heterodimer responsible for recognising mistakes introduced upon DNA replication. Thereafter, MLH1 and PMS2 are recruited, directing the daughter strand to BER (36). Microsatellite sequences are hotspots for the establishment of deletions and insertions due to the formation of secondary DNA structures at these sites during DNA replication (37). Thereby, MSI-H tumours are easily recognised when comparing the sizes of microsatellite sequences between tumour and germ line DNA (38).

MAP is caused by germ line inactivation of both copies of the *MUTYH* gene (26,27). The MUTYH protein is a BER glycosylase, involved in the repair of one of the most frequent and stable forms of oxidative damage: 8-oxo-7,8-dihydro-2'-

deoxyguanosine (8-oxoG). 8-OxoG, when used as a template, mismatches with adenines that can be removed by action of MUTYH. When this repair mechanism is impaired, the next round of replication results in G:C to T:A transversions (25). MAP patients normally present, at a young age, an elevated number of polyps (between 10 and 100) with diagnosis of malignant lesions being made at a mean age of 48 years old (29). Whether the inherited inactivation of a single copy of the *MUTYH* gene leads to an increased risk for cancer is still under debate (39–41).

Clinicopathological features of MSI-H and MAP CRCs

Several clinicopathological features of MSI-H and MAP carcinomas overlap. One of those relates to their location as they are often diagnosed at the proximal (right) side of the colon (42–45). During embryogenesis, the proximal colon (proximal to the splenic flexure of the colon) develops from the midgut, while the distal colon (distal to the splenic flexure) derives from the hindgut. These separate embryological origins imply distinct blood and lymph supply and drainage. For instance, the microvascular volume is greater in the proximal colon. Additionally, the proximal and distal colons are exposed to different dietary and digestive constituents, pH conditions and microbial flora (23). The preferential location of MSI-H and MAP carcinomas in the proximal colon suggests that there is a higher demand for the intervention of the MMR and BER systems in this part of the colon. This could be a consequence of an increased exposure of the proximal colon to external insults produced by chemical agents such as oxidising molecules when compared to the distal colon. Since the faecal material occurs in a liquid form at this stage, it might facilitate the interaction of certain mutagens with the colon epithelium, thereby inducing DNA damage. The colon epithelium is also prone to accumulate aberrant DNA methylation patterns during lifetime and some loci are specifically affected in the proximal colon (46,47). CpG island methylation is an effective mechanism by which cells either silence or activate gene transcription. CpG dinucleotides are conserved at promoter regions of the genes and hypermethylation of the latter usually correlates with decreased transcriptional activity and gene silencing (48). Virtually, all sporadic MSI-H cancers arise with promoter hypermethylation of the *MLH1* gene, but de novo methylation patterns are found throughout their whole genome (49,50). Therefore, MSI-H sporadic tumours are assumed to possess a methylator phenotype (51).

MSI-H and MAP carcinomas also diverge from the remaining spectrum of CRC in their histological presentation. MSI-H cancers are often poorly differentiated and both MSI-H and MAP CRCs are often mucinous (24,42). Notably, Lynch syndrome carcinomas display a less pronounced phenotype when compared to sporadic MSI-H tumours (52). The loss of the typical colonic crypt architecture implies the rearrangement of cell-to-cell interactions, favouring the loss of intercellular control mechanisms, characteristic of epithelial cells, mediated by adhesion molecules such as integrins and cadherins (13,53,54). The latter molecules are paramount in the perception of multicellularity by a cell in an organ context. Conversely, the loss of differentiation might expose tumour cells to increased interactions with the extracellular matrix composed of fibroblasts, immune cells, endothelial cells and others and moreover, it could favour tumour dissemination and invasion to adjacent tissues (15).

Genetics of MSI-H and MAP colorectal tumourigenesis

CRC genetics is intimately associated with the inactivation of the classical tumour suppressor *Adenomatous Polyposis*

Coli (*APC*) gene. Mutations in *APC* have been described to a lower extent in MSI-H carcinomas than in the remaining spectrum of CRC (42,55). The inactivation of the MMR and BER systems in MSI-H and MAP cancers is primarily seen as a generator of genetic instability and clonal diversity. Nevertheless, both DNA repair systems were shown to interact with tumour suppressing DNA damage response pathways (56,57). Accordingly, MMR and BER proteins might also function as tumour suppressors. Another hallmark of CRC genetics is the constitutional activation of the RAS/RAF/ERK signalling pathway, either by activating mutations in the KRAS GTPase or in the BRAF kinase. In this aspect, MSI-H and MAP carcinomas follow the CRC genetics dogma although in distinct manner. MSI-H sporadic tumours frequently present V600E BRAF activating mutations (in approximately half of cases) while those are absent in both Lynch syndrome and MAP CRC (58–60). These cancers often present mutations in the codon 12 of the *KRAS* gene (59,60). MAP tumours are further particularised by frequently displaying the characteristic G:C to T:A transversions at base position c.34 (42).

The 'mutome' of MSI-H CRCs has been described more extensively than the one of MAP tumours due to the recent discovery of the latter. Genetic research on MSI-H CRCs has focused on genes that contain microsatellite sequences in their coding regions as these are primary targets for the establishment of mutations in an MMR-deficient background. Accordingly, the *TGFBR2*, *IGFR2* and *BAX* genes are frequently affected by frameshift mutations in their microsatellite repeats in MSI-H colorectal tumours (61–63). The study of the *TGFBR2* gene assumes particular relevance as the TGF-β pathway is considered to play a major role in cancer progression (64). TGFβR2 is an extracellular receptor that mediates the binding of the TGF-β growth factors and activation of the intracellular SMAD signalling proteins (65). The *TGFBR2* gene possesses a microsatellite repeat of 10 adenines that is mutated in the majority of MSI-H CRCs (62). TGF-β pathway activation is generally considered to exert tumour suppressive effects, provided by growth inhibitory signals, but on the other hand, it has also been shown to promote tumour growth, angiogenesis, cellular plasticity and migration of tumour cells (64,66,67). The overgrowth of tumour cell clones with inactivating TGFβR2 mutations is thought to be associated to the insensitivity of those clones to its tumour suppressing effects (Figure 1) (68). Conversely, cancer cells may increase TGF-β production in order to modulate their microenvironment and cripple anti-tumour immune responses (69,70). Nevertheless, some reports suggested that despite the presence of inactivating mutations in *TGFBR2*, MSI-H CRC cells might still be sensitive to TGF-β growth factors (71,72). Mutations in other receptor genes of the TGF-β pathway such as *ACVR2A* and *BMPR2* are also common in MSI-H cancers (73,74). Although mutations in *SMAD4* were reported in a minority of MAP carcinomas, it is still unknown whether and how these tumours target the TGF-β pathway (42).

As previously mentioned, the "mutome" of MAP carcinomas still remains to be fully characterised. Interestingly, MAP carcinomas possess a characteristic form of genetic instability that differentiates them from MSI-H cancers and instead provides them with a CIN phenotype. MAP carcinomas often exhibit widespread loss of heterozygosity of large chromosomal regions without copy number alterations, a mechanism referred to as copy-neutral loss of heterozygosity (28). While loss of heterozygosity is frequent in CRCs with CIN, a dominant pattern of copy-neutral loss of heterozygosity is uncommon (75). The high prevalence of this form of loss of heterozygosity in MAP carcinomas

suggests that homologous recombination is a major DNA repair mechanism employed by these tumours. MUTYH deficiency could thus promote the usage of homologous recombination to resolve DNA damage that would otherwise be repaired by BER (76).

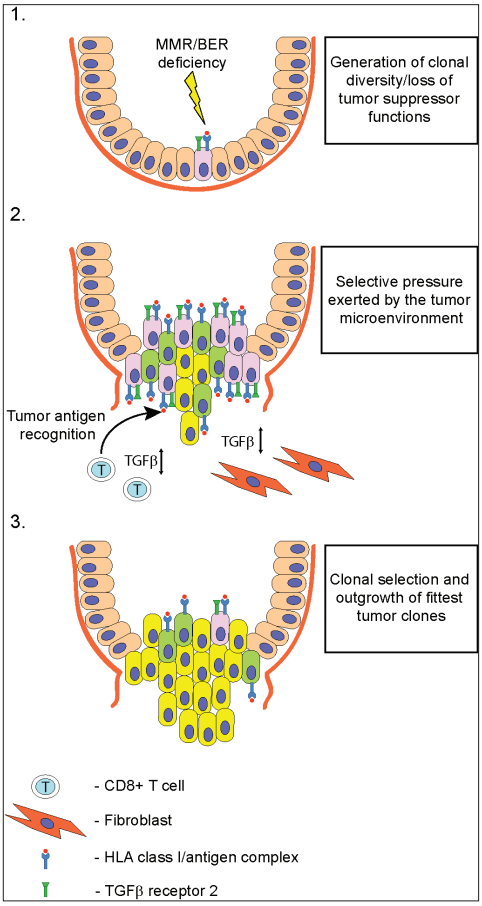

Figure 1. Diagram representing the clonal evolution occurring during MSI-H and MAP colorectal tumourigenesis. Clonal diversity is provided by the inactivation of the MMR and BER systems (1). A genetically heterogeneous population of tumour cells is then subjected to selective pressure, exerted by the tumour microenvironment (2). The particular immunogenic phenotype of MSI-H and MAP CRC evokes an immune reaction, mainly mediated by CD8+ T cells that favours the outgrowth of tumour cell clones that acquired immune-evasive phenotypes. Additionally, the desensitization of tumour clones to growth suppressive signals derived from the microenvironment constitutes a selective advantage (3).

Immunogenicity of MSI-H and MAP colorectal carcinomas

One of the most important characteristics of the adaptive immune system relates to its ability to distinguish between 'self' and 'non-self' antigens. This allows the host to deal specifically with viral or bacterial infections without targeting healthy host cells. Theoretically, tumour cells carrying a surplus of mutated proteins should be dealt with effectively by the immune system (77,78). Notwithstanding, during evolution, there was no selective pressure for the host to deal with cancer as this is an ageing disease mostly occurring after reproductive age (79). Nevertheless, a strong immune reaction, perceived in the form of a dense infiltration by activated T lymphocytes, is a hallmark of MSI-H CRCs (80,81). Other immune cells such as macrophages, dendritic cells or neutrophiles are also considered to play an important role in MSI-H CRC tumourigenesis (82,83). Evidence for an anti-tumour immune reaction is often detected at early stages of tumour development (84,85). More recently, MAP carcinomas were also shown to possess pronounced infiltration by immune cells when compared to other microsatellite stable CRCs (42). Moreover we, and others, have reported that MSI-H and MAP CRCs are particularly prone to lose human leukocyte antigen (HLA) class I expression (86–89). HLA class I expression, in human cells, is essential for competent immune surveillance. HLA class I molecules can be viewed as cellular informants that report to the immune system the mutation status of endogenous proteins (90). Antigen recognition is primarily mediated by CD8+ T cells that become activated in the presence of non-self antigens presented in an HLA class I context. When a neo-antigen is recognised by CD8+ T cells, the latter become cytotoxic (cytotoxic CD8+ T cells — CTLs) and have the ability to eliminate aberrant cells (91). Accordingly, HLA class I

loss is interpreted as a mechanism, adopted by tumours, to escape immune surveillance and thereby avoid tumour cell recognition and destruction (Figure 1) (92–94). Both MSI-H and MAP CRCs are thought to be more capable of triggering an immune response as a consequence of the inactivation of their respective DNA repair mechanisms. As the latter translates in the accumulation of mutations in coding regions of their genome, the probability that mutated antigens are presented to the immune system is higher than the one found in CIN CRCs (95). Interestingly, distinct molecular mechanisms underlie the loss of HLA class I expression in sporadic MSI-H and Lynch syndrome and MAP CRCs. HLA class I loss was often associated with genetic defects in antigen-processing machinery components in MSI-H sporadic tumours, while Lynch syndrome and MAP carcinomas frequently failed to express β2-microglobulin, the molecular chaperone necessary for cell surface expression of HLA class I antigens (86,89,96). Approximately one-third of MSI-H CRCs were also shown to lose HLA-DR expression (97,98). This HLA class II molecule is an important mediator of antigen presentation in antigen-presenting cells and its loss could constitute an additional immune-evasive mechanism in MSI-H CRCs.

The makeup and magnitude of the anti-tumour immune reaction were previously associated with clinical prognosis and tumour staging in CRC (99,100). MSI-H and MAP tumours have been reported to present an improved clinical behaviour when compared to the remaining spectrum of CRCs (101,102). This observation could be, in part, explained by the robust anti-tumour immune reaction provoked by these tumours (103). Notably, the group from von Knebel Doeberitz has reported that healthy Lynch carriers are able to mount an antibody-mediated response against frameshift peptides that are commonly mutated in MSI-H colorectal tumours (104,105). This observation suggests that at some point, in the lifetime of Lynch carriers, the host immune system encountered those mutated peptides. Whether this was also responsible for preventing the onset of malignancy is still speculative. Paradoxically, the immune system, as discussed, is also an important vector of selective pressure leading to the outgrowth of tumour cell clones with immune-evasive phenotypes. Although the loss of HLA class I provides tumour cells with an effective local adaptation mechanism, it might impair tumour migration and tumour spreading to other organs (106). A different component of the immune system is responsible for detecting whether cells carry 'self' markers such as HLA class I — "missing-self" recognition (107). This mission is primarily carried out by natural killer (NK) cells that become cytotoxic when target cells fail to present HLA class I, an inhibitory ligand for NK cell activation (108). NK cells are mostly present in the blood stream and thus do not affect local tumour growth but when tumour cells metastasise they might encounter NK cells and be eliminated (109). It is thought that tumour cells often compensate HLA class I loss by favouring the expression of additional NK-inactivating ligands and by losing antagonist ligands with NK-activating properties (110,111). We found a significant association between the presence of activated CD8+ T cells in Lynch carcinomas and early staging of the primary tumours. Conversely, we discovered an elusive immune cell population that is characteristic for non-metastasised Lynch CRCs (N. de Miranda, in preparation). Such complementary immune reaction by two types of immune cells countering tumour growth locally and at distance might explain the improved clinical prognosis of Lynch CRC patients and be further explored for their clinical management.

Management of MSI-H and MAP CRC patients

As discussed, MSI-H and MAP colorectal patients are thought to possess improved survival rates when compared to the remaining spectrum of CRC patients (101,102). This observation could be partly explained by the immunogenic character of MSI-H and MAP tumours. On the other hand, their DNA repair-deficient background could be responsible for the frequent generation of unviable tumour clones that have reached unsustainable states of genomic instability (103). Nevertheless, MSI-H CRC patients do not seem to benefit from adjuvant fluorouracil (5-FU) chemotherapy, which integrates the chemotherapeutic scheme (also including oxaliplatin) generally applicable to Stages III and IV CRCs (112,113). Additionally, still a considerable amount of MSI-H patients present advanced tumour stages at diagnosis and succumb from this disease. Therefore, novel therapeutic strategies that specifically target this group of tumours should be developed and their rationale could also be applied to the treatment of MAP carcinomas.

The immunogenic character of MSI-H and MAP carcinomas remains to be fully exploited and might hold a promising source of therapeutic opportunities. Vaccination and T cell-based immunotherapeutic approaches provide a way to prolong survival of certain groups of patients in clinical trials but treatment of advanced disease still remains a promise (114). This may be explained 2-fold: as demonstrated, advanced CRCs already acquired immune-evasive phenotypes and are therefore insensitive to therapeutic vectors that require antigen recognition, and the intrinsic nature of the clinical trials does not allow the selection of patients with cancers that present immunogenic features. The findings from von Knebel Doeberitz's group (104,105) have encouraged others to propose the application of prophylactic vaccination strategies in Lynch syndrome and MAP patients. The stimulation of an early and robust response of the immune system, based on commonly mutated peptides, could prevent or delay tumour onset at a stage that antigen presentation still occurs in cancer cells. From the diagnostic point of view, the monitoring of serum responses to tumour antigens could be utilised for improved surveillance of Lynch and MAP carriers. On the other hand, the recruitment of Lynch and MAP patients for such trials is difficult as they are already included in effective colonoscopic surveillance schemes (115,116).

The DNA repair-deficient background of MSI-H and MAP colorectal carcinomas might also encourage the investigation of the efficacy of targeting DNA repair mechanisms based on the concept of synthetic lethality (117). MMR and BER mechanisms cooperate in the processing of mutations and the targeting of one system in the genetic background of deficiency of the other could promote the generation of an unsustainable state of genetic instability in tumour cells. Our group reported the extremely mild phenotype of a Lynch patient that in addition to possessing a germ line *MSH6* mutation was also carrier of compound heterozygous mutations in the *MUTYH* gene. A genetic background of synthetic lethality that impedes the establishment of a second hit in the *MSH6* gene when BER is inactivated might offer an explanation for this mild phenotype (118).

Future perspectives

With the current advances in genomic technology, we are expected to comprehend further the genetics of MSI-H and MAP CRC tumourigenesis. Moreover, the definition of the landscape of the cancer genome of these tumours might deliver novel therapeutic targets, including the definition of a set of commonly mutated antigens that could be used for prophylactic vaccination of

Lynch syndrome and MAP carriers. The description of mutations in MSI-H CRCs has been strongly biased towards microsatellite repeats as their screening is less technically demanding and strongly supported by the type of DNA repair deficiency characteristic of MSI-H tumours. Nevertheless, since the processing of nucleotide mismatches in an MMR-deficient background is also impaired, a surplus of this type of mutations is also expected to be found in MSI-H cancers. Mismatches that translate into amino acid substitutions could be more easily applicable for vaccination strategies as they are less likely to affect protein expression as opposed to frame shifts that introduce early STOP codons and affect mRNA and protein stability. The definition of common mutation targets in MAP carcinomas would simultaneously shed light on the genetics of tumourigenesis of these cancers and provide potential therapeutic targets. In the meanwhile, the high frequency of G:T transversions observed in codon 12 of the *KRAS* gene could be exploited for the testing serum responses in MAP patients against KRAS mutated peptides. Finally, the close association between MSI-H and MAP tumour progression with the loss of HLA class I expression suggests that individuals might be at a different risk for developing CRC depending on their ability to present tumour antigens to the immune system. The extremely polymorphic character of the HLA class I system and variation of haplotypes among the population might conceal distinct susceptibilities to the development of these diseases.

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