Mismatch repair and MUTYH deficient colorectal cancers: at the crossroad of genomic stability and immune escape

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Mismatch repair and MUTYH deficient colorectal cancers: at the crossroad of genomic stability and immune escape

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Kettlewell found that in unpolluted areas, more of his light-colored moths had survived. In soot-blacked areas, more of the dark-colored moths had survived. Thus Kettlewell showed that in each environment the moths that were better camouflaged had the higher survival rate. It was logical to conclude that when soot darkened the tree trunks in the area, natural selection caused the dark-colored moths to become more common. Today Kettlewell's work is considered to be a classic demonstration of natural selection in action.

-BIOLOGY ("The Elephant Book"), Miller & Levine, p.298. -

To my parents
and
in memory of Prof. Noé Gonçalves de Miranda

Contents:

Abbreviations:

BMP	Bone morphogenetic protein
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CTL	Cytotoxic T cell
FAP	Familial adenomatous polyposis
HLA	Human leukocyte antigen
HNPCC	Hereditary non-polyposis colorectal cancer
MAP	MUTYH-associated polyposis
MHC	Major histocompatibility complex
MSI	Microsatellite instability
MSI-H	Microsatellite instability - high
NK	Natural killer
TGF-β	Transforming growth factor beta

General Introduction

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Chapter 1

1. COLORECTAL CANCER

1.1. Epidemiology and etiology of colorectal cancer

Colorectal cancer is the third most frequently diagnosed cancer and the fourth most common cause of cancer-related deaths worldwide. Its incidence is estimated at 17.2 per 100,000 individuals, although higher prevalence is observed in the so-called "developed countries" (1). The overall five-year survival rate for colorectal cancer is higher than 50% but the individual patient prognosis is highly dependent on tumor-staging at diagnosis (2, 3). For instance, patients affected by localized lesions (stages 0 and I) present five-year survival rates higher than 90% (2). The majority of colorectal cancers (approximately 95%) arise in a sporadic context, while autosomal dominant and recessive cancer syndromes are responsible for the remainder (Figure 1). Nevertheless, familial aggregation of colorectal cancers, not explained by known cancer syndromes, is observed in approximately one-third of the so-called "sporadic cases" (4). The identification of genetic predisposition factors in these families constitutes one of the major remaining challenges in colorectal cancer research (5, 6). Tobacco smoking, high intake of saturated fat and red meat, and alcohol consumption constitute major environmental factors that have been associated with an increased risk for colorectal cancer (7).

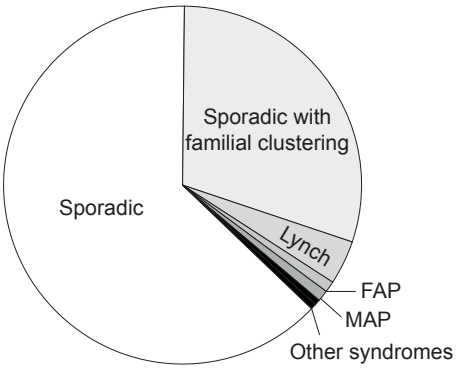

Figure 1. Spectrum of colorectal cancers according to their etiology (FAP - Familial Adenomatous Polyposis syndrome; MAP - MUTYH-associated polyposis syndrome).

1.2. Genetics of oncogenesis

Colorectal cancer is a heterogeneous disease where different oncogenic pathways can support cancer development (8, 9). Classically, colorectal cancers have been divided according to the type of genetic instability that is observed in tumors (Figure 2). Extensive accumulation of nucleotide insertions and deletions at DNA microsatellite sequences (short nucleotide repeats) are observed in 15-20% of colorectal cancers. Such phenotype, denominated microsatellite instability-high (MSI-H), is caused by a defective DNA mismatch repair system (10, 11). Almost invariably, MSI-H sporadic colorectal cancers display DNA hypermethylation of the *MLH1* gene promoter, thereby silencing its expression, as well as widespread methylation of gene promoters throughout the genome (10, 11).

Lynch syndrome, previously denominated hereditary non-polyposis colorectal cancer (HNPCC) syndrome, is the hereditary counterpart of MSI-H colorectal cancers and affects carriers of germline mutations in mismatch repair genes, where *MLH1*, *MSH2*, *MSH6,* and *PMS2* are most commonly affected. Lynch syndrome is an autosomal dominant genetic condition where one defective allele of a mismatch repair gene is inherited. Cancer development in carriers generally involves the somatic inactivation of the second copy of the gene (10, 11). The mismatch repair system is a caretaker of the genome that is essential for the repair of nucleotide mismatches and small base insertions and deletions (12). Microsatellite DNA sequences are hotspots for the accumulation of mutations, resulting from the frequent slippage of DNA polymerases at these sites (13). This is proposed to result

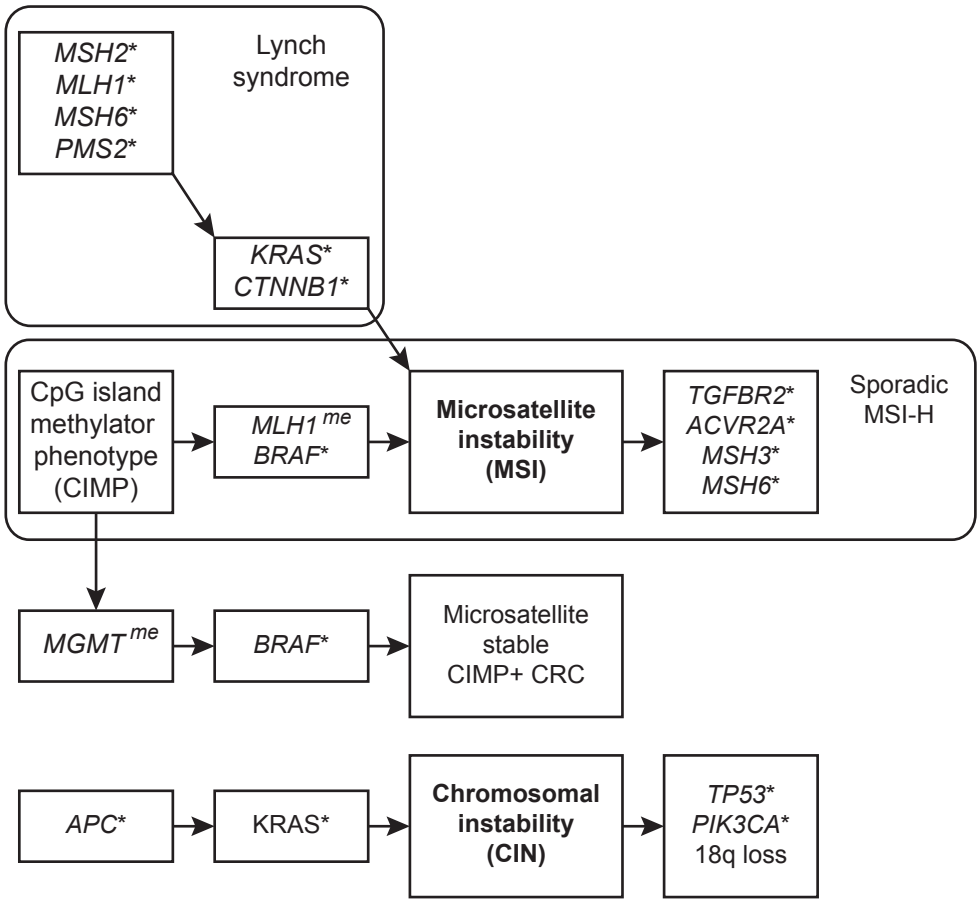

Figure 2. Simplified scheme representing the most frequent (epi-) genetic alterations occurring during colorectal carcinogenesis in different genetic pathways. (* - mutations; me - methylation; CRC - colorectal cancer).

from the formation of loop DNA structures in single stranded microsatellites and from inefficient proofreading exonuclease activity by the DNA polymerase (14, 15). Large chromosomal aberrations are rare in MSI-H colorectal cancers and their cells generally possess peridiploid DNA contents, similar to the one of a healthy somatic cell (16). MSI-H colorectal cancers develop more frequently in the colon ascendens and are further characterized by a poorly differentiated and mucinous histology and a dense intraepithelial, lymphocytic infiltrate (8, 17, 18).

Most colorectal cancers (80-85%) are mismatch repair proficient and do not display microsatellite instability (MSI). Instead, the majority of microsatellite stable colorectal cancers present gross chromosomal aberrations that translate into aneuploid DNA contents in tumor cells (19). Recurrent chromosomal aberrations in colorectal cancer include gains of chromosomes 7, 8q, 13, and 20q and losses of 4q, 8p, and 18q (20-22). The generation of chromosomal instability (CIN) has been associated with the loss of function of the *Adenomatous Polyposis Coli* (*APC*) gene, a classical tumor suppressor in colorectal cancer (23). Truncating mutations in *APC* occur in the majority of colorectal cancers with CIN and are considered to be one of the

initiating events in colorectal tumorigenesis (24, 25). APC is part of a protein complex that controls the availability of β-catenin, a key signal transducer of the canonical Wnt signaling (26). Loss of APC promotes the stabilization and nuclear accumulation of β-catenin that, upon association with specific transcription factors, activates the transcription of proto-oncogenes such as *MYC* and *CCND1* (26, 27). APC defects were also shown to disturb kinetochore function and chromosomal segregation during mitosis, thereby supporting APC's role in the propagation of CIN (23). Activation of Wnt signaling has also been suggested to promote the so-called stemness of cancer cells that, thereby, can overcome replicative senescence (28, 29). Germline mutations in *APC* cause familial adenomatous polyposis (FAP), an autosomal dominant disease that is responsible for less than 1% of all colorectal cancers (30). Although common, truncating somatic mutations in *APC* are less frequent in MSI-H colorectal cancers when compared to tumors with CIN (31). Interestingly, MSI-H colorectal cancers, particularly the ones associated with Lynch syndrome, display relatively frequent mutations in the β-catenin gene (*CTNNB1*) (31, 32). Such mutations were suggested to increase the stability of β-catenin and, thereby, to produce an effect similar to the loss of APC (33). Although alterations in the Wnt signaling pathway constitute a hallmark in colorectal cancer development, biallelic inactivation of *APC* or activating mutations in *CTNNB1* are only present in approximately 80% of tumors. The comprehensive characterization of the genomic landscape of colorectal cancers identified less frequent mutation targets such as *SOX9*, *TCF7L2*, *AXIN2*, *FBXW7*, *ARID1A,* and *FAM123B*, which, cumulatively, might explain Wnt activation in the remaining proportion of cases (24).

Another form of (epi-) genetic instability can be recognized in a subset of colorectal cancers and it refers to the widespread methylation of CpG islands at gene promoters (34). The CpG island methylator (CIMP) and the MSI phenotypes are largely overlapping but CIMP-positive, MSI-negative tumors still account for approximately 8% of colorectal cancers (35). Of note, in a sporadic context, CpG methylation changes, often accompanied by mutations in *BRAF*, are considered to precede the onset of MSI (36). Furthermore, CIN can accompany CIMP in a substantial proportion of cases (37, 38). Interestingly, a fraction of colorectal cancers simultaneously lack CIN, MSI, and CIMP (37). The type of genetic instability observed in different tumors has been shown to correlate with the patients' survival and response to therapy. An improved patient prognosis has been associated with the MSI phenotype, while worse patient survival was reported for CIMP-positive tumors that lacked MSI (37, 39). Paradoxically, MSI colorectal cancers appear to be less sensitive to fluorouracil (5-FU), the standard chemotherapeutic adjuvant in colorectal cancer therapy (40, 41).

In addition to FAP and Lynch syndrome, a number of other cancer syndromes are responsible for the onset of colorectal cancer in a hereditary setting. MUTYH-associated polyposis (MAP) constitutes the only known colorectal cancer syndrome that is inherited in a recessive manner. It is caused by germline mutations in the gene that encodes for the MUTYH DNA glycosylase (42). Although MAP patients display a milder phenotype than FAP patients, most carriers of biallelic mutations in *MUTYH* develop numerous polyps at a young age that, eventually, progress to malignant lesions (42). The MUTYH protein is part of the DNA base-excision repair pathway and is involved in the repair of one of the most common forms of oxidative damage, the oxidation of guanine to 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG). In the absence of MUTYH, cells display a distinctive mutation signature that is characterized by the abundance of G:C

to T:A transversions, which results from the mispairing of 8-oxoG with adenines during replication (43). Similar to MSI-H cancers, MAP patients were reported to have better overall survival than sporadic colorectal cancer patients (44). Furthermore, MAP tumors most often develop in the proximal colon and frequently present mucinous histologies and high amounts of intraepithelial lymphocytic infiltrate (45).

A number of human syndromes have been associated with the finding of hamartomatous and/or hyperplastic polyps in affected individuals (46). However, for several of those, the "a priori" risk for colorectal cancer development is unknown. On the other hand, the risk for colorectal cancer has been clearly assessed in the autosomal dominant Peutz-Jeghers syndrome (PJS) and in the juvenile polyposis syndrome (JPS), which are caused by germline mutations in the *STK11/LKB1* gene and the *BMPR1A* or *SMAD4* genes, respectively (47). Although mutations in *BMPR1A* and *SMAD4* explain a considerable proportion of juvenile polyposis cases, the genetic basis of disease is still elusive in a large number of patients. It was recently established that patients suffering from the PTEN hamartoma tumor syndrome, comprising Cowden and Bannayan-Riley-Rubalcaba syndromes, are also at increased risk for colorectal cancer (48). Finally, a recent whole genome sequencing approach identified high-penetrance variants that confer susceptibility to colorectal polyposis and cancer in the *POLE* and *POLD1* genes (49). Those variants were located in the proofreading domains of the polymerases and compromised the repair of mismatches introduced during DNA replication.

1.3. Mutation landscape of MSI-H and MAP colorectal cancers

MSI-H colorectal cancers are notorious for the accumulation of insertions and deletions at microsatellite DNA sequences throughout the genome. Accordingly, genes containing microsatellite repeats within their coding regions are often targeted by mutations in those tumors. The *TGFBR2* and *ACVR2A* genes, which contain ten and eight adenine microsatellites, respectively, are found mutated in the majority of MSI-H colorectal cancers (50-52). Interestingly, the *MSH3* and *MSH6* mismatch repair genes also carry microsatellite sequences within their sequences that are targeted by MSI (24). This secondary targeting of mismatch repair genes constitutes an additional source of DNA repair deficiency that contributes to the high mutation load of MSI-H cancers (53-55). Similar to the inactivation of the *APC* gene, the constitutional activation of the MAPK signaling pathway is one of the primary events in colorectal cancer tumorigenesis. It occurs mainly through the establishment of activating mutations in the KRAS GTPase or the BRAF serine/threonine kinase (Figure 2), in a mutually exclusive manner (56). Mutations in the *BRAF* gene are most frequent in sporadic MSI-H cancers and absent in Lynch-associated cancers (57, 58). In turn, mutations in the *KRAS* gene are more common in colorectal cancers with CIN and the ones developing in patients with Lynch syndrome (Figure 2) (59-61). As discussed previously, in addition to their hypermutated genomes, most MSI-H sporadic cancers display a methylator phenotype, responsible for the altered expression of a myriad of genes (Figure 2) (24).

The majority of MAP carcinomas display mutations in the *APC* and *KRAS* genes that are postulated to derive directly from the MUTYH-associated, base-excision repair deficiency (62). G>T transversions at GAA triplets are frequent in *APC* and nearly all *KRAS* mutations found in MAP carcinomas are restricted to a c.34 G>T transversion, an uncommon substitution in the remaining spectrum of colorectal cancers. Mutations in *TP53* and *SMAD4* are also encountered in a substantial proportion of MAP carcinomas

but are not restricted to G>T transversions, suggesting that they might occur at a later stage in tumorigenesis (45). In agreement with the fact that the base-excision repair system is not directly involved in the repair of small insertions and deletions, MSI is rarely observed in MAP carcinomas (45, 62). Interestingly, MAP tumors display a distinctive form of chromosomal instability characterized by the widespread presence of chromosomal copy-neutral loss of heterozygosity (63).

2. TGF-β SIGNALING PATHWAY: A MULTIFACETED REGULATOR OF CARCINOGENESIS

The transforming growth factor-β (TGF-β) signaling pathway regulates cell proliferation, differentiation, apoptosis and migration (64). Abnormalities in this pathway compromise tissue homeostasis and may support carcinogenesis (65, 66). Signal transduction is initiated with the binding of a TGF-β ligand to the TGF-β type 2 transmembrane serine/threonine kinase receptor TGFβR2, which becomes activated and phosphorylates the type 1 TGF-β serine/threonine kinase receptor TGFβR1 (67). A type 3 TGF-β receptor (TGFβR3) facilitates the interaction between TGF-β ligands and the serine/threonine kinase receptors (Figure 3) (68). Upon activation, TGFβR1 phosphorylates a receptor-regulated Smad (Smad2, Smad3) that forms a heterocomplex with the co-Smad, Smad4, in the cytoplasm (Figure 3) (69, 70). This complex translocates to the nucleus where it modulates the expression of gene targets together with additional transcription factors (Figure 3) (71). Alternative ligands (e.g. Activins) and receptors (e.g. ACVR2A, ALK4) can also convey TGF-β signaling to Smad2 and Smad3 (70). The bone morphogenetic protein (BMP) pathway operates in an analogous way to TGF-β but makes use of different ligands, receptors, and intracellular Smad proteins, except for Smad4, which operates as a co-Smad in both the TGF-β and BMP pathways (69).

As discussed previously, the *TGFBR2* and *ACVR2A* genes are fated to mutate in MSI-H colorectal cancers due to the presence of microsatellite repeats within their protein-

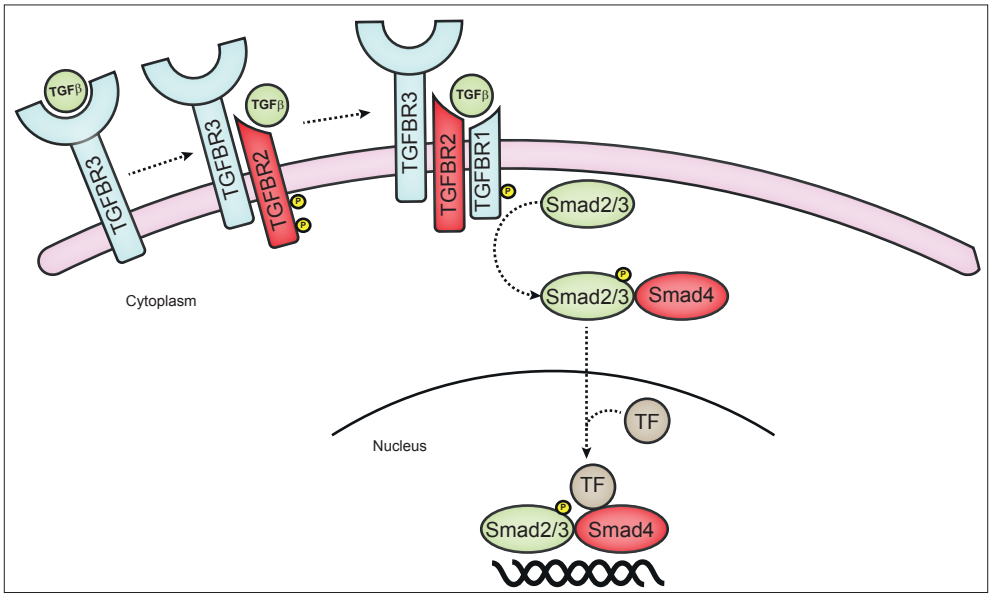

Figure 3. The TGFβ signaling pathway (adapted from Meulmeester et al. (72)). The molecules more often affected by mutations in colorectal cancer are depicted in red. (TF - transcription factor).

coding sequences (50-52). Mutations often target both alleles and result in frameshifted, early-truncated proteins that are unable to transduce TGF-β signaling. Mutations in TGF-β receptor genes are uncommon in microsatellite stable tumors that instead target the Smad proteins, most often Smad4. The *SMAD4* gene is found mutated in up to 15% of microsatellite stable colorectal cancers and its locus (18q21.1) is targeted by loss of heterozygosity in the majority of CIN colorectal cancers (21, 73, 74). Since the *SMAD2* gene is located in the same chromosomal region, it is also affected by loss of heterozygosity. Mutations in *SMAD2* and *SMAD3* occur in a minority of colorectal cancers (75).

Disruption of the TGF-β pathway leads to the decreased expression of TGF-β target genes such as the cell cycle regulators *CDKN1A* (p21) and *CDKN2B* (p15), thereby providing a growth advantage to tumor cells (76, 77). Accordingly, loss of SMAD4 expression has been associated with advanced disease stages and poor prognosis in colorectal cancer patients (78, 79). On the other hand, a dual role has been attributed to TGF-β in the sense that activation of this pathway might also promote malignant behavior. High levels of TGF-β ligand at primary tumors were correlated with metastatic disease and tumor recurrence in colorectal cancer (80, 81). Furthermore, TGF-β production by cancer cells was shown to dampen anti-tumor immune responses and to promote the colonization of tumor metastasis through its activity on stromal cells (82, 83). By acquiring defects in TGF-β signalling mediators, tumor cells can modulate their microenvironment through TGF-β production without suffering from its growth suppressive effects.

3. TUMOR IMMUNOLOGY

3.1. The immune system: unable but equipped?

Cancer development is accompanied by massive changes at cellular and tissue level that, theoretically, could be detected and dealt with by the immune system. Nevertheless, reports on immune system-mediated, spontaneous tumor rejections, in humans, are scarce. As most cancer-related deaths occur after reproductive age, and are thus not involved in natural selection, the contribution of this disease for the shaping of the immune system is considered to be limited. Nevertheless, both the innate and adaptive immune systems are equipped with mechanisms to detect and eliminate anomalous cells. Moreover, the increased risk for malignancies in patients receiving immune suppressants partially supports a role for the immune system as a tumor suppressor (84). It should be noted, however, that the use of immune suppressants also impairs the clearing of infections by oncogenic viruses and thus, the increased cancer risk observed in these patients is not exclusively attributable to an impaired anti-tumor immune response.

The genetic and epigenetic alterations that occur in cancer cells lead to changes in their protein repertoire that include the production of mutated proteins and the abrogation of proteins that would normally be expressed in their non-transformed counterparts. The former may constitute novel antigens for which central T cell tolerance was not imposed (85). They could trigger anti-tumor immune responses that would eventually lead to the destruction of cancer cells ("non-self recognition") (86, 87). The activation of anti-tumor immune responses requires the uptake of tumor antigens by professional antigen presenting cells such as dendritic cells (Figure 4). The high turnover of cancer tissues guarantees an abundant source of tumor antigens but

also of molecular "danger signals" that are essential for dendritic cell activation (88). Upon activation, dendritic cells migrate to tumor-draining lymph nodes where they induce proliferation and activation of antigen-specific CD4+ and CD8+ T cells (Figure 4). The activation of CD8+ T cells occurs through the presentation of antigens by Human Leukocyte Antigen (HLA) class I molecules (cross-presentation) while antigen presentation to CD4+ T cells is mediated by HLA class II molecules (89). Once activated, CD8+ T cells acquire cytotoxic capacity and the ability to eliminate cancer cells that express the same tumor antigen that led to their activation. The killing of tumor cells may occur through the release of lytic granules by cytotoxic CD8+ T cells (CTLs) that contain the pore-forming protein Perforin and Granzyme A and B proteases but also by the Fas-FasL cell death pathway, provided that tumor cells express the Fas receptor (90, 91). Expression of HLA class I molecules by tumor cells is an essential condition for the recognition of tumor antigens by CTLs (92, 93).

In addition to their role in presenting "non-self" antigens to immune cells, HLA class I molecules are fundamental for recognition of the "self". Their absence from the cell surface ("missing-self") evokes the action of natural killer (NK) cells, another lymphocyte with cytotoxic potential (94, 95). HLA class I molecules constitute ligands for NK cell receptors that contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) at their cytoplasmic tail. These motifs become phosphorylated upon HLA class I binding and subsequently suppress NK cell activation (96). In addition, activating signals are also required to trigger NK cell-mediated cytotoxicity, generally transduced by the NKG2D receptor at NK cells. Those signals are provided by ligands such as MICA, MICB or ULBP, which are upregulated in target cells as a consequence of cellular stress (97-99). It is considered that the balance between inhibitory and activation signals provided by target cells ultimately determines the action of NK cells.

The combination of T cell-mediated recognition of tumor antigens and the detection of anomalous cells by NK lymphocytes could constitute effective anti-tumor barriers. In support of this, recognition of tumor antigens by autologous T cells has been widely demonstrated in cancer patients (100, 101), while a variety of tumors have been shown to express NK cell-activating ligands (102, 103). Thus, not surprisingly, the presence of tumor-infiltrating immune cells constitutes a relevant prognostic indicator in cancer patients (104, 105). Remarkably, Galon and colleagues discovered that qualitative and quantitative profiles of immune cell infiltration in colorectal cancer were better prognostic indicators than tumor staging, although a potential overrepresentation of MSI-H tumors in the study cohort was not accounted for (106). The presence of high numbers of tumor-infiltrating lymphocytes has generally been associated with improved clinical outcomes in colorectal cancer patients (107). Reports were most concordant when analyzing specifically the infiltration by cytotoxic T cells expressing granzyme B (108, 109), suggesting a major role for antigen-driven anti-tumor immune response. The potential role of other T cell subsets such as regulatory T cells (Tregs) in the progression of colorectal cancer has not been clearly established (107). As for NK cells, they are relatively infrequent in colorectal cancer tissues but the expression of NKG2D ligands in cancer cells has been associated with improved patient prognosis (103, 110). NK cells appear to be particularly important in controlling tumor metastases by eliminating circulating tumor cells in the blood stream (111, 112).

3.2. Immune escape: too fast, too furious

The mechanisms underlying cancer cell

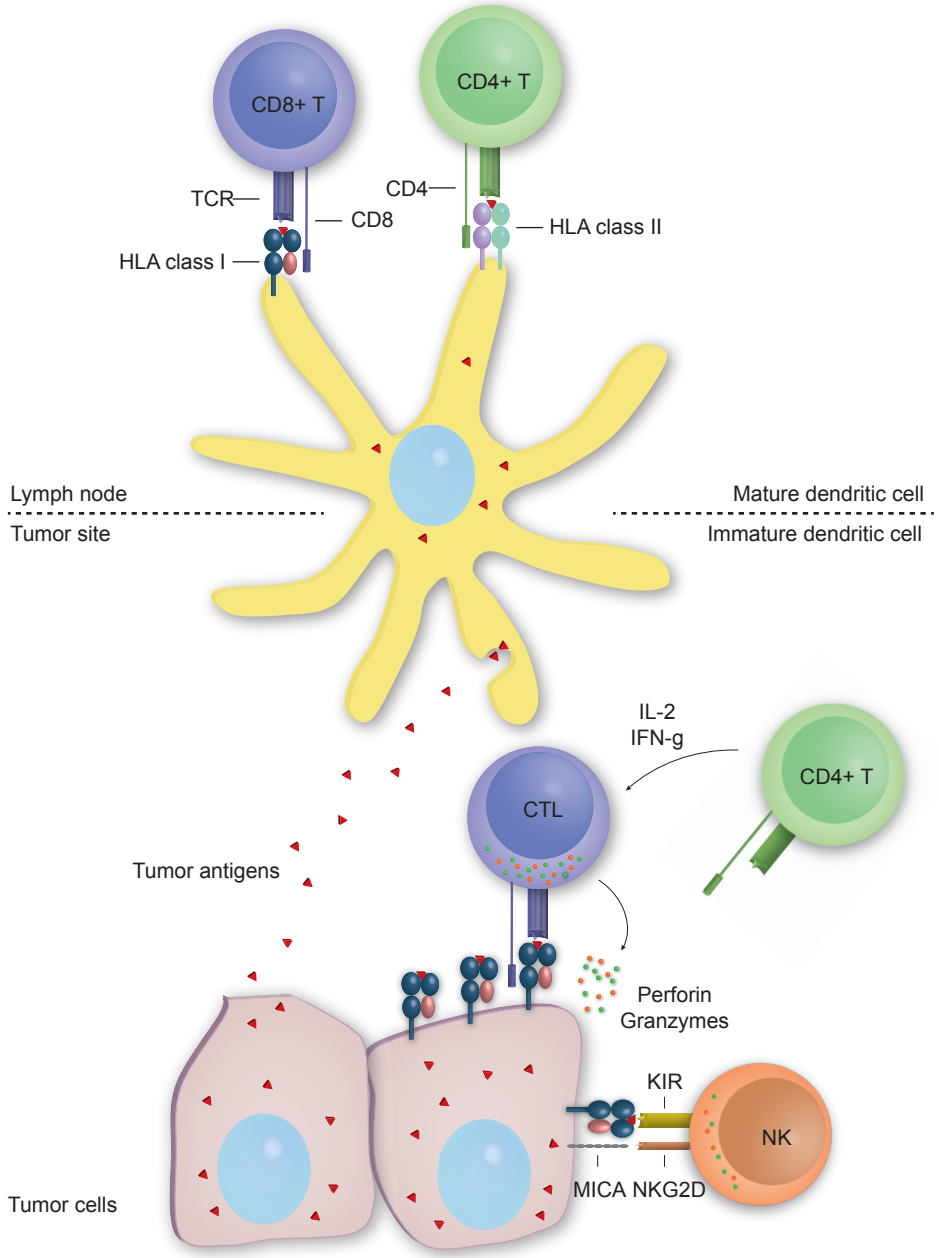

Figure 4. Dendritic cells play a central role in mediating anti-tumor immune responses. The high cellular turnover of tumor tissues guarantees an abundant source of tumor antigens for dendritic cells. After picking up the antigens and transporting them to the draining lymph nodes, dendritic cells activate naïve CD4+ and CD8+ T cells through the presentation of HLA/antigen complexes. CD8+ T cell activation occurs through HLA class I while CD4+ T cell activation is mediated by HLA class II. Additional co-stimulatory signals are required for T cell activation and are provided by the interaction between B7 ligands present on antigen presenting cells and the CD28 receptor on T cells. Once activated, CD8+ T cells gain cytotoxic capacity and the ability to eliminate target cells that present their specific antigen.

resilience to the action of the immune system, also in the context of immunotherapy, have been a major object of study for tumor immunologists throughout the years. Anti-tumor immune responses constitute strong vectors of selection that contribute towards the shaping of clonal evolution in cancer. One of the most common and functionally interpretable immune evasive mechanisms is the loss of HLA class I expression by tumors. By losing HLA class I expression, cancer cells are excused from presenting tumor antigens to CTLs, thereby avoiding detection and destruction (113). Furthermore, loss of HLA class I is also expected to result in failure of therapeutic approaches based on CD8+ T cell recognition such as vaccination with tumor antigens or adoptive transfer of autologous T cells (114). On the other hand, abrogation of HLA class I expression would support NK cell-mediated recognition in the presence of activating signals and thus, further escape mechanisms are expected to accompany HLA class I loss (115, 116). Cellular stress, such as the one derived from DNA damage, was shown to result in increased expression of NK cell activating ligands (117). Therefore, and providing that DNA damage response mechanisms are in place, tumor cells should upregulate the expression of NK cell activating ligands as a consequence of their chronic exposure to replicative damage and/or chromosomal instability. Not surprisingly, a considerable proportion of human cancers lack NKG2D ligands and are, thereby, resistant to NK cell-mediated lysis, even when HLA class I expression is abrogated (103, 118, 119). Absent or low expression of NKG2D ligands has been generally correlated with increased malignant behavior of tumors (103, 120, 121), but conflicting findings underline the complexity of anti-tumor immunity (122, 123). An additional escape mechanism to NK cells is provided by the release of soluble forms of NKG2D ligands by tumor cells, which induce the internalization and destruction of NKG2D, thereby impairing NK cell function (124). Furthermore, acquired expression of the non-classical HLA-G antigen and the loss of HLA class II expression have also been reported in cancer cells (125, 126). As a corollary of the accumulated evidence on the selection of immune evasive traits during cancer progression, Douglas Hanahan and Robert A. Weinberg have recently acknowledged immune escape as a hallmark of cancer in an updated version of their seminal review (127). The aforementioned phenotypes are conceptually concordant with Darwinian models of evolution that imply the elimination of "less-fit" tumor clones (128). The generation of clonal diversity is fundamental for the emergence of immune escape phenotypes and other traits. The latter is assured by the impairment of DNA repair and damage response mechanisms that support the accelerated evolutionary process that accompanies tumorigenesis. Nevertheless, there is a high probability that carcinogenic processes not always lead to the generation of tumor variants with immune evasive properties and that, occasionally, tumors are indeed swiftly eliminated by the immune system in asymptomatic individuals.

3.3. HLA genes and the HLA class I antigen presenting pathway

The HLA system is the human counterpart of the major histocompatibility complex (MHC), a unifying feature in vertebrate organisms that plays a key role in the immune system. The MHC class I and class II loci are comprised of the most polymorphic genes known in vertebrates in spite of the strong selective pressure imposed by the evolutionary "arms race" between hosts and pathogens. Instead, a positive selection is in place for the maintenance of MHC variability, derived from the fact that the immune system has to deal with a myriad of pathogens (129).

The HLA class I molecule is a heterodimer

consisting of a variable heavy chain (α) and the non-polymorphic β2-microglobulin molecule, encoded by a gene located on chromosome 15. Three loci encode for different HLA class I heavy-chains on chromosome 6p: *HLA-A*, *HLA-B* and *HLA-C*. These are generally defined as the classical HLA class I genes for which more than 5000 alleles are currently known (130). The *HLA-E*, *HLA-F* and *HLA-G* genes comprise the non-classical HLA class I genes, which are considerably less polymorphic than the classical HLA class I genes. Their proteins are not involved in general antigen presentation but, instead, they bind peptides derived from the classical HLA class I molecules themselves (131). Similar to the classical HLA class I molecules, they can also modulate NK cell activity (132). HLA class II molecules are composed of two variable chains (α and β), encoded by six main HLA class II genes also located on chromosome 6p: *HLA-DPA1*, *HLA-DPB1*, *HLA-DQA1*, *HLA-DQB1*, *HLA-DRA* and *HLA-DRB* (130).

Each HLA class I (classical) or class II allelic variant is able to present a pool of peptides that display affinity to that specific allele. This affinity is defined by anchor residues located at the extremities of a peptide sequence that directly bind the HLA molecule. Since the sequences within anchor residues are relatively free to vary, each HLA class I molecule can present a broad range of peptides (133). Cross-presentation-apart, the peptides presented in the context of HLA class I are derived from endogenous proteins that have either reached the end of their functional life or resulted from defective transcription or translation (Figure 5). Those peptides are generated in the cytosol by the 26S proteasome which is composed of a 20S core barrel protein complex with protease activity, sandwiched by two 19S caps (134). The LMP2 (PSMB9), LMP7 (PSMB8) and LMP10 (PSMB10) proteins are core subunits of the 20S complex in the immunoproteosome, a modified

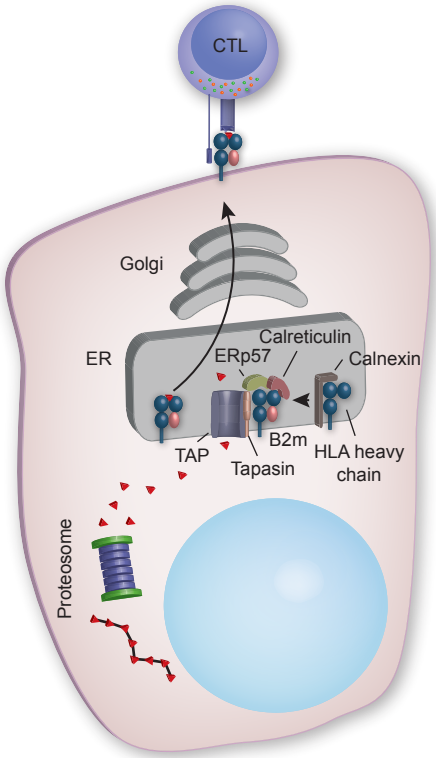

Figure 5. HLA class I antigen processing machinery.

proteasome form that is particularly effective in generating peptides for HLA class I in the presence of inflammatory signals (135). Recently, the cytosolic endopeptidases nardilysin and TOP were also shown to complement the proteosome's activity and to be essential for the generation of specific CTL epitopes (136). In order to be loaded onto HLA class I molecules, peptides must be transported by the transporter associated with antigen processing (TAP) proteins TAP1 and TAP2 into the lumen of the endoplasmic reticulum (Figure 5). The TAP proteins associate with HLA class I molecules through their interaction with Tapasin, a HLA class I chaperone. Additional chaperones such as Calnexin, Calreticulin and ERp57 are involved in stabilizing HLA class I and in assisting the loading of peptides onto this molecule (137). Often, peptides require further trimming in the endoplasmic

reticulum before loading onto HLA class I. This task is performed by the ERAP1 and ERAP2 aminopeptidases (138). Since the peptides generated by the 26S proteasome are highly unstable in the cytosol, only a small fraction of those reach the cell surface in complex with HLA class I (Figure 5) (139). The HLA class II antigen presenting pathway deals with peptides derived from exogenous proteins that are processed by endocytic pathways (134).

3.4. The HLA system: around the dogmas

Traditionally, HLA class I molecules have been considered to be expressed on nearly every nucleated cell of the human body, except for few "immune privileged" sites (e.g. brain, cornea, liver, and testis). However, a number of studies support that additional tissues present non-detectable or reduced HLA class I expression (115, 140, 141). We (de Miranda and Morreau, unpublished), and others (142), have also observed that HLA class I expression is often higher in colorectal cancers than in the normal mucosa, which might derive from an overall increase in protein expression in tumors or from a natural response to cellular stress. Therefore, in certain contexts, the lack of HLA class I expression in tumors might not represent a loss but rather the inability of tumor cells to induce HLA class I expression. Nevertheless, since normal colorectal tissue consistently displays immune-reactivity to anti-HLA class I antibodies, we refer to HLA class I loss throughout the thesis.

In the opposite direction, there is a generalized misconception that HLA class II expression is restricted to antigen-presenting cells such as B cells, dendritic cells and macrophages. On the contrary, HLA class II expression can be induced in a variety of cells including epithelial cells, endothelial cells, and fibroblasts in the presence of inflammatory signals (143, 144). Additionally, a variety of tumors have been shown to acquire expression of HLA class II molecules during tumorigenesis (126, 145, 146). These observations are of great relevance as HLA class II molecules are known to mediate the presentation of tumor-specific antigens (147, 148). Local activation of CD4+ T cells at tumor sites might support a more effective CTL response (149) but also the triggering of Th1 and Th2 inflammatory responses that engage macrophages and eosinophils, respectively (150). Furthermore, a subset of CD4+ T cells appears to possess cytotoxic capacity and the ability to eliminate target cells presenting tumor antigens in a MHC class II context (151, 152).

4. OUTLINE OF THE THESIS

In this thesis, we compiled five studies where we report some of the genetic and molecular alterations that accompany the tumorigenesis of mismatch repair and MUTYH deficient colorectal cancers, with particular focus on immune escape mechanisms.

Both sporadic and hereditary mismatch repair deficient colorectal cancers are characterized by the presence of a conspicuous intraepithelial lymphocytic infiltrate, indicative of an anti-tumor immune response. We hypothesized that those tumors would be particularly prone to adopt immune evasive strategies, such as the loss of HLA class I expression, in order to escape from immune cell-mediated recognition and destruction. In **chapter 2**, we studied the expression of HLA class I, and associated antigen processing machinery molecules, in a well-characterized set of sporadic and Lynch colorectal cancers. We compared the frequencies of HLA class I loss between mismatch repair-deficient and proficient colorectal cancers and dissected the molecular mechanisms that underlie HLA class I defects in sporadic and hereditary mismatch repair deficient tumors.

Following the discovery of the MUTYH-

associated polyposis (MAP) syndrome and the reported histopathological similarities with mismatch repair deficient tumors, we speculated that MAP colorectal cancers might also present a high frequency of HLA class I alterations. In **chapter 3**, we characterized the expression of HLA class I and associated antigen processing machinery molecules in a cohort of MAP colorectal cancers and compared our findings to the ones reported in chapter 2.

The outgrowth of tumor clones lacking HLA class I expression is likely to result from the immune system-mediated destruction of HLA class I-positive cancer cells. In addition to providing an effective immune escape mechanism from cytotoxic T cells, the loss of this essential immune recognition molecule may also alter the capacity of cancer cells to invade surrounding tissues or to disseminate at distance (metastases). **In chapter 4**, we investigated a potential correlation between the type and density of lymphocytic infiltration in Lynch colorectal cancers with their HLA class I phenotype and clinicopathological stage. By relating the density of intraepithelial lymphocytic infiltrate with distinct HLA class I phenotypes we sought to establish a link between the agent of selection and the selected traits, respectively.

In chapter 5, we have studied one of the most common genetic alterations found in MSI-H colorectal cancers: the accumulation of frameshift mutations in the *TGFBR2* gene. TGFβR2 is a fundamental receptor for the transduction of TGF-β signaling in cells. Despite the fact that biallelic truncating mutations in *TGFBR2* occur in the majority of MSI-H cancers, some studies have reported that TGF-β signaling is still active in these tumors. We have attempted to replicate the latter findings in a cohort of MSI-H tumors and in a panel of colorectal cancer cell lines. Furthermore, we provide a mechanistic explanation for the retained sensitivity to TGF-β observed in *TGFBR2* mutants.

The alterations observed in HLA class I expression and in TGF-β pathway components in colorectal cancers are tightly connected to the role of the microenvironment in selecting the "most fit" tumor phenotypes. In colorectal tumors that develop in a background of mismatch or base-excision repair deficiency, the relation between tumor genotypes, phenotypes, and the environmental agents of natural selection is particularly evident and fascinating. These relations are discussed in a review paper that comprises **chapter 6**.

A few concluding remarks and future perspectives are presented in **chapter 7**.

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HNPCC versus sporadic microsatellite-unstable colon cancers follow different routes toward loss of HLA class I expression

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ABSTRACT

Abnormalities in Human Leukocyte Antigen (HLA) class I expression are common in colorectal cancer. Since HLA expression is required to activate tumor antigen-specific cytotoxic T-lymphocytes (CTL), HLA class I abnormalities represent a mechanism by which tumors circumvent immune surveillance. Tumors with high microsatellite instability (MSI-H) are believed to face strong selective pressure to evade CTL activity since they produce large amounts of immunogenic peptides. Previous studies identified the prevalence of HLA class I alterations in MSI-H tumors. However, those reports did not compare the frequency of alterations between hereditary and sporadic MSI-H tumors neither the mechanisms that led to HLA class I alterations in each subgroup.

To characterize the HLA class I expression among sporadic MSI-H and microsatellite-stable (MSS) tumors, and HNPCC tumors we compared immunohistochemically the expression of HLA class I, β2-microglobulin (β2m), and Antigen Processing Machinery (APM) components in 81 right-sided sporadic and 75 HNPCC tumors. Moreover, we investigated the genetic basis for these changes.

HLA class I loss was seen more frequently in MSI-H tumors than in MSS tumors ($P < 0.0001$). Distinct mechanisms were responsible for HLA class I loss in HNPCC and sporadic MSI-H tumors. Loss of HLA class I expression was associated with β2m loss in HNPCC tumors, but was correlated with APM component defects in sporadic MSI-H tumors ($P < 0.0001$). In about half of the cases, loss of expression of HLA class I was concordant with the detection of one or more mutations in the *B2M* and APM components genes.

HLA class I aberrations are found at varying frequencies in different colorectal tumor types and are caused by distinct genetic mechanisms. Chiefly, sporadic and hereditary MSI-H tumors follow different routes toward HLA class I loss of expression supporting the idea that these tumors follow different evolutionary pathways in tumorigenesis. The resulting variation in immune escape mechanisms may have repercussions in tumor progression and behavior.

INTRODUCTION

During cancer development, tumor cells may elicit cytotoxic T-lymphocyte (CTL)-mediated immune responses–partly a consequence of accumulated gene mutations that are translated into altered peptides (1). Tumor cell expression of HLA class I-antigen complexes is essential for CTL recognition of aberrant peptides and subsequent activation (2). Consequently, alteration of HLA class I cell surface expression provides an effective mechanism by which tumors can escape immune detection (3,4). Multiple mechanisms have been shown to underlie defects in HLA class I expression by tumor cells. They include mutations in the individual HLA class I genes *HLA-A*, *-B* and *-C*, located on chromosome 6p21.3) (5); mutations in β2-microglobulin (β2m) (6-9), molecule required for cell surface expression of HLA class I antigens; and defects in components of the HLA class I-associated antigen-processing machinery (APM) (9-11). The APM consists of proteasome components delta, MB1 and Z; the immunoproteasome components LMP2, LMP7 and LMP10; peptide transporters TAP1 and TAP2; and chaperones Calnexin, Calreticulin, ERp57, and Tapasin. The immunoproteasome generates peptides mostly, although not exclusively from endogenous proteins, TAP1 and TAP2 facilitate peptide translocation from the cytosol into the lumen of the endoplasmic reticulum, where the peptides are loaded onto the HLA class I molecules with the aid from the several chaperones

(12).

Chromosomal instability (CIN) and microsatellite instability (MIN) are the two major forms of genetic instability in colorectal cancer. Combined with distinct somatic mutation patterns and epigenetic modifications, CIN and MIN lead to the development of sporadic colorectal cancer (13). MIN sporadic tumors, which constitute approximately 15% of all colorectal cancer cases and up to 40% of the tumors localized on the right side (preceding the splenic flexure) of the colon (14), have a phenotype resulting from the epigenetic inactivation of the mismatch repair gene *MLH1*. Its inactivation destroys a cell's ability to repair base-base mismatches and small insertions or deletions in repetitive stretches, leading to an accumulation of frameshift mutations that get translated into abnormal peptide sequences. When these mutations are accumulated to large extent in the cell genome the tumors are said to possess high-microsatellite instability (MSI-H) (15). Hence, it is expected that genes containing microsatellite sequences within their coding regions are more susceptible to somatic mutations, as seen in the *TGFBR2* gene. *TGFBR2*'s third exon contains a microsatellite repeat of 10 adenines that is frequently targeted by frameshift mutations in MSI-H tumors (16). MSI-H is also the hallmark of hereditary non-polyposis colorectal cancer (HNPCC), in which germline mutations of *MLH1*, *MSH2*, *MSH6* and *PMS2* can be found. HNPCC constitutes approximately 2–4% of all CRC cases (17). Tumors with MSI-H are thought to be more able to stimulate a CTL-mediated immune response due to their frequent generation of the aberrant frameshift peptides (18). Therefore, these tumors are subjected to a greater selective pressure which favors the outgrowth of tumor cells with the ability to escape from recognition and destruction by host immune system.

Various studies have identified HLA alterations in colorectal cancer (19-21), including the prevalence of HLA class I alterations in MSI-H tumors (8,22). However, the latter studies did not compare the frequency of alterations between hereditary and sporadic MSI-H tumors neither the mechanisms that led to HLA class I alterations in each subgroup. It was suggested that MSI-H sporadic and hereditary tumors follow parallel evolutionary pathways during tumorigenesis in terms of both genotype and phenotype (23). As far as HLA class I defects are concerned it was never investigated whether these different tumors present distinct escape mechanisms from the immune system. In the present study, we compared the frequency of defects in HLA class I expression in right-sided sporadic (MSI-H and microsatellite-stable (MSS) subgroups) colon tumors and in HNPCC tumors and studied the mechanisms underlying any abnormalities in these subgroups.

RESULTS

HLA class I, β2m and APM component expression

In order to compare the expression of HLA class I in sporadic MSI-H and MSS right sided tumors (RST) and HNPCC MSI-H cases, we used an antibody panel recognizing monomorphic determinants expressed on HLA class I heavy chains, β2m and APM components (Figure 1).

In total, we identified loss of HLA class I expression in about 34.6% of the RST and 42.7% of the HNPCC cases. The frequency of alterations differed significantly between the sporadic MSS and MSI-H RST. The lack of HLA class I expression was more frequent in MSI-H sporadic cases than in MSS cases ($P < 0.0001$), as it was 16.7% in the latter group, but 60.6% in the former (Table 1).

Subsequently, we have investigated the frequency of a concomitant loss of HLA class I expression with that of either the β2m molecule or of any APM component. In the

sporadic subset, loss of HLA class I expression was more often associated with that of one of the APM components, occurring in about 37% of HLA-negative tumors regardless of their mismatch repair status (Table 1). β2m loss was only found in one HLA class I negative MSI-H sporadic tumor (case 65) that interestingly also presented loss of the APM molecules TAP2, Calreticulin and Tapasin (Figure 2). In contrast, loss of HLA class I expression in HNPCC cases was more frequently associated with that of β2m (Table 1), as it was found in 46.9% of the HLA class I-negative tumors. In contrast loss of any APM component was observed in only 6% of these cases (h38, h49) which also showed loss of β2m expression.

In sporadic RST, the simultaneous loss of more than one APM molecule per case was frequent (Figure 2). Only 3 out of 10 cases lost a single APM component. The TAP2 molecule was most frequently lost (6 cases), followed by TAP1, Tapasin and LMP2 (5 cases), Calreticulin (4 cases), LMP7 (2

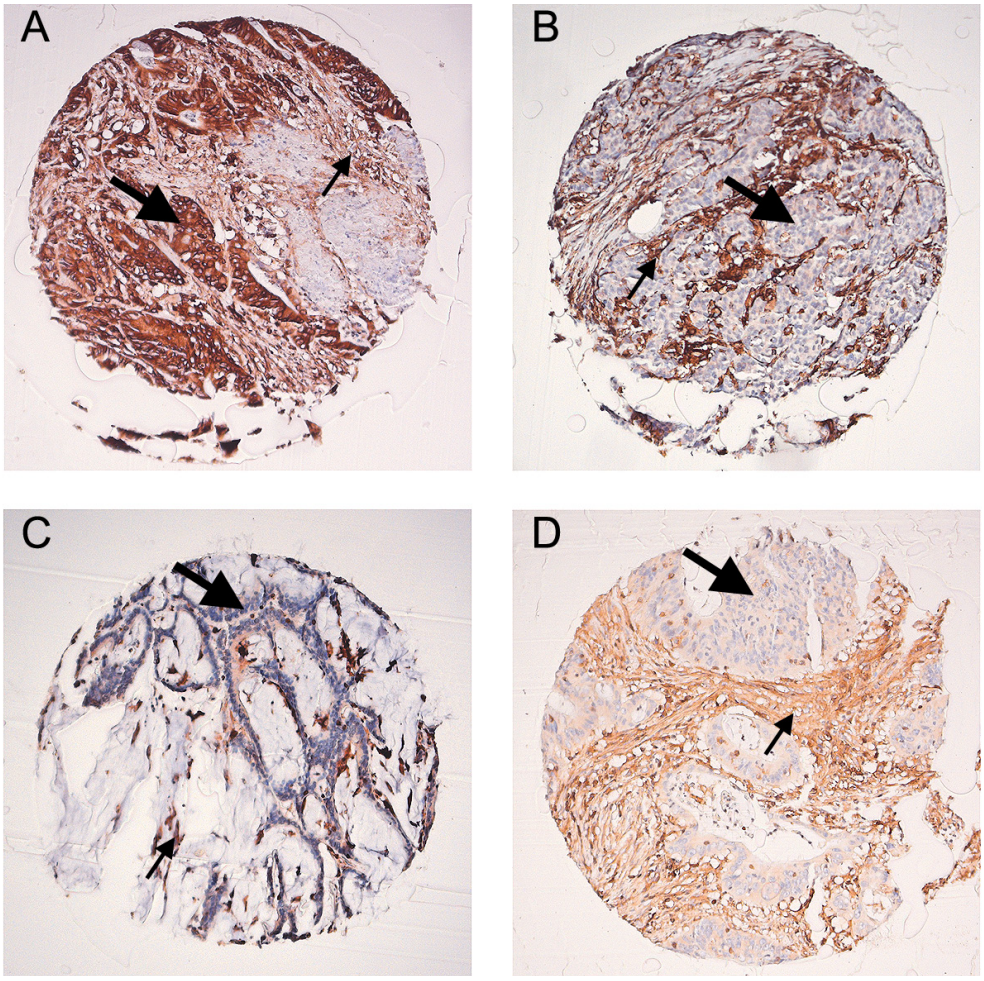

Figure 1. Example of immunohistochemical analysis performed on the RST and HNPCC tumors (Amplification 10×). A, Positive expression of HLA class I antigens detected with the HCA2 antibody. The epithelial (large arrow) membranous expression of HLA class I antigens is identical to the lymphocytic infiltrate (small arrow). B, Loss of expression of HLA class I identified with the HCA 2 antibody. The lymphocytic infiltrate (small arrow) was used as a positive control to determine the loss of expression on the epithelial cells. C, Loss of expression of β2m in a HNPCC case. D, Loss of expression of one of the APM members (Tapasin) in a RST case.

Table 1. HLA, APM and β2m expression in RST and HNPCC colon cancer.

			HLA †	APM loss ‡	β2m loss ‡
RST N = 81	MSS N = 48 (59,3%)	+	40	0	0
		-	8 (16.7%)	3 (37.5%)*	0
	MSI-H N = 33 (41,7%)	+	13	0	0
		-	20 (60.6%)	7 (35.0%)*	1 (5.0%)*
HNPCC N = 75	MSI-H	+	43	0	0
		-	32 (42.7%)	2 (6.3%)*	15 (46.9%)*

* – The percentage of cases that lost APM or β2m expression is relative to the number of HLA negative cases of each subset, † – HLA expression differs significantly between sporadic MSI-H and MSS tumors ($P < 0.0001$) and between HNPCC and sporadic MSS tumors ($P < 0.005$) ‡ – The association of HLA class I loss with that of β2m and of APM components differs significantly ($P < 0.0001$) between sporadic RST and HNPCC tumors.

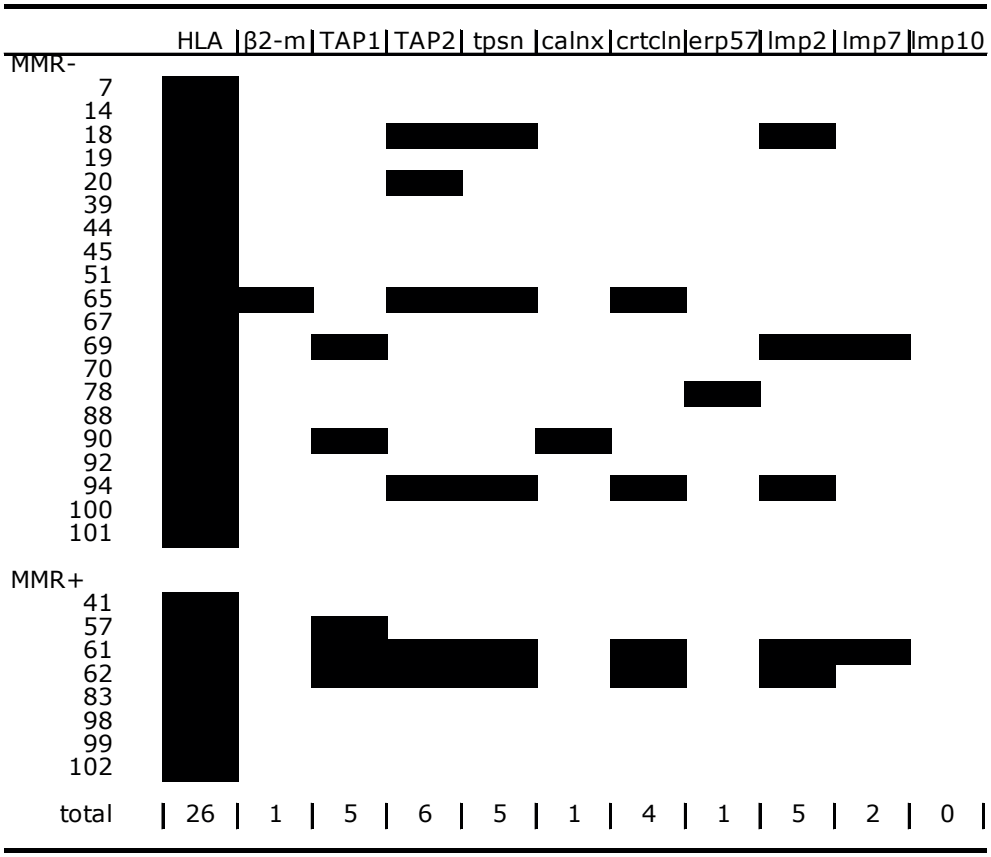

Figure 2. Loss of expression of β2m and different APM members was detected by immunohistochemistry in the RST that presented with HLA loss. The shadowing (in black) is indicative for loss of expression of the respective molecules. (tpsn – Tapasin, calnx – Calnexin, crtcln – Calreticulin).

cases), and Calnexin and ERp57 (1 case). Loss of the LPM10 protein was detected in neither sporadic RST nor HNPCC tumors. The HNPCC cases h38 and h49 lost the expression of TAP2 and LMP7 respectively.

LOH and frameshift analysis

Polymorphic markers around the classical HLA genes (*A*, *B* and *C*), *TAP 1* and *TAP2* genes were used to study LOH and reveal possible chromosomal aberrations that could relate to loss of HLA class I expression (Figure 3A). In HNPCC cases, LOH analysis was only performed around the HLA genes since loss of the TAP1 and TAP2 proteins was rarely associated with HLA class I loss. LOH was more frequent in the MSS tumors (50%) than in the MSI-H sporadic (20%) and HNPCC (6%) tumors with loss of HLA class I expression ($P < 0.05$) (Figures 4, 5). Furthermore, the patterns of LOH in the MSS cases might indicate loss of the entire 6p21.3 region, in contrast to the MSI-H cases (hereditary and sporadic forms) where LOH seems to be limited.

Frameshift mutation screening of the microsatellite sequences present in the coding regions of the HLA class I, APM components and *B2M* genes was performed on all cases with aberrant HLA class I expression (Figures 3B,C). However specific genes were only analyzed when lack of expression of the encoded proteins was detected by immunohistochemistry. Of the classical HLA class I genes only *HLA-A* and *-B* were analyzed since *HLA-C* does not carry any microsatellite repeat in its coding region. Ten RST cases and 20 HNPCC control cases with normal expression of β2m and APM components were screened for frameshift mutations and none was detected.

Frameshift mutations were mainly found in the MSI-H cases (both sporadic and hereditary forms). At least one mutation in an APM component gene was found in 6 of 7 sporadic MSI-H tumors that lost expression of one or more APM components. The

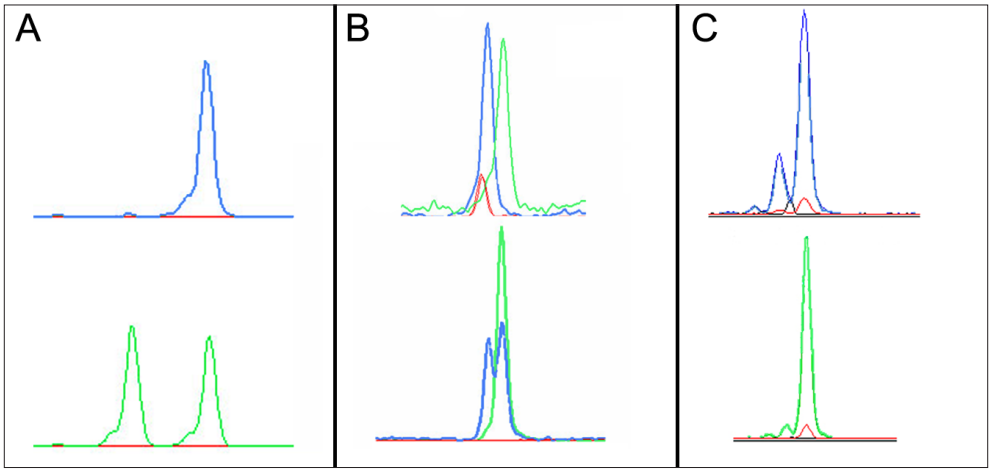

Figure 3. Genetic analysis performed on tumors that have lost HLA class I expression. The different peaks correspond to different sizes from the PCR-amplified products. Peaks corresponding to the normal samples are represented in green whereas tumor samples are represented in blue. A, LOH analysis performed on the RST 41 sample with the polymorphic marker C141. The total loss of a normal allele (on top) illustrates the technical advantage of using flow cytometric sorting to identify LOH events. B, Frameshift mutations identified in different members of the APM machinery. On top a homozygous deletion in the sample RST 65 on the 4th exon of the *Tapasin* gene is shown. On the bottom, a heterozygous deletion in the sample RST 18 on the second exon of the *TAP2* gene is shown. C, Frameshift mutation identified in one HNPCC case (h4) in the 2nd exon of the *B2M* gene. Because flow sorting was not performed in the HNPCC cases, we cannot determine whether the frameshifts are homo- or heterozygous due to contamination with normal DNA.

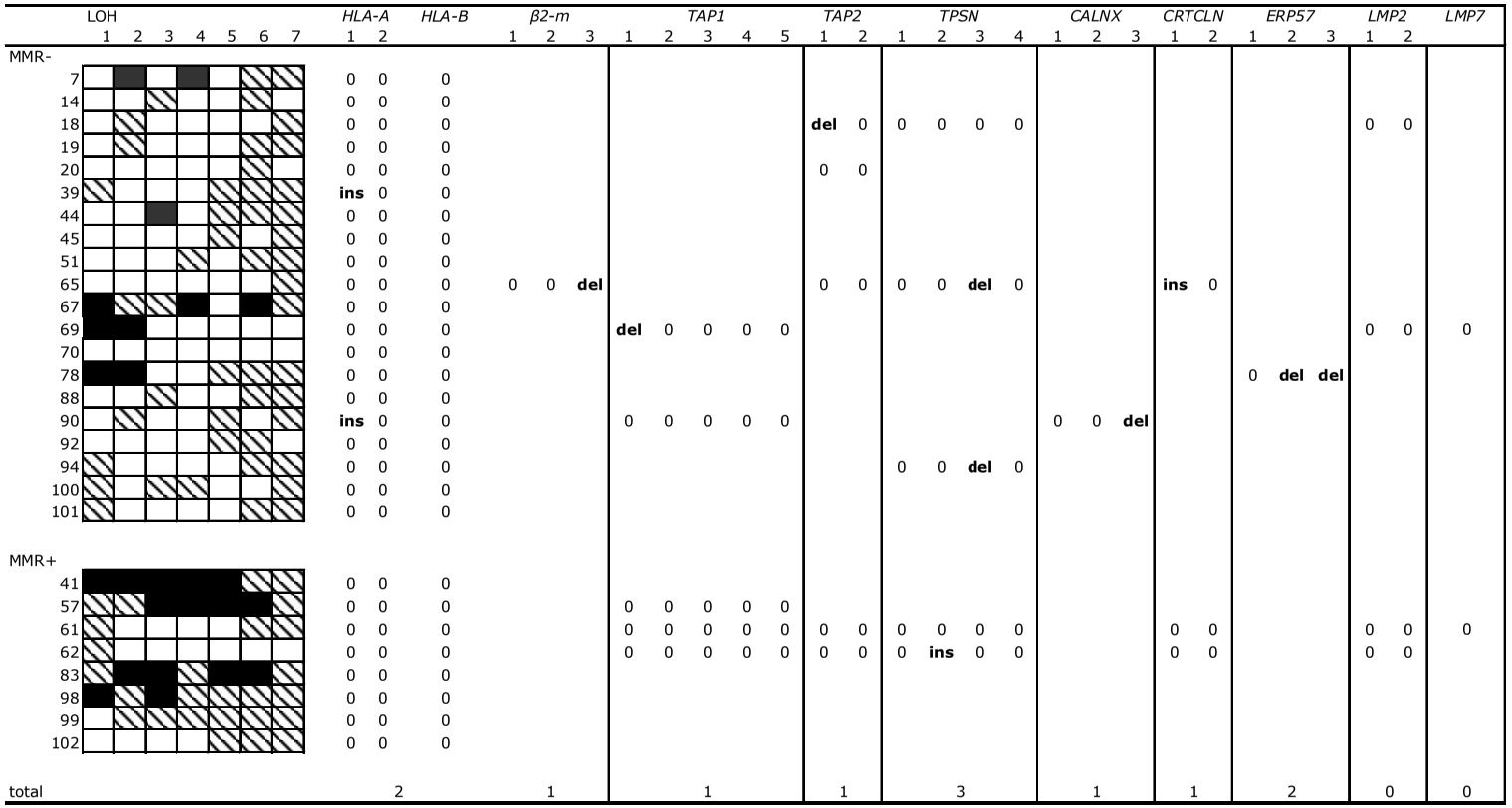

Figure 4. LOH and frameshift analysis was performed on sporadic RST that lost HLA class I expression. Only the tumors that presented with loss of one of the APM molecules or β2m were subjected to fragment analysis in their respective genes. The following repeats were analyzed for frameshift mutations: *HLA A*: 1 – 4th exon 7(C), 2 – 5th exon 3 (GGA); *HLA – B*: 3(GA) & 3(CA); *B2M*: 1 – 1st exon 4(CT), 2 – 2nd exon 4(GA) & 5(A), 3 – 2nd exon 5(A); *TAP1*: 1 – 1st exon 5(C), 2 – 3rd exon 5(T), 3 – 8th exon 5(G), 4 – 10th exon 5(G), 5 – 11th exon 6(G) & 5(A); *TAP2*: 1 – 2nd exon 6(C), 2 – 9th exon 5(G); *Tapasin*: 1 – 2nd exon 5(G), 2 – 3rd exon 5(C), 3 – 4th exon 6(C), 4 – 5th exon 5(G); *Calnexin*: 1 – 7th exon 5(A), 2 – 8th exon 5(A), 3 – 11th exon 8(T); *Calreticulin*: 1 – 3rd exon 5(G), 6th exon 5(C); *ERp57*: 1 – 5th exon 6(T), 2 – 6th exon 6(A), 3 – 13th exon 6(C); *LMP2*: 1 – 2nd exon 5(G), 2 – 6th exon 5(G); *LMP7*: 1st exon 6(C) (key: ins – insertion; del – deletion; 0 – no mutation). LOH analysis of the 6p chromosome was also performed with the following markers: 1 – MOGc, 2 – D6S510, 3 – C125, 4 – C141, 5 – D6S2444, 6 – TAP1, 7 – M2426 (Key: Black – Loss of heterozygosity; Striped – non informative marker; White – Retention of heterozygosity).

single sporadic MSI-H case that lost β2m expression also presented with a single frameshift mutation in the *B2M* gene. Of the 13 sporadic MSI-H cases in which loss of HLA class I expression was associated neither with APM component nor with β2m loss as detected by immunohistochemical staining, one presented with a frameshift in an HLA gene (case 39, Figure 4, Table 2) while 3 other cases showed LOH of the markers adjacent the HLA genes (cases 7, 44 and 67). One frameshift mutation was found in the *Tapasin* gene in a MSS case (case 62). From 15 HNPCC tumors that lost β2m expression at least one mutation was found in 8 cases (Figure 5). Three of the latter showed 2 mutations localized in different stretches. In the remaining 17 HNPCC cases that solely lost HLA class I expression, only 4 showed genetic abnormalities. LOH was found in the HLA region in cases h16, h56 and h120 (data not shown). A frameshift mutation in one of the HLA genes (*HLA - B*) was found in one case (h99, Figure 5). In neither of the 2 HNPCC cases that immunohistochemically lost the expression of one of the APM components an APM frameshift mutation was found.

DISCUSSION

Abnormalities in HLA class I cell surface expression are commonly observed in tumors and are interpreted as a mechanism

	LOH 1	2	3	HLA-A 1	2	HLA-B	*β2*-m 1	2	3	Criteria for HNPCC
h4		■		0	0	0	**del**	**del**	0	*hMLH1* mutant
h8			■	0	0	0	0	0	0	*hMLH1* mutant
h26				0	0	0	0	0	0	MSI-H, no methylation on *hMLH1*, IHC MSH6 neg
h38				0	0	0	**del**	0	0	*hMLH1* mutant
h45				0	0	0	0	0	0	MSI-H, no methylation on *hMLH1*
h49				0	0	0	0	0	0	*MSH2* mutant
h54				0	0	0	**del**	0	**ins**	*hMLH1* mutant
h87				0	0	0	**del**	0	0	*hMSH6* mutant
h93				0	0	0	0	0	0	*hMSH6* mutant
h97				0	0	0	0	0	0	*hMLH1* mutant
h99				0	0	**ins**	0	0	0	*hMLH1* mutant
h103				0	0	0	0	0	**ins**	MSI-H, no methylation on *hMLH1*, IHC MSH6 neg
h118				0	0	0	0	0	**ins**	*PMS2* mutant
h119				0	0	0	0	0	**del**	*hMLH1* mutant
h123				0	0	0	0	**del**	**del**	*hMSH6* mutant
Total				0		1		11		

Figure 5. LOH and frameshift analysis was performed on HNPCC tumors that simultaneously lost HLA class I and β2m expression. LOH markers: (see legend from Figure 4 for key). Frameshift markers:*HLA A*: 1 – 4th exon 7(C) 2 – 5th exon 3 (GGA); *HLA – B*: 3(GA) & 3(CA); *B2M*: 1 – 1st exon 4(CT), 2 – 2nd exon 4(GA). & 5(A), 3 – 2nd exon 5(A) (key: ins – insertion; del – deletion; 0 – no mutation; IHC -immunohistochemistry).

Table 2. Frameshift mutations description in RST.

RST	Mutation description
18	*TAP2* (341–346)del1 – het
39	*HLA-A* (621–627)ins1 – het
65	*B2M* (341–345)del1 – hom; *Tapasin* (1217–1222)del1 – hom; *Calreticulin* (418–422)ins1 – hom
69	*TAP1* (362–366)del1 – hom
78	*ERp57* (675–680)del2 – hom; *ERp57* (1459–1464)del1 – hom
90	*HLA-A* (621–627)ins1 – het; *Calnexin* (1476–1483)del1 – het
94	*Tapasin* (1217–1222)del1 – het

ins – insertion; del – deletion; hom – homozygous; het – heterozygous; the numbers before and after del/ins indicate the location of the microsatellite repeat containing the frameshift mutation and the number of affected nucleotides respectively.

by which tumor cells evade the host immune system (1). In colorectal cancer, especially in MSI-H tumors, the high degree of lymphocytic infiltrate in some cases may suggest an active immune response during tumor development (24,25). Moreover, MSI-H tumors might cause increased immune reactivity as a consequence of the high amounts of aberrant frameshift peptides they generate (8,18). A selective pressure by CTLs upon these tumors would favor the outgrowth of tumor cells that lost HLA class I expression at the cell surface allowing them to surpass the action of the immune system.

Applying immunohistochemistry on tissue arrays, we compared HLA class I expression in both sporadic RST (MSI-H and MSS subgroups) and HNPCC tumors. RST were chosen because of the high percentage of MSI-H cases in this specific tumor type (43). Indeed, immunohistochemical staining with monoclonal antibodies showed that HLA class I loss was frequent in the MSI-H cases analyzed when compared to their MSS counterpart. This finding supports the hypothesis that MSI-H tumors face greater selective pressure to lose HLA class I expression, as described by Kloor *et al.* (8). However, we have shown for the first time that distinct molecular mechanisms underlie HLA class I loss in sporadic MSI-H and HNPCC colon cancers. In the latter, HLA class I loss was preferentially associated with that of β2m, while in the former HLA class I loss was associated with that of one or more APM components ($P < 0.0001$).

We investigated the genetic abnormalities underlying the HLA class I loss of expression. They included LOH on chromosome region 6p21.3 (encompassing HLA class I and TAP genes), mutations in APM components and mutations in *B2M*.

Loss of heterozygosity at 6p21.3 was most prevalent in MSS tumors. This is consistent with the observation that these tumors frequently possess gross chromosomal aberrations and are often aneuploid (13). Moreover, since LOH events in MSS tumors normally comprise large areas of a chromosome, LOH on 6p21 might not be a direct consequence of selective pressure directed to the loss of HLA expression but instead to other genes within the same chromosomal region. The general absence of LOH in MSI-H tumors suggests that this is not the major mechanism by which the cells abrogate HLA class I expression.

The genome's coding regions contain multiple microsatellite repeats, which are considered hotspots for mutations in mismatch repair-deficient tumors (26). Such repeats are also present within the exons of the APM components, *B2M*, *HLA-A* and *HLA-B* genes. In about half of the MSI-H cases, loss of expression of HLA class I was concordant with the detection of one or more mutations in these genes. We have discovered novel mutations in the antigen presenting machinery genes; *Tapasin*, *Erp57*, *Calreticulin* and *Calnexin* in colorectal cancer. Previous reports associated the loss of HLA class I expression in MSI-H tumors with defects on β2m molecule (7,9). However, the authors did not distinguish the sporadic/hereditary nature of the tumors that were studied. We cannot exclude that the MSI-H cases included in these studies were mainly HNPCC tumors.

The reason sporadic MSI-H tumors would target APM members for inactivation and HNPCC would target the β2m chaperon is unclear. One possibility worth further exploration is that the various mutations suggest different immune-escape mechanisms for thwarting distinct anti-tumor responses. HNPCC tumors can have an age of onset before the 5th decade of life while sporadic MSI-H tumors appear generally around the 7th decade of life (26); one would therefore predict that the alertness and robustness of the immune system would be higher in HNPCC patients leading to a stronger, or at least different selective pressure on the latter. Furthermore it has

been recently suggested that the JC polyoma virus plays a role in the oncogenicity of colon tumors with an identical phenotype to sporadic MSI-H tumors (27). Although speculative, the presence of the JC virus might be implicated in a different immune response between sporadic MSI-H and HNPCC tumors.

The advantages of different escape mechanisms (loss of APM members vs. abrogation of β2m) are not understood. The only known function of APM members is facilitating the expression of HLA classical molecules in complex with endogenous peptides. Thus, one would expect that only these HLA molecules would be affected by failure of the antigen processing machinery. On the other hand, it is accepted that cell surface expression of non-classical HLA molecules (e.g. HLA -G, -E) also depends on β2m, so the function of these highly specialized molecules would be compromised if β2m were mutated or lost. These molecules might play an important role in regulation of immune cell activity by inhibiting or activating its function. Therefore, MSI-H sporadic tumors that have lost expression of both HLA and an APM component and HNPCC tumors with lost β2m expression might behave differently or present a different kind of interaction with cells from the immune system. For instance, Yamamoto *et al.* have described a correlation between *B2M* mutations and unfavorable prognosis in colorectal cancer (28).

We separately analyzed the presence of the characteristic *BRAF* V600E somatic mutations in the RST cohort (data not shown). Forty-percent of MSI-H sporadic tumors presented with this mutation which was absent in the MSS tumors. It was previously described that this mutation is also absent in HNPCC tumors (29). V600E was distributed equally between tumors that lost vs. retained expression of HLA class I in the sporadic MSI-H cases.

CONCLUSION

HLA class I aberrations are found at varying frequencies in different colorectal tumor types and are caused by distinct genetic mechanisms. Chiefly, sporadic and hereditary MSI-H tumors follow different routes toward HLA class I loss of expression supporting the idea that these tumors follow different evolutionary pathways in tumorigenesis. The resulting variation in immune escape mechanisms may have repercussions in tumor progression and behavior.

METHODS

Patient material and tissue microarrays

Two tissue microarrays were constructed from formalin-fixed, paraffin-embedded tissues as described previously (30). One array, previously described (31), included colorectal tumor specimens from 129 suspected HNPCC patients with MSI-H colon tumors of which 75 cases were analyzed in the present study after confirmation of their HNPCC status: 73.3% (n = 55) of the latter possessed a germline pathogenic mutation in *MLH1* (n = 24), *MSH2* (n = 18), *MSH6* (n = 12) or *PMS2* (n = 1), the remaining were MSI-H, without methylation of the *MLH1* promoter and/or with immunohistochemical loss of the MSH2/MSH6 heterodimer and/or possessed a very young age at diagnosis of colon cancer (<50 yrs old). All cases possessed a positive family history for MSI-H tumors. The second tissue array included 3 tumor tissue cores from 81 sporadic right-sided colon cancer cases resected between 1990 and 2005 at the Leiden University Medical Center (Leiden, The Netherlands) and at the Rijnland Hospital (Leiderdorp, The Netherlands). The 81 patients in the latter array consisted of 47 females and 34 males with a mean age of 71.15 years (SD= 9.958). Approximately 60% (n = 48) of these cases were classified as MSS while the remaining (n = 33) possessed a MSI-H phenotype. The microsatellite instability status of the tumors was determined according to recommendations of the National Cancer Institute/ICG-HNPCC (15). Moreover all MSI-H sporadic cases have lost the expression of the MLH1/PMS2 heterodimer as assessed by immunohistochemistry. The sporadic status of the MSI-H right-sided tumors (RST) was confirmed by methylation analysis of the *MLH1* promoter using a methylation-specific MLPA assay as previously described (32). All MSI-H sporadic cases presented with hypermethylation at the *MLH1* promoter.

The present study falls under approval by the Medical

Ethical Committee of the LUMC (protocol P01–019). Cases were analyzed following the medical ethnical guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences (33).

Immunohistochemistry

Standard three-step, indirect immunohistochemistry was performed on 4-µm tissue sections transferred to glass slides using a tape-transfer system (Instrumedics, Hackensack, NJ), including citrate antigen retrieval, blockage of endogenous peroxidase and endogenous avidin-binding activity, and di-aminobenzidine development.

The following primary antibodies were used: the mAb HCA2 which recognizes β2m-free HLA-A (except -A24), -B7301 and -G heavy chains (34,35) ; the mAb HC10, which recognizes a determinant expressed on all β2m-free HLA-B and C heavy chains and on β2m-free HLA-A10, -A28, -A29, -A30, -A31, -A32 and -A33 heavy chains (supernatant kindly provided by Dr. J. Neefjes, NKI, Amsterdam, The Netherlands and Dr. H. L. Ploegh, MIT, Boston, MA) (34,36); TAP1 specific mAb NOB1; LMP2-specific mAb SY-1; LMP7-specific mAb HB2; LPM10-specific mAb TO-7; Calnexin-specific mAb TO-5; Calreticulin-specific mAb TO-11; Tapasin-specific mAb TO-3; ERp57-specific mAb TO-2 (37-39); TAP2-specific mAb (BD Biosciences Pharmingen, San Diego, CA); rabbit anti-β2m polyclonal Ab (A 072; DAKO Cytomation, Glostrup, Denmark); anti-MLH1 (clone G168–728; BD Biosciences) and anti-PMS2 (clone A16-4; BD Biosciences). Secondary reagents used were biotinylated rabbit anti-mouse IgG antibodies (DAKO Cytomation), goat anti-rabbit IgG antibodies (DAKO Cytomation), and biotinylated-peroxidase streptavidin complex (SABC; DAKO Cytomation).

Loss of expression was defined by complete lack of staining in membrane and cytoplasm (HCA2, HC10, and anti-β2m), in the nucleus (anti-MLH1 and anti-PMS2), in the peri-nucleus/endoplasmic reticulum (NOB1, anti-TAP2, TO-2, TO-3, TO-5, TO-7, and TO-11), or in the cytoplasm (SY-1, HB2, and TO-7), but with concurrent staining in normal epithelium, stroma or infiltrating leukocytes. HLA class I expression was considered to be lost when one of the HLA class I antigen-specific antibodies gave a negative result alongside a positive internal control (lymphocytic infiltrate).

Flow cytometric sorting

The flow cytometric sorting procedure, including tissue preparation, staining and flow cytometry analysis was performed as described previously (40). Briefly, 2 mm diameter punches from selected areas of formalin-fixed paraffin embedded colorectal carcinomas were digested enzymatically in a mixture of 0.1% collagenase I-A (Sigma-Aldrich, St Louis, MO, USA) and 0.1% dispase (Gibco BRL, Paisley, UK). After determination of cell concentration, one million cells were incubated with 100 µl of mAb mixture directed against keratin and vimentin containing clones MNF116 (anti-keratin; IgG1; DAKOCytomation, Golstrup, Denmark), AE1/AE3 (anti-keratin; IgG1; Chemicon International Inc, Temecula, CA, USA), and V9-2b (anti-vimentin; IgG2b; Department of Pathology, LUMC (41)). Next day, cells were incubated with 100 µl of premixed FITC and RPE-labelled goat F(ab')2 anti-mouse subclass-specific secondary reagents (Southern Biotechnology Associates, Birmingham, AL, USA). After washing, cells were incubated with 10 µM propidium iodide (PI) and 0.1% DNase-free RNase (Sigma). The next day cells were analyzed by flow cytometry. A standard FACSCalibur (BD Biosciences) was used for the simultaneous measurement of FITC, RPE, and PI. Tumor and normal cell populations were flow-sorted using a FACSVantage flow-sorter (BD Biosciences) using the FACSCalibur filter settings. Sorting was only performed on samples included in the RST array due to shortage of material from the HNPCC cases. DNA from flow-sorted tumor material was isolated as described by Jordanova et al. (42). DNA from non-sorted material was isolated using Chelex extraction as described previously (43).

LOH and fragment analysis

Markers for loss of heterozygosity (LOH) analysis were chosen from the dbMHC database (44) to map the chromosome 6p21.3 region between HLA-A and TAP2. They were MOGc, D6S510, C125, C141, D6S2444, TAP1 and M2426. A "linker" sequence of 5'-GTTTCTT was added to the 5' terminus of all reverse primers (45). LOH was defined as allelic imbalance >2 in the HNPCC cases (non-sorted) and allelic imbalance >5 in the sorted RST (46).

To detect frame-shift mutations in the *HLA-A*, *HLA-B*, *B2M*, *LMP2*, *LMP7*, *LMP10*, *TAP1*, *TAP2*, *Calnexin*, *Calreticulin*, *ERp57* and *Tapasin* genes, 28 pairs of primers (Table 3) were constructed surrounding non-polymorphic microsatellite regions within the coding regions.

Statistics

Significance values were calculated using the software package SPSS 10.0.7 (SPSS Inc., Chicago, IL, USA).

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Table 3. Primers used in fragment analysis.

Gene	Forward primer	Reverse primer	Microsatellite Repeat
HLA A 4thex	CCTGAATTTTCTGACTCTTCCCGT	GTTTCTTTCCCGCTGCCAGGTCAGTGT	7(C)
HLA A 5thex	CCATCGTGGGCATCATTG	GTTTCTTTCAGTGAGACAAGAAATCTC	3(GGA)
HLA B 2ndex	GCTTCATCTCAGTGGGCTAC	GTTTCTTCTCGCTCTGGTTGTA	3(GA) + 3(CA)
β2m 1stex	GGCTGGGCACGCGTTTAAT	GTTTCTTAGGGAGAGAAGGACCAGAG	4(CT)
β2m 2ndex (1)	TACCCTGGCAATATTAATGTG	GTTTCTTGATAGAAAGACCAGTCCTTGC	4(GA) + 5(A)
β2m 2ndex (2)	CTTACTGAAGAATGGAGAGAG	GTTTCTTGACTACTCATACACAACTTTCA	5(A)
TAP1 1stex	TAAATGGCTGAGCTTCTCGC	GTTTCTTAGAGCTAGCCATTGGCA	5(C)
TAP1 3rdex	ACAGCCACTTGCAGGGAG	GTTTCTTTATGAACAGTACATGGCGTAT	5(T)
TAP1 8thex	CTGCCCTGCTGCAGAATCTG	GTTTCTTCAAGCCACCTGCTTCCAT	5(G)
TAP1 10thex	CTCTGCAGAGGTAGACGAGG	GTTTCTTATTAAGAAGATGACTGCCTCAC	5(G)
TAP1 11thex	AGCACCTCAGCCTGGTGGA	GTTTCTTGCAGGTCTGAGAAGGCTTTC	6(G) + 5(A)
TAP2 2ndex	TTCCTCAAGGGCTGCCAGGAC	GTTTCTTGCTCCAAGGGGCTGAAG	6(C)
TAP2 9thex	CCTACGTCCTGGTGAGGTGA	GTTTCTTCTGGCTGTGCAGGTAGC	5(G)
Tapasin 2ndex	TTGGTTCGTGGAGGATGC	GTTTCTTCCTAGAGACTCACCGTGTAC	5(G)
Tapasin 3rdex	CTTCCTTCTCTACACTCAGACC	GTTTCTTAGGACTGGGCTGGATATGC	5(C)
Tapasin 4thex	CCTGTCTTCCTCAGTGGTAC	GTTTCTTGAGCAGATGTCCCTTACCC	6(C)
Tapasin 5thex	TGCTCATTTCGTCCTCTTTCC	GTTTCTTGTTCCCACTCCACCTCCAG	5(G)
Calnexin 7thex	GAAGGATCAGTTCCATGACAAG	GTTTCTTCTGCATCTGGCCTCTTAGC	5(A)
Calnexin 8thex	TCTGCTCAATGACATGACTCC	GTTTCTTTGAAGACAGTTCCCCAAGAC	5(A)
Calnexin 11thex	AACCTTTCAGAATGACTCCTTTTAG	GTTTCTTCAAGCAGCAAACACGAACC	8(T)
Calreticulin 3 rdex	CTACCGTCCCGTCTCAGG	GTTTCTTTCTGTCTGGTCCAAACTATTAGG	5(G)
Calreticulin 6 thex	GACAAGCCCGAGCATATCC	GTTTCTTCACCTTGTACTCAGGGTTCTG	5(C)
ERp57 5thex	CACTTATTGCTTCTTCCTTGTG	GTTTCTTAATACTTGGTCAGGAGATTCAAC	6(T)
ERp57 6thex	CTTCTGCTATCTGCCTACTGAG	GTTTCTTTCAAGCAAATAAATCCCAGACAAG	6(A)
ERp57 13thex	ACTTTTAAGCTGATCTTTCTGTTTT	GTTTCTTTTAGAGATCCTCCTGTGCCTT	6(C)
LMP2 2ndex	GAGGGCATCAAGGCTGTTC	GTTTCTTGCAGACACTCGGGAATCAG	5(G)
LMP2 6thex	CCCTCTCTCCAACTTGAAACC	GTTTCTTTGTAATAGTGACCAGGTAGATGAC	5(G)
LMP7 1stex	GGCTTTCGCTTTCACTTCC	GTTTCTTGAGATCGCATAGAGAAACTGTAG	6(C)

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MUTYH-associated polyposis carcinomas frequently lose HLA class I expression—a common event amongst DNA-repair-deficient colorectal cancers

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ABSTRACT

Human leukocyte antigen (HLA) class I expression defects frequently occur in colorectal cancers bearing mismatch repair (MMR) deficiencies and are interpreted as immune evasion mechanisms to avoid cancer cell recognition and elimination by the immune system. MMR-deficient tumours are thought to be more prone to lose HLA class I expression, due to their frequent generation of aberrant peptides which can stimulate a cytotoxic T-cell-mediated response. MUTYH-associated polyposis (MAP) is a colorectal cancer syndrome caused by defects in the MUTYH DNA repair enzyme. Impairment of MUTYH activity could lead to a surplus of mutated peptides which would be presented to cytotoxic T-cells through the HLA class I molecules.

We have studied the frequency of HLA class I expression defects in MAP carcinomas and have compared it to those observed in MMR-deficient and -proficient colorectal tumours. Immunohistochemical detection of the expression of HLA class I, β2-microglobulin (β2m), and antigen-processing machinery molecules was performed in 37 primary MAP carcinomas and nine metastases resected from 29 MAP patients. Furthermore, we sequenced the *B2M*, *TAP1*, and *TAP2* genes.

Defects in HLA class I expression were detected in 65% of primary MAP carcinomas, affecting 72% of patients. HLA class I expression abnormalities were often concomitant with β2m expression loss and mutations in the *B2M* gene. Loss of HLA class I expression is thus a frequent event in MAP carcinomas, similarly to MMR-deficient colorectal tumours.

The extensive mutagenic background of these tumours most likely triggers a strong selective pressure, exerted by the immune system on the tumour, which favours the outgrowth of tumour cell clones with an immune evasive phenotype. Our data provide additional evidence for a link between DNA repair deficiencies and altered HLA class I phenotypes in colorectal cancer.

INTRODUCTION

During cancer development, tumour cells may elicit cytotoxic T-lymphocyte-mediated immune responses—partly a consequence of accumulated gene mutations that are translated into altered peptides (1, 2). Tumour cell expression of the human leukocyte antigen (HLA) class I-antigen complexes is essential for T-cell recognition of aberrant peptides and subsequent activation (3). Consequently, alteration of HLA class I cell surface expression provides an effective mechanism by which tumours can escape detection by immune cells (4, 5). Multiple mechanisms have been shown to underlie defects in HLA class I expression by tumour cells; they include mutations in the individual HLA class I genes, *HLA-A*, *-B*, and *-C*, located on chromosome 6p21.3 (6); loss of heterozygosity (LOH) at 6p21.3 (7); mutations in β2-microglobulin (β2m) (8, 9), the molecule required for the cell surface expression of HLA class I antigens; and defects in components of the HLA class I-associated antigen-processing machinery (10–12). The antigen-processing machinery consists of the proteasome components delta, MB1, and Z; the immunoproteasome components LMP2, LMP7, and LMP10; the peptide transporters TAP1 and TAP2; and the chaperones calnexin, calreticulin, ERp57, and tapasin. The immunoproteasome mostly, although not exclusively, generates peptides from endogenous proteins. TAP1 and TAP2 facilitate peptide translocation from the cytosol into the lumen of the endoplasmic reticulum, where the peptides are loaded onto the HLA class I molecules with the aid of several chaperones (13).

We and others have previously described

a higher frequency of HLA class I defects in colorectal cancers presenting with DNA mismatch-repair (MMR) deficiency, in comparison with MMR-proficient tumours (12, 14, 15). Furthermore, we found that distinct molecular events underlie HLA class I loss, depending on the aetiology of the tumours; Lynch syndrome-related cancers presented with mutations in the β2m molecule, while sporadic microsatellite-unstable tumours mainly showed alterations in the antigen-processing machinery components (12).

Microsatellite instability is a form of genetic instability that occurs in about 15% of colorectal cancers. Microsatellite-unstable sporadic tumours have a phenotype resulting from the epigenetic inactivation of the MMR gene *MLH1 (*16). Microsatellite instability is also the hallmark of Lynch syndrome-related tumours, in which germline mutations of the MMR genes *MLH1*, *MSH2*, *MSH6*, and *PMS2* can be found (17). Their inactivation destroys a cell's ability to repair base–base mismatches and small insertions or deletions in repetitive stretches, leading to an accumulation of frameshift mutations that become translated into abnormal peptide sequences (18). These tumours are therefore thought to be more able to stimulate a cytotoxic T-cell-mediated immune response, due to their frequent generation of aberrant peptides (19, 20). Consequently, these tumours are probably subjected to a selective pressure that favours the outgrowth of tumour cells with the ability to escape from recognition and destruction by the host immune system.

Another colorectal cancer syndrome in which DNA repair deficiencies are implicated is MUTYH-associated polyposis (MAP) (21). MAP is a recessive inheritable disease caused by bi-allelic *MUTYH* inactivating germline mutations (22). Carriers may develop 10–500 polyps until the fifth decade of their lives and most of these patients will present with a colorectal cancer (23, 24). The MUTYH protein is a base excision repair enzyme involved in the repair of one of the most frequent and stable forms of nucleotide oxidative damage: 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG). This structure readily mispairs with adenosine residues, leading to G : C > T : A transversions after DNA replication (25). MUTYH acts by scanning the daughter DNA strand for any mispaired adenines, either with guanines or 8-oxodGs, and subsequently excising them (26). Defects in this repair mechanism incapacitate the cell's ability to reverse such mispairs, leading to an accumulation of mutations. The *APC* and *KRAS* genes are frequently mutated in MAP tumours, although not exclusively presenting the typical transversions (21, 27, 28).

We hypothesized that, similarly to MMR-deficient tumours, MAP tumours could be more prone to stimulate a cytotoxic T-cell-mediated immune response, due to their frequent generation of aberrant peptides. Hence, these tumours could also be subjected to a strong selective pressure favouring the outgrowth of cancer cells that acquire an immune evasive phenotype.

We aimed to study the occurrence of HLA class I expression deficiencies in MAP carcinomas, as well as the expression of HLA class I chaperones and antigen-processing machinery components. Furthermore, we investigated the occurrence of mutations in β2m or antigen-processing machinery components when their expression was lost.

RESULTS

HLA class I, β2m, and antigen-processing machinery components' expression

Defects in HLA class I expression were detected in 24/37 primary carcinomas and in all nine tumour metastases (seven of which derived from patient 29), comprising 72% of the patients included in the study (Table 1). The HCA2 and HC10 antibodies detect the expression of HLA-A and HLA-B, -C,

Table 1. Expression of HLA class I, β2m, and antigen-processing machinery components in MAP carcinomas.

MAP carcinomas		HLA class I expression	β2m loss	APM loss
Primary tumors	+	13 (35.1%)	0	0
n = 37	−	24 (64.9%)	14 (58.3%)	4 (16.7%)
Metastases	+	0	0	0
n = 9*	−	9	8	1

– altered expression in tumour cells,* Seven of the nine metastases belonged to one patient.

respectively. Within the group of tumours with HLA class I defects, six primary carcinomas (25%) presented with total loss of HLA class I (concomitant loss of HCA2 and HC10); another two carcinomas (8%) presented with loss of solely the HLA-A molecule (as assessed with HCA2 antibody); and the remaining carcinomas (67%) presented heterogeneous patterns with alternating positive and negative tumour areas for HLA class I expression (Figure 1). All seven metastases from patient 29 expressed HLA class I, although no membranous expression was detectable with the HCA2 antibody (Figure 1).

We also investigated the concurrent loss of expression of β2m and antigen-processing machinery components with that of HLA class I. Loss of β2m expression was detected in 58% of carcinomas with HLA class I expression abnormalities (Table 1); four of these presented with total loss of HLA class I (Figure 2B), two exclusively lost HLA-A expression, and eight cases possessed a heterogeneous pattern of HLA class I expression (Figures 2C and 2D) normally accompanied by a similar pattern of β2m expression. This heterogeneous pattern was frequently recognized within one tissue core, as represented in Figures 2C and 2D. Notably, the seven tumour metastases from patient 29, presenting with a peri-nuclear pattern of expression for HLA class I, showed total absence of β2m expression, further confirming the existence of HLA class I expression abnormalities (Figures 2E and 2F).

Concurrent loss of antigen-processing machinery components and HLA class I expression was observed in five cases: four primary tumours (17% of tumours with HLA class I expression abnormalities) and one tumour metastasis (Table 1). TAP1 and/or TAP2 were lost in all of these cases, while tapasin was found to be abrogated in patient 26 (Figure 1). Except for the latter, all other tumour tissues failed to present a clear internal positive control, thus not allowing the confirmation of loss of expression of those molecules. Loss of expression of the antigen-presenting machinery components and β2m was mutually exclusive.

Fourteen adenomas, from ten patients, were analysed for HLA class I and β2m expression. Only three (21%) displayed aberrant HLA class I expression which was always heterogeneous throughout the adenomatous tissue. All three belonged to patients with HLA class I deficiencies in at least one of their carcinomas. Loss of expression of β2m was found in one of the adenomas with HLA class I deficiencies.

B2M, *TAP1*, and *TAP2* sequencing

We sequenced the coding regions of the *B2M*, in all tumours for which DNA was available, and *TAP1* and *TAP2* genes in the tumours that lacked expression of these molecules. All mutations are listed in Figure 1. Mutations in *B2M* were identified in eight primary carcinomas. In one case (patient 1, tumour 1), the mutation was not related to loss of either β2m or HLA class I expression. Two other tumours conserved β2m expression

Patient	Tumor	Metastases	HCA2	HC10	2M	APM	2m mutation	APM mutation
1	1		+	+	+	+	c.17 C>T (Ala6Val)	
	2		0+het	+	0+het	+		
	3		0+het	0+het	0+het	+		
	4		0+het	0+het	+	+		
		1	0+het	+	0+het	+		
2			0+het	+	+	+	c.323C>A (Ser107Stop)	
3			+	+	+	+		
4			0+	0+	0+het	+		
5			+	+	+	+	-	
6			0+het	0+het	0+het	+	-	
7			0+het	0+het	0+het	+		
8			+	+	+	+		
9			+	+	+	+		
10			+	+	+	+		
11			+	+	+	+	-	
12			+	+	+	+	-	
13			0+het	0+het	+	+	-	
14			+	+	+	+		
15			0+het*	+	0+	+	-	
16			0+het	+	+	00 (TAP2)	-	-
17	1		+	+	+	+	-	
	2		0+het	0+het	0+het	+	-	
18			0+het	0+het	+	+	-	
19			0+het	0+	+	+		
20			0+	0+	0+	+	-	
21	1		0+	+	+	00 (TAP2)		-
	2		+	+	+	+		
22	1		0+het	0+het	+	+	c.199G>A(Glu66Lys)	
	2		0+het	0+het	0+het	+	c.4T>G(Ser2Ala) c.14T>A(Val5Glu)	
	3		0+	0+	0+	+	c.330delC	
23	1		0+het	+	0+het	+		
	2		+	+	+	+	-	
24	1		0+	+	0+	+	c.37_38insCC	
25	1		0+	0+	+	00 (TAP1)		-
		1	0+	0+	+	00 (TAP1,TAP2)	c.148T>A (Phe49Ile)	c.416G>A (Trp139Stop)
26	1		0+	0+	+	0+ (TAP1, TAP2, Tpsn)	-	-
27	1		+	+	+	+	-	
	2		0+het	+	0+	+	c.68-2A>G	
28	1		0+	0+	0+	+	-	
29		1	0+het*	+	0+	+	c.1A>T (Met1Leu)	
		2	0+het*	+	0+	+	c.1A>T (Met1Leu)	
		3	+*	+	0+	+	c.1A>T (Met1Leu)	
		4	0+het*	+	0+	+	c.1A>T (Met1Leu)	
		5	+*	+	0+	+	c.1A>T (Met1Leu)	
		6	0+het*	+	0+	+	c.1A>T (Met1Leu)	
		7	0+het*	+	0+	+	c.1A>T (Met1Leu)	

Figure 1. Expression of HLA class I was detected with HCA2 (HLA-A) and HC10 (HLA-B, HLA-C) antibodies. Additionally, the expression of the chaperone β2-microglobulin and the antigen-processing machinery components were determined. + = positive; 0+ = loss of expression with internal positive control; 00 = loss of expression without internal positive control; 0+ het = heterogeneous expression, with positive and negative tumour cells; +* = non-membranous (cytoplasmic HLA class I expression); 0+ het* = heterogeneous expression, with positive (cytoplasmic HLA class I expression) and negative tumour cells. *B2M* mutations were investigated in all cases where DNA was available, and *TAP1* and *TAP2* mutations were screened when one of these molecules was not expressed. – = No mutations found with the complete gene sequence available.

but presented deficiencies in HLA class I expression (patient 2 and patient 22, tumour 1). The remaining all had detectable defective β2m expression. Eight metastases with HLA class I loss of expression carried a mutation in the *B2M* gene. All seven belonging to patient 29 presented with the same mutation in the starting codon of the *B2M* gene (c.1A > T). Starting-codon, splice-site or frameshift mutations were associated with loss of β2m expression. The carcinoma from patient 2 displayed a truncating mutation in the 3' end of the gene which did not lead to abrogation of β2m expression. Amino-acid substitutions were associated with retained β2m expression and in one case, a heterogeneous pattern of expression (patient 22, tumour 2). The complete *B2M* sequence was available for six additional tumours without HLA classI/β2m aberrations and no mutations were found. Only one mutation was found in the peptide transporters. Patient 25 presented an early

STOP codon in the third exon of the *TAP2* gene. The usage of formalin-fixed material for DNA extraction did not always allow full gene sequencing for all cases, implying a possible underestimation of the number of samples carrying mutations.

DISCUSSION

HLA class I expression defects have been frequently reported in several cancers (5, 6, 10, 11, 29). These are interpreted as mechanisms adopted by tumours to escape immune surveillance and thereby avoid tumour cell recognition and destruction. We, and others, have previously described a predisposition for MMR-deficient colorectal tumours to lose HLA class I expression (12, 14, 15). MMR-deficient tumours are thought to be more competent at triggering an immune response, due to their increased mutagenic potential, when compared with MMR-proficient tumours, which translates into an accumulation of frameshift peptides that might work as tumour neo-antigens (8, 19, 20). The immune reaction might function as a selective pressure that favours the outgrowth of tumour cell clones that have lost HLA class I expression. In the present work, we investigated the occurrence of HLA class I expression deficiencies in MAP carcinomas. Patients with MAP carry biallelic mutations in the *MUTYH* gene, which prevents the cells from accumulating point mutations derived from DNA oxidative damage. Like Lynch syndrome and sporadic MSI-H tumours, one would expect that MAP carcinomas would be competent at triggering immune responses and would be similarly subjected to a selective pressure, imposed by the immune system, favouring the outgrowth of cells with absent HLA class I expression.

We identified HLA class I expression abnormalities in the majority of MAP carcinomas analysed, both in the primary carcinomas and in the tumour metastases. In total, 72% of patients carried carcinomas (68% of primary tumours) that exhibited HLA class I expression deficiencies. Previously, we described that approximately half of MSI-H sporadic tumours and Lynch syndrome-related tumours had defects in HLA expression, while colon mismatch-repair-proficient tumours presented HLA class I expression abnormalities in only a minority of the carcinomas analysed (17%) (12). Put together, these observations provide additional evidence of the prevalence of HLA class I expression alterations in tumours carrying defects in DNA-repairing molecules. Furthermore, we analysed 14 adenomas from the same cohort of patients for HLA class I and β2m expression but these were seldom, as was previously reported for colorectal adenomas (30). The increased mutagenic load and cytotoxic T-cell infiltration of carcinomas probably constitute an environment with greater selective pressure for immune evasive events than at adenoma stages.

Intriguingly, the majority of defects found in MAP carcinomas were in the form of heterogeneous patterns of HLA class I expression within the tumours, suggesting that MAP carcinomas are composed of different clones with respect to HLA class I expression. Such a phenotype was not observed in MSI-H tumours (data not shown). We previously reported that MAP carcinomas frequently display multiclonality for DNA content (31, 32), further supporting our observations. One piece of circumstantial evidence for increased immunogenicity of MMR-deficient tumours is the dense infiltration of the epithelial compartments of MSI-H carcinomas by T-cells, a hallmark of the histopathology of MMR-deficient tumours (33, 34). We also observed a higher incidence of moderate/marked infiltration by T-cells in the epithelium of MAP carcinomas, when compared with microsatellite-stable tumours (data not shown).

In the work of Dierssen *et al*., we dissected the most common mechanisms by which

HLA class I expression was altered in sporadic MSI-H and hereditary (Lynch syndrome) MMR-deficient tumours (12). We found that *B2M* mutations occurred preferentially in Lynch syndrome tumours, while sporadic MSI-H tumours presented more frequently with deficiencies at the level of the antigen-processing machinery

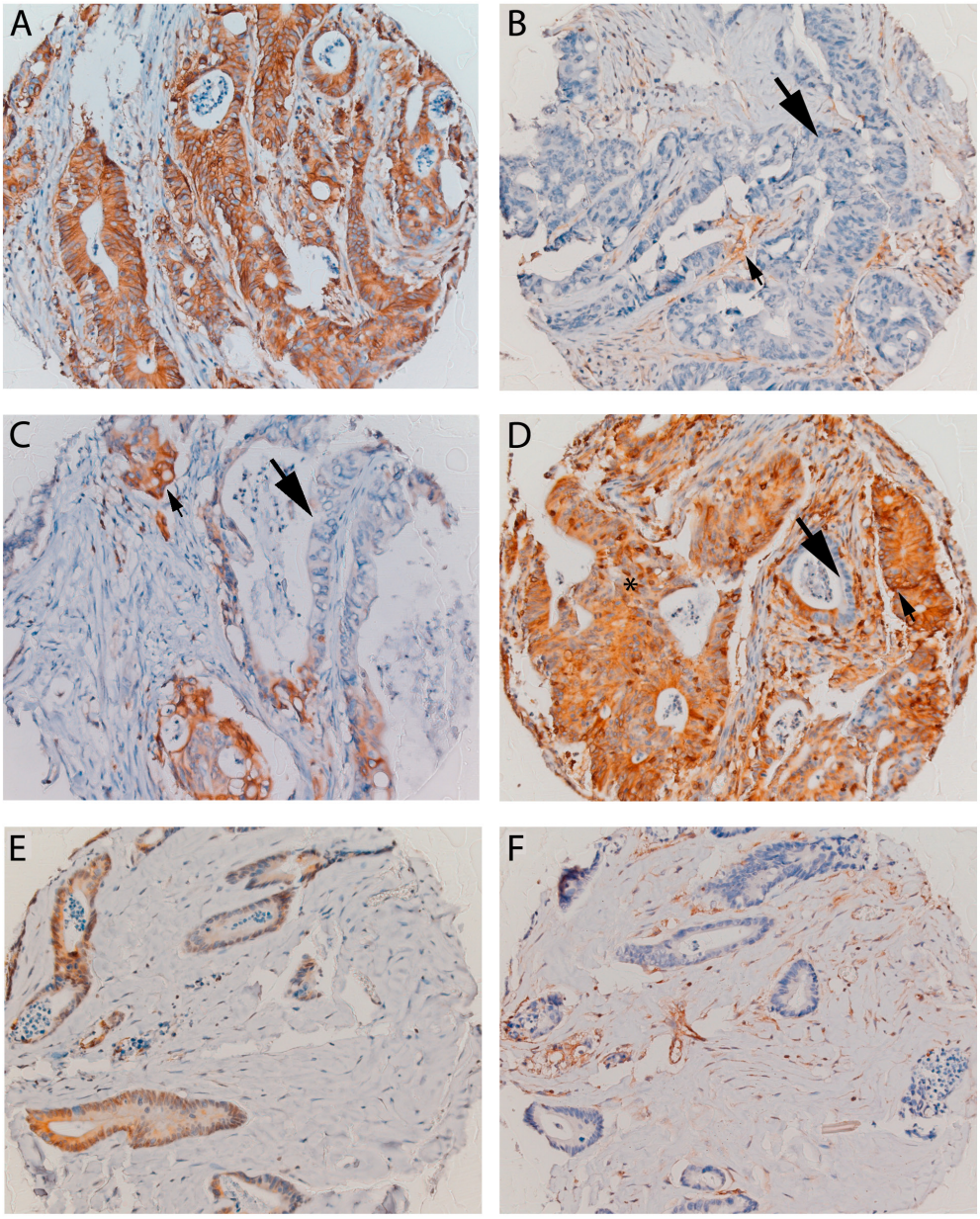

Figure 2. (A) Immunohistochemical detection of HLA class I expression with HCA2 antibody in the carcinoma from patient 11, displaying membranous expression in tumour cells. (B) HLA class I-negative carcinoma (large arrow) with positive internal control (small arrow) (patient 28). (C, D) Heterogeneous pattern of HLA class I expression; focal loss of HLA class I expression (large arrow) presented together with positive membranous expression (small arrow) (C, patient 17, tumour 2; D, patient 22, tumour 1). (E) Cytoplasmic accumulation of HLA class I in a case with β2m loss of expression, depicted in F (patient 29, metastases 4).

components. Kloor *et al.* also reported that mutations in the *B2M* gene occurred more frequently in Lynch syndrome-derived tumours than in sporadic MSI-H tumours (9). We investigated whether the loss of any of these molecules was coupled to HLA class I expression deficiencies in MAP carcinomas; expression of β2m was absent in 58% of MAP primary carcinomas with altered HLA class I expression, while at least one of the antigen-processing machinery components was not detected in 18% of MAP carcinomas with HLA class I alterations. Although speculative, it is interesting to underline that carcinomas derived from both MAP and Lynch syndromes preferentially lose β2m expression coupled to HLA class I deficiencies. A functional explanation for these observations remains elusive, but perhaps distinct reactions (both qualitative and quantitative) by the immune system, depending on the age of onset of the tumours, could condition the type of mechanisms that lead to HLA class I expression deficiencies.

Middeldorp *et al.* reported extensive chromosomal recombination events in MAP carcinomas (31). These frequently translate into LOH with maintenance of chromosome copy number (copy neutral LOH) throughout the genome. LOH at chromosome 6p21 (HLA locus) and 15q22–23 (*B2M* locus) was frequently detected, but no association with HLA class I or β2m expression was found (data not shown). In cases with maintenance of HLA class I expression, LOH at 6p might conceal an alternative HLA class I phenotype, not identified by immunohistochemistry: loss of one parental haplotype and amplification of the other. Each HLA class I haplotype has distinct affinities to different antigens, based on their peptide sequence. Therefore, the loss of one haplotype excuses the tumour cell from presenting a specific pool of peptides to the immune system. Moreover, the maintenance of one parental haplotype allows recognition of the 'self' by the immune cells and might impede the destruction of tumour cells by natural killer (NK) cells (4). No polymorphisms in β2m have been associated with different efficiencies in peptide presentation; it is therefore difficult to determine whether LOH in 15q, in cases without HLA class I abnormalities, has any effect on HLA class I/antigen presentation.

The high frequency of alterations in HLA class I expression in MMR-deficient and in MAP carcinomas constitutes a strong handicap for the employment of T-cell-based immunotherapy on advanced tumours (35). On the other hand, the fact that the immune system is able to recognize tumour antigens during cancer development reveals an opportunity for the development of vaccination strategies based on frequently altered peptides across different MAP carcinomas (36, 37). Additionally, memory T-cell responses could be used in the diagnostic setting, if they occurred at an early stage of tumour development, thus adding possibilities for the development of less invasive screening techniques, especially in carriers of hereditary syndromes.

We are the first to report HLA class I abnormalities in MAP carcinomas. It provides additional evidence that tumours carrying defects in DNA base repair mechanisms are more prone to undergo immune escape mechanisms. With this work, all known syndromes and subsets of sporadic colorectal tumours arising in the context of DNA repair deficiencies have now been linked with higher frequencies of HLA class I abnormalities, when compared with DNA base repair-proficient tumours.

METHODS

Patient material

Patients were recruited throughout The Netherlands. Germline DNA mutation analysis of the whole *MUTYH* gene was performed on lymphocytic DNA or DNA from formalin-fixed, paraffin-embedded normal tissue as described previously (38, 39). Informed consent was obtained according to protocols approved by the LUMC ethics review board (02–2004). A tissue microarray was constructed as reported previously (40) and contained

carcinoma tissue from 37 primary carcinomas and nine metastases (one lymph node and eight distant metastases), derived from 29 MAP patients, represented by more than four (mean = 4.5, SD = 1.7) 0.6-mm-diameter tissue cores. Such a number of tissue cores was shown to be sufficient to account for tumour

Table 2. Primer sequences utilized to sequence the *B2M*, *TAP1* and *TAP2* genes.

Primer	Forward	Reverse
B2M ex1	GGCTGGGCACGCGTTTAA	GGAGAGGAAGGACCAGAG
B2M ex2.1	TACCCTGGCAATATTAATGTGT	GATAGAAGACCAGTCCTT
B2M ex2.2	CTTACTGAAGAATGGAGAGAG	GATAGAAAGACCACTCCTTGC
B2M ex3	ATTCTGCCAGCTTATTTCTAACC	GGGTTATCATGTTAGAGCTGTC
TAP1 ex1.1	CGATTTCGCTTTCCCCTAA	AGCTAGCCATTGGCACTCG
TAP1 ex1.2	GCCAGTAGGGGAGGACTCG	GCACCAGCAGGGAGAATATG
TAP1 ex1.3	ACTGCTACTTCTCGCCGACT	CGCAGCTAATGGCTTCAAA
TAP1 ex1.4	CAACGGTTGGCTCCAAGA	GACTTCCCCAGTGCAGTAGC
TAP1 ex1.5	GACTTGCCTTGTTCCGAGAG	GACGCACAGGGTTTCCAG
TAP1 ex1.6	CGCCTTCGTTGTCAGTTATG	TTTGATTCCTGTCCCAGTCC
TAP1 ex2	TGTGCGTGACCTCTCTTCTC	ACCCTTCCAACTCCCTCATTTGC
TAP1 ex3	CACACCCTGATCCCCTTTTT	GGGGAGATCAAAGCAGATGT
TAP1 ex4.1	GGCATCCTGGCTCATTGTTA	GACACTGATCCCCAGAGCAT
TAP1 ex4.2	ACGTCCACCCTGAGTGATTC	TCTGGGAGATGAGGGTCTGT
TAP1 ex5.1	CCGTGTGACATCTCTTGTCC	ATAGGCCACAGCCTCCTTCT
TAP1 ex5.2	GGAATCTCTGGCAAAGTCCA	ACGGCATCTTAAGGACAAGG
TAP1 ex6	TTGTGTCCCCTCCAGATTTC	ATCACACTGGGGAGTGAAGG
TAP1 ex7.1	GCTGGCAAGCAGACTACCTC	GACAAGGCCCTCCAAGTGTA
TAP1 ex7.2	CTGTGGGCTCCTCAGAGAAA	GCCAGTGGAATACAGGGAGT
TAP1 ex8	GCCTTGGTAGCCTCTTATCG	CAAGCCACCTGCTTCCATAC
TAP1 ex9	CCCATGTGCCTTGTTCTATG	GGGTGGTGAGATGAGTGGAG
TAP1 ex10	GTTCTCATCTTGGCCCTTTG	TTGGGTGGGATATAGCCATT
TAP1 ex11.1	CTCTGACGGTCCGATGTCTT	CCCTTTTTCTCCATGAGCTG
TAP1 ex11.2	CAGGCTGACCACATCCTCTT	GAAAAGGGAGGGAGATGGAG
TAP2 ex2.1	CACCCCCATGATTTCTCATC	AATCCCAGCAGCCCTCTTAG
TAP2 ex2.2	GCTGTGGGGGCTGCTAAA	TCAGAACAGCCCACAGTGAC
TAP2 ex2.3	GGGGCTCAGCTGGTCACT	CCTCCAACTCACAACGTCCT
TAP2 ex3	GGGCACCAGCACATTCTT	AGATTTGGGGCTAGCAAATG
TAP2 ex4	GCTTGCTCCTCTGTTTCACC	TGGGAATCTCAGACCTGGAC
TAP2 ex5	CCTCACCTGCTCTGTCCTTC	CCCTTACATGCACGCTCAC
TAP2 ex6.1	AGCATCTCACTGGCTGGAGT	GCGACAGACTTCATGCTCCT
TAP2 ex6.2	CAGACCGTTCGCAGTTTTG	GGCTCCTTTCACAACCACTC
TAP2 ex7	GAAGATTCCCAGCCTCATCTC	CCCAGGAGTCCACAAAGAAA
TAP2 ex8	TCTCCCATTCCTGTTTTCCA	GGTTTCCTCCCTCTTTCAGG
TAP2 ex9	GGCAGGCCCTTAACTCTTTT	CTGTCTTCTCCCTCCTCACC
TAP2 ex10	ATAGTCCTCTGCCCCTGTCC	TTTACTGAAGGAGCAAGCTTACAA
TAP2 ex11	GCACTTGTCCCTCCTTGTGT	CCTGCCCTCTCACGGTACT
TAP2 ex12	TGGTGTCCATCTCATTCCTG	CGGGAATAGAGGTCCTGTCC

18mer M13 derived sequences were added to the 5' end the primer (Fw – TGTAAAACGACGGCCAGT, Rv – CAGGAAACAGCTATGACC)

heterogeneity and thus produce reliable results (41). The tissue array also included 14 adenomas resected from the above patients. The cohort's age mean was 51.2 years old (SD = 9.9). The majority of primary carcinomas were localized in the right-sided colon (73%) and the remaining had a sigmoid/rectum localization. Tumours were staged according to Dukes' classification: one was Dukes' A, 22 B, six C, and six D (three patients; one patient presented with four primary tumours and one distant metastasis). Patient samples were handled according to the medical ethical guidelines described in the Code Proper Secondary Use of Medical Sciences (http://www.federa.org).

Immunohistochemistry

Standard three-step, indirect immunohistochemistry was performed on 4 μm tissue sections transferred to glass slides using a tape-transfer system (Instrumedics, Hackensack, NJ, USA), including citrate antigen retrieval (citrate buffer, pH 6.0), blockage of endogenous peroxidase and endogenous avidin-binding activity, and diaminobenzidine development.

The following primary antibodies were used: the monoclonal antibody (MAb) HCA2, which recognizes β2m-free HLA-A (except -A24), -B7301, and -G heavy chains (42, 43); the MAb HC10, which recognizes a determinant expressed on all β2m-free HLA-B and -C heavy chains and on β2m-free HLA-A10, -A28, -A29, -A30, -A31, -A32, and -A33 heavy chains 33, 35; TAP1-specific MAb NOB1; LMP2-specific MAb SY-1; LMP7-specific MAb HB2; LPM10-specific MAb TO-7; calnexin-specific MAb TO-5; calreticulin-specific MAb TO-11; tapasin-specific MAb TO-3; ERp57-specific MAb TO-2 (45–47); TAP2-specific MAb (BD Biosciences Pharmingen, San Diego, CA, USA); rabbit anti-β2m polyclonal Ab (A 072; DAKO Cytomation, Glostrup, Denmark); and anti-granzyme B (clone 11F1; Novocastra Laboratories, Newcastle upon Tyne, UK). Secondary reagents used were biotinylated rabbit anti-mouse IgG antibodies (DAKO Cytomation), goat anti-rabbit IgG antibodies (DAKO Cytomation), and biotinylated-peroxidase streptavidin complex (SABC; DAKO Cytomation).

All antigen-processing machinery components' antibodies, except TAP2 MAb, were kindly provided by Dr Soldano Ferrone (Roswell Park Cancer Institute, Buffalo, NY, USA).

Loss of expression was defined as a complete lack of staining in the membrane and cytoplasm (HCA2, HC10, and anti-β2m), in the peri-nucleus/endoplasmic reticulum (NOB1, anti-TAP2, TO-2, TO-3, TO-5, TO-7, and TO-11), or in the cytoplasm (SY-1, HB2, and TO-7), but with concurrent staining in normal epithelium, stroma or infiltrating leukocytes.

***B2M*, *TAP1*, and *TAP2* sequencing**

The *B2M*, *TAP1*, and *TAP2* genes were screened for mutations in their coding regions. DNA was isolated from three 0.6-mm-diameter tissue cores retrieved from a tissue block after evaluation of the corresponding haematoxylin and eosin-stained tissue slide. After dewaxing, the tissue cores were incubated overnight at 56 °C in 0.3 ng/μl proteinase K and purified the next day with the Genomic Wizard kit (Promega, Leiden, The Netherlands). PCR was performed in a 25 μl volume containing 20 μM of primer pair and 12 ng of sample DNA. 18mer M13-derived oligos were added to the 5' end of each primer in order to facilitate sequence analysis. Primer sequences are presented in Table 2. Amplified products were sequenced at The Leiden Genome Technology Center (Leiden, The Netherlands).

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Infiltration of Lynch colorectal cancers by activated immune cells associates with early staging of the primary tumor and absence of lymph node metastases

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ABSTRACT

Lynch syndrome colorectal cancers often lose human leukocyte antigen (HLA) class I expression. The outgrowth of clones with immune evasive phenotypes is thought to be positively selected by the action of cytotoxic T cells that target HLA class I–positive cancer cells. To investigate this hypothesis, we related the type and density of tumor lymphocytic infiltrate in Lynch colorectal cancers with their HLA class I phenotype and clinicopathologic stage.

HLA class I expression was assessed by means of immunohistochemistry. Characterization of tumor-infiltrating lymphocytes was carried out by using a triple immunofluorescence procedure that allowed the simultaneous detection of CD3-, CD8-, and granzyme B (GZMB)-positive cells. Additional markers were also used for further characterization of an elusive CD3–/CD8–/GZMB+ cell population.

We discovered that high tumor infiltration by activated CD8+ T cells correlated with aberrant HLA class I expression and associated with early tumor stages ($P < 0.05$). CD8+ T cells were most abundant in HLA class I heterogeneous tumors ($P = 0.02$) and frequent in HLA class I–negative cases ($P = 0.04$) when compared with HLA class I–positive carcinomas. An elusive immune cell population (CD45+/CD8–/CD56–/GZMB+) was characteristic for HLA class I–negative tumors lacking lymph node metastases ($P < 0.01$).

The immune system assumes an important role in counteracting the progression of Lynch colorectal cancers and in selecting abnormal HLA class I phenotypes. Our findings support the development of clinical strategies that explore the natural antitumor immune responses occurring in Lynch syndrome carriers.

TRANSLATIONAL RELEVANCE

Lynch syndrome–associated colorectal cancers are known to evoke a strong reaction from the immune system of patients. We discovered that early staged tumors were more densely infiltrated by activated CD8+ T cells than tumors diagnosed at advanced stages. Moreover, we observed an immune cell population that was specifically associated with nonmetastasized Lynch syndrome colorectal cancers. Those cells lacked most common lymphocytic surface antigens. Altogether, our findings support that the immune system plays a major role in counteracting colorectal cancer progression in patients with Lynch syndrome. We propose the development of novel clinical strategies, inspired by the natural antitumor immune response occurring in patients with Lynch syndrome. Considering that Lynch colorectal cancers rapidly acquire immune evasive phenotypes, special relevance should be given to prophylactic approaches.

INTRODUCTION

Expression of human leukocyte antigen (HLA) class I/antigen complexes, in human cells, is essential for a competent immune surveillance (1). CD8+ T cells are capable of recognizing and eliminating target cells that present non–self-antigens in an HLA class I context (2). Accordingly, HLA class I loss is interpreted as a mechanism, adopted by tumors, to escape immune surveillance and thereby avoid tumor cell recognition and destruction (3). We, and others, previously reported that the majority of DNA mismatch- and base excision repair–deficient colorectal cancers lose HLA class I expression (4–7). Those include sporadic mismatch repair–deficient as well as Lynch syndrome colorectal cancers and MUTYH-associated polyposis (MAP) colorectal cancers, respectively. The frequency of HLA class I deficiencies in these tumors was

found to be considerably higher than the one observed for DNA repair–proficient colorectal cancers (5, 7). Both mismatch- and base excision repair–deficient colorectal cancers are thought to be particularly prone to evoke antitumor immune responses due to their pronounced mutator phenotypes (8). Such immune reaction will act as a vector of selective pressure that favors the outgrowth of tumor clones that acquired immune evasive phenotypes.

Multiple mechanisms have been shown to underlie defects in HLA class I expression by tumor cells; they include mutations in the individual HLA class I genes, *HLA-A*, *-B*, and *-C*, located on chromosome 6p21.3 (9); loss of heterozygosity at 6p21.3 (10); mutations in β2-microglobulin (*B2M*; ref. 11), the molecular chaperone required for the cell surface expression of HLA class I antigens; and defects in components of the HLA class I–associated antigen-processing machinery (12, 13). For unknown reasons, β2m defects were preferentially associated with HLA class I loss in hereditary colorectal cancers (Lynch syndrome and MAP), whereas sporadic mismatch repair–deficient tumors were frequently affected by antigen-processing machinery defects (5, 14).

The loss of HLA class I expression, in mismatch- and base excision repair–deficient colorectal cancers, constitutes a strong handicap for the employment of T-cell–based immunotherapeutic approaches (15, 16). On the other hand, evidence for T-cell–mediated antitumor immune responses could support the development of prophylactic vaccination strategies based on peptides that are frequently mutated in the aforementioned colorectal cancers (17, 18). This approach is of particular importance for individuals carrying an increased risk for developing colorectal cancer at an early age. Lynch syndrome is an autosomal, dominant disease caused by the germline inactivation of one copy of either *MLH1*, *MSH2*, *MSH6*, or *PMS2* mismatch repair genes (19). Patients with Lynch syndrome have an increased lifetime risk of developing colorectal cancer, as well as other cancer types, when compared with the general population (20). Currently, endoscopic surveillance constitutes the most effective approach to increase life expectancy of affected individuals (21).

The development of T-cell–based prophylactic vaccination strategies for patients with Lynch syndrome requires additional evidence that T cells are the drivers of immune selection in Lynch colorectal carcinomas. It has been previously reported that colorectal cancers carrying HLA class I defects are more densely infiltrated by T cells (22–24). However, these observations were not reproduced specifically in cohorts of mismatch repair–deficient tumors. Accordingly, those reports might carry a possible bias: Because both pronounced lymphocytic infiltration and HLA class I loss are hallmarks of mismatch repair–deficient tumors it is difficult to establish a causal relation between the presence of lymphocytes and HLA class I abnormalities on consecutive series of colorectal cancers (8, 25, 26). Therefore, the current study was conducted on a homogeneous cohort of genetically proven Lynch colorectal carcinomas. We sought to characterize and quantify the lymphocytic infiltration present in Lynch colorectal carcinomas and relate it with their HLA class I expression status and clinicopathologic stage.

RESULTS

Lymphocytic infiltration of Lynch carcinomas associates with the HLA class I phenotype

Expression of HLA class I and β2m was assessed in 90 Lynch colorectal carcinomas. Altogether, 83% of tumors presented HLA class I defects (Table 1). Membranous HLA class I expression was completely lost in 58 tumors, whereas a heterogeneous pattern

of HLA class I staining was observed in 17 carcinomas (Figure 1). The latter presented fields where tumor cells conserved membranous expression of HLA class I together with additional tumor areas where HLA class I expression was completely lost (Figures 1C and 1D). HLA class I alterations were accompanied by aberrant β2m expression in 51% of cases (Table 1). There was no difference in the distribution of HLA class I phenotypes between Lynch colorectal carcinomas with mutations in different mismatch repair genes (data not shown).

Qualitative and quantitative characterization of intraepithelial lymphocytic infiltration by means of triple immunofluorescence was possible for 83 tumors. In a few cases (n = 7), the staining procedure was not successful due to poor fixation and/or age of the tissue. The combination of CD3, CD8, and GZMB markers allowed the discrimination between CD3+/CD8− cells (presumably CD4+ and γδ T cells), CD3+/CD8+ cells (CD8+ T

Table 1. HLA class I phenotypes and density of lymphocytic infiltration in Lynch colorectal cancers.

			Infiltrate density, n cells/mm2							
			CD3+/CD8−		CD8+ T cells (CD3+/CD8+)		Activated CD8+ T cells (CD3+/CD8+/GZMB+)		CD3−/CD8−/GZMB+	
HLA class I status	Tumors, n (%)	% β2M loss	Median	Mean	Median	Mean	Median	Mean	Median	Mean
Positive	15 (17)	—	6.13	20.78	39.10	62.86	16.85	31.96	1.03	3.30
Heterogeneous	17 (19)	26	8.73	19.56	72.88	111.9	51.74[a]	67.97	1.86	3.78
Negative	58 (64)	59	11.98[a]	23.51	60.58	90.39	35.33[a]	54.38	4.96[a]	23.03

[a] Medians differ significantly from the HLA class I–positive group.

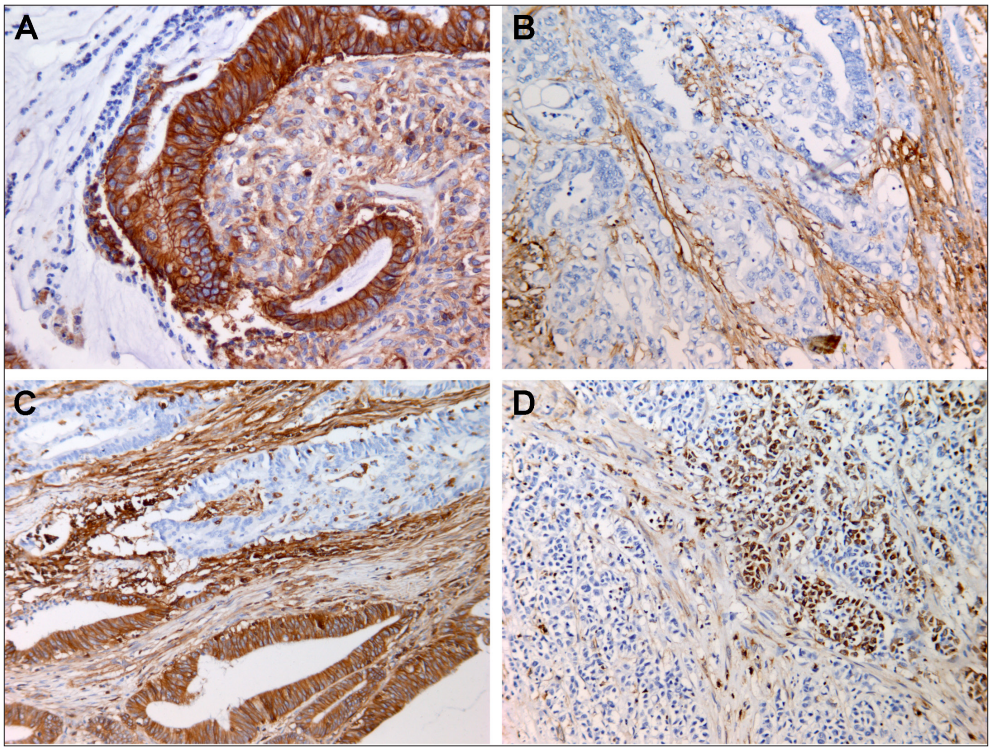

Figure 1. Three different HLA class I phenotypes were observed in Lynch colorectal carcinomas. Tumors were either HLA class I positive, with clear membranous expression of the HLA class I molecules (A); HLA class I negative, with total absence of HLA class I expression in tumors cells and respective positive internal control provided by stromal cells (B); and HLA class I heterogeneous, with HLA class I positive and negative tumor *foci* (C, D).

cells), and CD3+/CD8+/GZMB+ (activated CD8+ T cells; Figures 2A–D). No significant difference was detected between distinct HLA class I phenotypes for total CD8+ T-cell density (Figures 3A). However, HLA class I–negative and heterogeneous tumors were

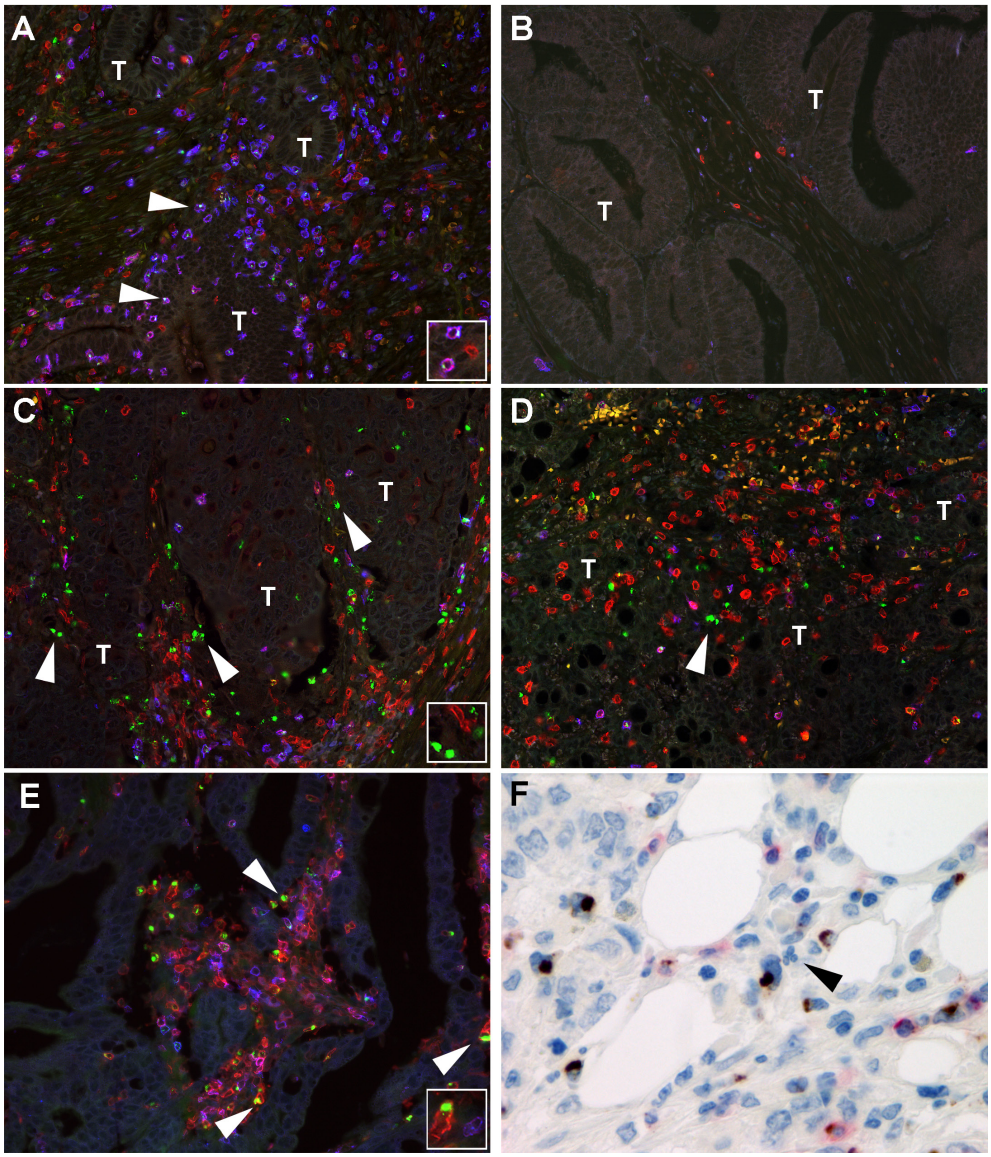

Figure 2. Density of lymphocyte infiltration in tumor fields (T) was determined by means of triple immunofluorescence. Antibodies against CD3 (red), CD8 (blue), and GZMB (green) were simultaneously used to identify CD3+/CD8– cells (presumably CD4+ or γδ T cells), total CD8+ T cells (CD3+/CD8+), and activated CD8+ T cells (CD3+/CD8+/GZMB+; A–D). CD4+ or γδ T cells are represented in red, whereas CD3+/CD8+ cells (purple) correspond to CD8+ T cells. GZMB positivity in CD3+/CD8+ cells (green/white) reveals activated CD8+ T cells (A, arrowheads). Density of lymphocyte infiltration varied greatly among tumors (A and B). A large number of GZMB-positive cells, without colocalization of CD3 and CD8, were detected in some tumors (C and D, arrowheads). We attempted to characterize those cells by using a set of markers in combination with CD8 (blue) and GZMB (green). CD45 (red) was the only marker that clearly associated with the CD8–/GZMB+ cells (E, arrowheads). By means of bright-field double staining, we excluded that CD8/GZMB+ cells were multinucleated granulocytes (F, arrowhead).

more densely infiltrated by activated CD8+ T cells than HLA class I–positive tumors (P = 0.02 and P = 0.04, respectively; Figure 3B). CD3+/CD8– cells (CD4+ and γδ T cells) were more frequent in HLA class I–negative tumors when compared with HLA class I–positive (P = 0.01; Figure 3C). Among HLA class I–negative tumors, only the density of CD3+/CD8– cells (CD4+ and γδ T cells) was related to the β2m expression status. β2m-negative cases were more densely infiltrated by these type of cells (P = 0.01; data not shown).

A population of cells with a CD3–/CD8–/GZMB+ phenotype was detected in 82% of cases at various frequencies (Figures 2C and D). These cells were often localized in the stromal compartment, immediately adjacent to the tumor fields, but less frequently within the epithelium. CD3–/CD8–/GZMB+ cells were mostly restricted to HLA class I–negative tumors (P = 0.004; Figure 3D).

The amount of CD8- and GZMB-positive cells relates to tumor stage of Lynch carcinomas

As the interaction between tumor and immune cells might have implications for tumor progression and dissemination, we compared the density of lymphocytic infiltration between different pathologic stages (TNM classification).

There was a clear correlation between the T staging of the primary tumor and the presence of CD8+ T cells, independently of their activation status (provided by GZMB staining). The total amount of CD8+ T

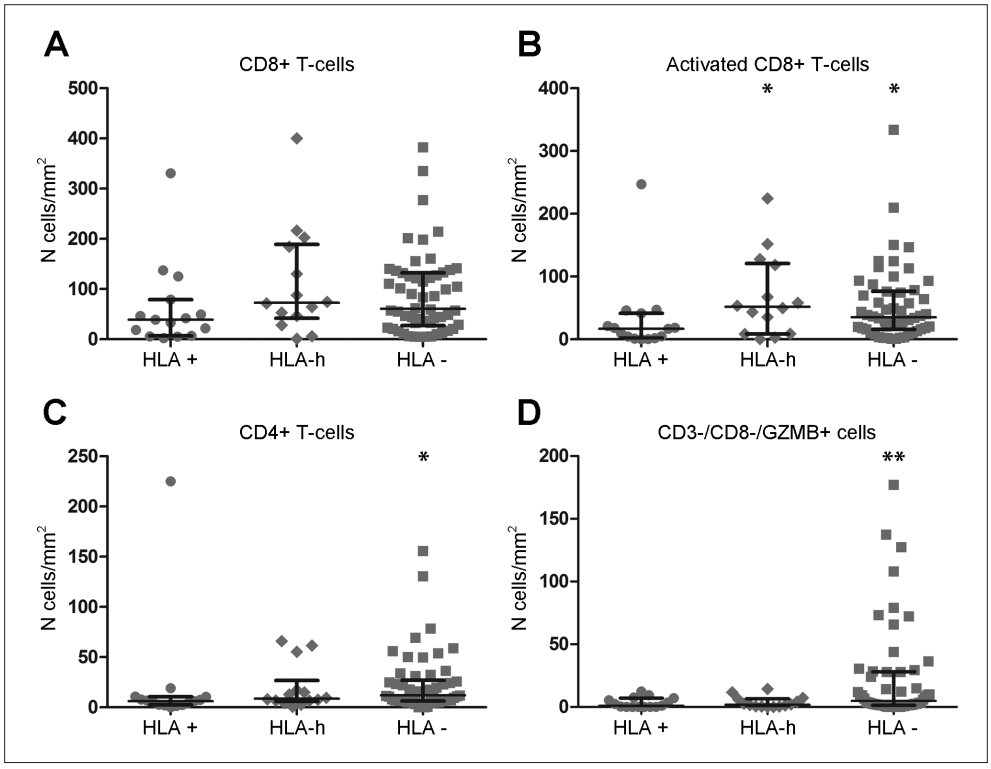

Figure 3. Total CD8+ T cells were not distributed differently between tumors with different HLA class I phenotypes (A). Activated CD8+ T cells were more frequent both in HLA class I–negative (HLA–) and heterogeneous (HLA-h) tumors than HLA class I–positive cases (B). CD3+/CD8– (CD4+ and γδ T cells) were also more frequent in HLA class I–negative tumors (C). GZMB+ cells lacking CD3 or CD8 markers were characteristic for HLA class I–negative carcinomas (D). *, $P < 0.05$; **, $P < 0.01$.

cells was gradually smaller with increasing tumor stage ($P < 0.05$; ANOVA, Figure 4A). This difference was most striking when the analysis was restricted to HLA class I–negative tumors ($P = 0.002$; Figure 4B). The same trend was observed for activated CD8+ T cells in all tumors ($P = 0.04$) and HLA class I–negative cases ($P = 0.004$; Figures 4C and D). No other lymphocyte population correlated with T staging.

Remarkably, abundance of the elusive CD3–/CD8–/GZMB+ cell population not only correlated with HLA-negative tumors but was also characteristic for lymph node–

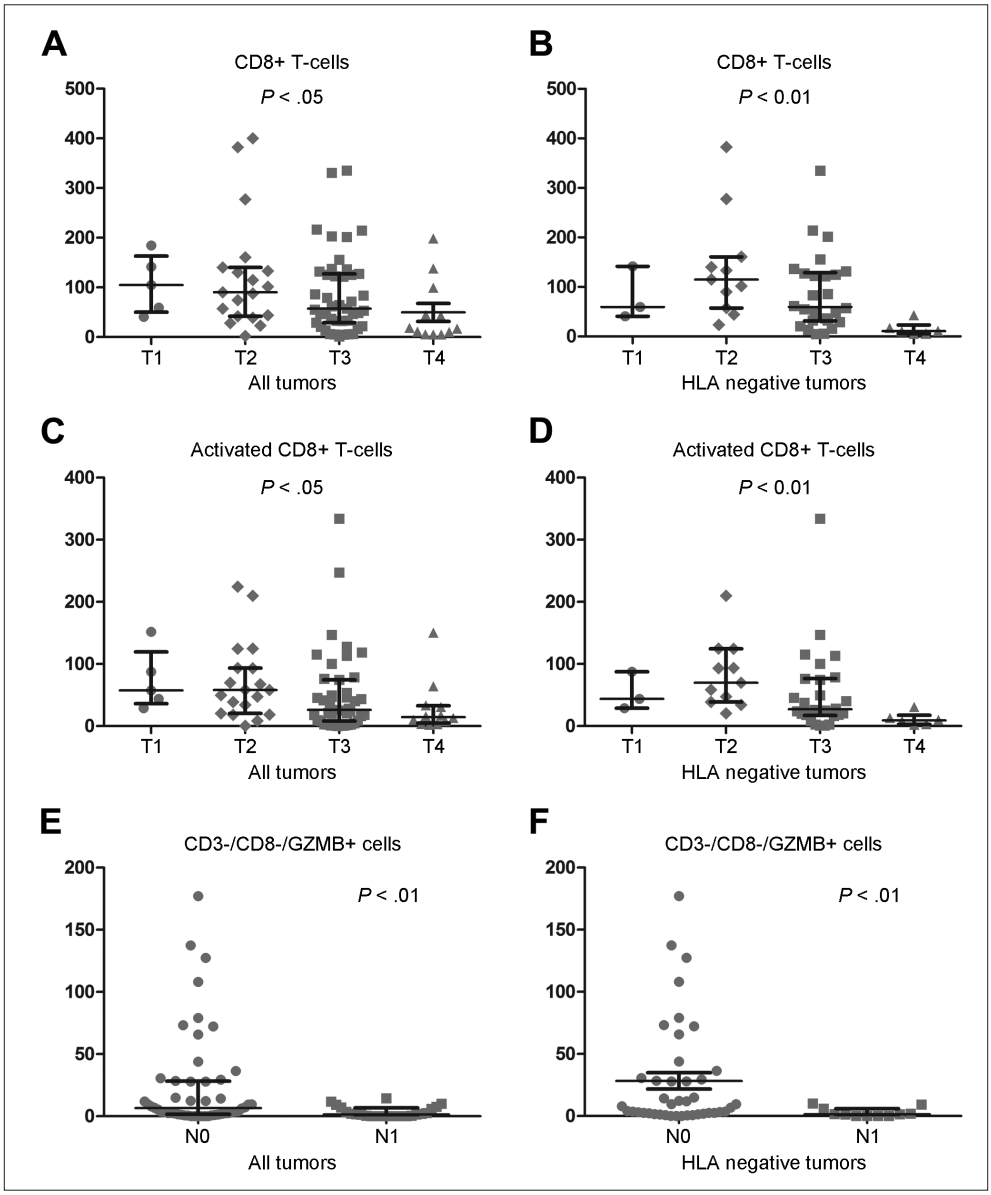

Figure 4. The density of lymphocytic infiltration was related to tumor stage both for the primary tumor (T) and lymph node status (N). Total CD8+ T cells and activated CD8+ T cells were more frequent in earlier stages of the primary tumor (A–D). The elusive CD3–/CD8–/GZMB+ cell population was almost exclusively present in lymph node–negative (N0) carcinomas (E and F).

negative carcinomas ($P < 0.01$; Figures 4E and F). All Lynch carcinomas with more than 10 CD3−/CD8−/GZMB+ cells per mm2 of tumor area did not present lymph node metastases. Furthermore, the only 2 cases presenting metastases at distant organs also carried lower numbers of CD3−/CD8−/GZMB+ cells (0 and 7 cells/mm2). None of the CD3+ lymphocyte populations related to the lymph node status of tumors.

Characterization of the CD3−/CD8−/GZMB+ cell population

The potential clinical significance of the CD3−/CD8−/GZMB+ cell population in the progression of Lynch carcinomas compelled us to further characterize these cells. Their clear association to HLA class I loss and lymph node–negative tumors supported the investigation of additional markers with a focus on natural killer (NK) cells. CD2, CD16, CD45, CD56, CD57, CD68, CD117, NKp46, and TCR-γ expression was assessed simultaneously with CD8 and GZMB markers (antibody description in Table 2).

The only marker that clearly associated with the CD3−/CD8−/GZMB+ cell population was CD45 (Figure 2E), thus confirming the hematopoietic nucleated lineage of these cells. CD56+ cells were rare and located nearby blood vessels (Figure 5A). CD16 cells were abundant but did not colocalize with GZMB positivity (Figure 5B). CD57+/CD8− cells were often found in the stromal compartment of tumors, and some displayed positivity for GZMB but CD57 failed to colocalize with the majority of CD8−/GZMB+ cells (Figure 5C). All other investigated markers did not colocalize with these cells, despite the presence of internal positive controls for the majority (data not shown). No NKp46+ cells were found in tumor tissues. We detected NKp46 expression in tonsil tissues to rule out a failure of the staining procedure.

As GZMB expression could be derived from granulocytes, we conducted a bright-field, double staining of CD8 and GZMB together with hematoxylin counterstaining to discern the nuclear morphology of CD8−/GZMB+ cells. The nuclei from CD8−/GZMB+ cells were nonlobated and easily discriminated from polymorphonuclear granulocytes (Figures 2F and 5D).

This CD45+/CD8−/CD56−/GZMB+ cell population presented consistently throughout the staining procedures a higher amount of GZMB+ granules when compared with activated CD8+ T cells. Morphologically, these cells also appeared to be considerably larger than T lymphocytes (Figures 2F and 5D).

DISCUSSION

We characterized the quality and density of lymphocytic infiltrate, in a cohort of Lynch colorectal cancers, in the context of the HLA class I expression status of tumors

Table 2. Description of primary antibodies utilized for the characterization of tumor infiltrating lymphocytes.

Antigen	Antibody	Host/Isotype	Dilution	Company	Antigen expression	Secondary antibody [a]
CD3	ab828	Rabbit polyclonal	1:200	Abcam	T cells	goat anti-rabbit IgG-Alexa Fluor 546
CD8	4B11[b]	Mouse IgG2b	1:200	Leyca Mycrosystems	CD8+ T cells	goat anti-mouse IgG2b-Alexa Fluor 647
CD8	c8/144B	Mouse IgG1	1:400	DAKO	CD8+ T cells	goat anti-mouse IgG1-Alexa Fluor 546
Granzyme B	GrB-7	Mouse IgG2a	1:400	DAKO	Cytotoxic T cells; NK cells	goat anti-mouse Ig2a-Alexa Fluor 488
CD2	AB75	Mouse IgG1	1:10	DAKO	T and NK cells	goat anti-mouse IgG1-Alexa Fluor 546
CD16	DJ130c	Mouse IgG1	1:200	Ab Serotech	NK cells, neutrophils, monocytes, macrophages	goat anti-mouse IgG1-Alexa Fluor 546
CD45	2B11+PD7	Mouse IgG1	1:100	DAKO	Hematopoietic nucleated cells except plasma cells	goat anti-mouse IgG1-Alexa Fluor 546
CD56	123C3	Mouse IgG1	1:100	DAKO	NK cells, activated T cells	goat anti-mouse IgG1-Alexa Fluor 546
CD57	HNK-1 hybridoma	Mouse IgM	1:20	ATCC	NK cells, T cells	goat anti-mouse IgM-Alexa Fluor 546
CD68	KP1	Mouse IgG1	1:200	DAKO	Monocytes, macrophages	goat anti-mouse IgG1-Alexa Fluor 546
CD117	A4502	Rabbit polyclonal	1:50	DAKO	Mast cells, lymphoid progenitors	goat anti-rabbit IgG-Alexa Fluor 546
NKp46	195314	Mouse IgG2b	1:100	R&D systems	NK cells	goat anti-mouse IgG2b-Alexa Fluor 647
TCR-γ	γ3.20	Mouse IgG1	1:25	Thermo Fisher	γδ T cells	goat anti-mouse IgG1-Alexa Fluor 546

[a] All secondary antibodies were employed at a 1:200 dilution and purchased from Invitrogen; Rb - rabbit; Mo - Mouse; Go – goat. [b] The c8/144B anti-CD8 clone was only used in combination with the NKp46 marker due to isotype matching between latter that the anti-CD8 4B11 clone.

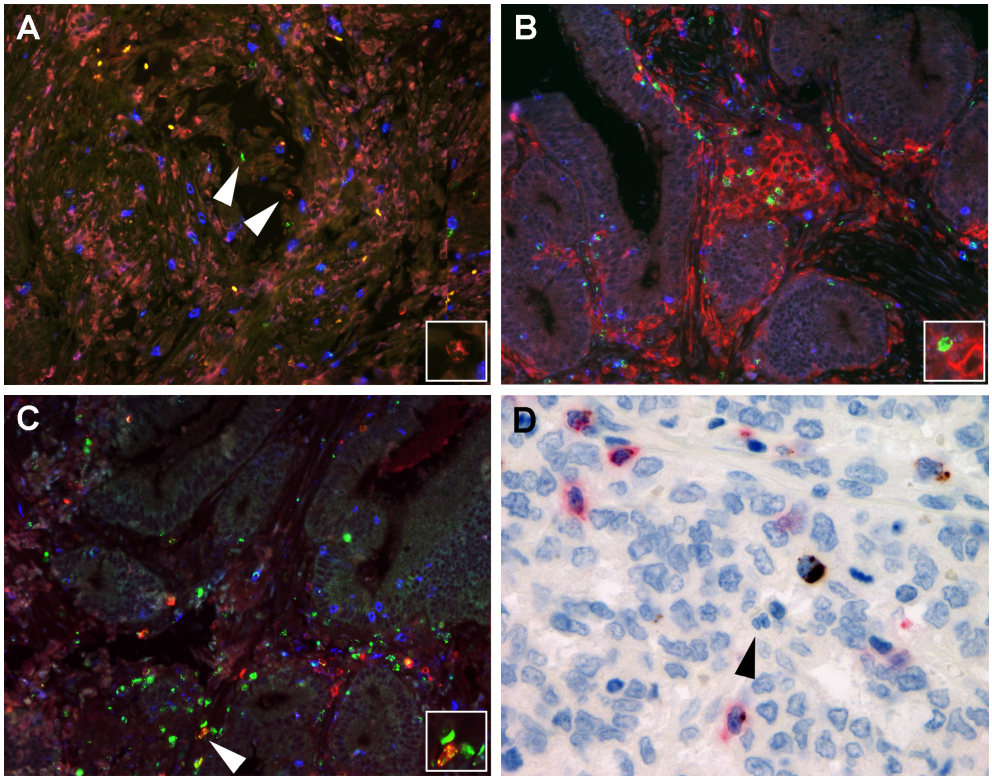

Figure 5. The characterization of the CD3-/CD8-/GZMB+ cell population was made through the employment of various markers (red) together with CD8 (blue) and GZMB (green). CD8-/GZMB+ cells failed to co-localize with CD56 (A, arrows), CD16 (B) and CD57 (C). Few CD57+ cells displayed GZMB positivity (C, arrow). By means of bright-field double-staining we excluded that CD8-/GZMB+ cells were multi-nucleated granulocytes (D, arrow).

and their clinicopathologic stage. We aimed to establish a relation between the makeup of the antitumor immune response and the HLA class I phenotype of tumors to support the notion of immune selection.

Both CD3+/CD8– cells (presumably CD4+ and γδ T cells) and activated CD8+ T cells were more frequent in HLA class I–negative tumors than HLA class I–positive cases. Moreover, tumors presenting heterogeneous patterns of HLA class I expression were infiltrated by remarkably high numbers of activated CD8+ T cells. It is tempting to speculate that these tumors were undergoing an active immune selection process, revealed by the presence of a heterogeneous HLA class I phenotype and the high affluence of activated CD8+ T cells. It is less evident why tumors that lost HLA class I expression remained infiltrated by activated CD8+ T cells as their effector function is dependent on the expression of HLA class I (27). Their persistent activation status might be supported by inflammatory signals derived from a past immune response against HLA class I–positive tumor cells. The elevated number of CD3+/CD8– cells (CD4+ and γδ T cells) in HLA class I–negative tumors alludes to the presence of T cells with helper and regulatory functions, capable of sustaining CD8+ T-cell activation (28, 29). On the other hand, the activation of CD8+ T cells might derive from the presence of antigen-presenting cells which conserve the capability of presenting tumor antigens through an HLA class II route (30). Colorectal tumor cells were also shown to express HLA class II and might thereby promote the migration

and activation of CD8+ T cells (31). Michel and colleagues described that approximately one third of microsatellite instability-high (MSI-H) colorectal tumors presented membranous expression of HLA class II (32). We found a similar distribution for HLA-DR expression, the most abundantly expressed HLA class II molecule, in a subset of the current cohort. Its expression was associated with a higher density of infiltration by CD8+ T cells and CD3−/CD8−/GZMB+ cells (data not shown).

An elusive CD3−/CD8−/GZMB+ cell population was highly specific for HLA class I–negative tumors. Those cells were infrequent in tumors with heterogeneous expression of HLA class I and nearly absent in HLA class I–positive tumors. Strikingly, the presence of CD3−/CD8−/GZMB+ cells, in primary tumors, was highly predictive for the absence of lymph node metastases. Altogether, these observations compelled us to further characterize this population with focus on NK cell markers.

NK cells are CD3-negative cells possessing cytolytic activity, which can be triggered by the absence of HLA class I expression in target cells (33). They, thus, constitute an important component of the innate immune system, responsible for dealing with cells lacking important host markers–missing self hypothesis (34). Accordingly, NK cells have been regarded as promising vectors for the treatment of HLA class I–negative cancers (35). It is thought that tumor cells compensate for HLA class I loss by favoring the expression of additional NK-inactivating ligands and by losing antagonist ligands with NK-activating properties (36). Expression of HLA-G, an NK cell inhibitory ligand, was previously associated with a worse prognosis in colorectal cancer (37). We investigated its expression in a fraction of tumors composing the current cohort. HLA-G expression was never membranous and only detected in 16% of tumors (data not shown).

Several molecules have been proposed to be characteristic for NK cells or, at least, expressed by certain NK cell subsets (36). Our investigation included CD56, NKp46, and CD16, among others. All these markers were absent in the CD3−/CD8−/GZMB+ cell population, thus not supporting their classification as NK cells. Several other markers were investigated, which in turn excluded that these cells were mast cells or other granulocytes, macrophages, or γδ T cells. The only marker that clearly associated with the CD3−/CD8−/GZMB+ cell population was CD45, which confirmed their hematopoietic-nucleated lineage.

Considering that tumor staging is still the most important prognostic factor in colorectal cancer, the findings presented in this work might be of great relevance. We found a clear association between the type and density of immune cells, present in primary tumors, and their clinicopathologic stage. Furthermore, different immune cell populations related to the local invasive behavior of tumors or with their disseminating capacity. Our results support a role for CD8+ T cells in counteracting a local invasion of the mucosa by cancer cells but not in preventing the migration of tumor cells to adjacent lymph nodes. Conversely, the elusive CD45+/CD8−/CD56−/GZMB+ cell population was characteristic of tumors without lymph node metastases but did not relate to the extent of invasion of the primary tumor. Such a combination of immunologic responses might contribute to the improved survival of Lynch syndrome carriers when compared with patients with sporadic colorectal cancer (38, 39). The makeup and magnitude of the immune responses observed in patients were previously associated with the clinical prognosis and staging of colorectal cancer (40). Moreover, Galon and colleagues proposed that the type, density, and location of immune cells were better predictors of patient outcome than staging (41). However, the majority of studies did not discriminate between mismatch

repair–deficient and mismatch repair–proficient colorectal cancers. Hence, the current study is the first to focus specifically on Lynch colorectal carcinomas.

While immune cells are important drivers of tumor selection, they could also hold the key for novel therapeutic or preventive interventions. The prophylactic vaccination of patients with Lynch syndrome with recurrent tumor antigens could elicit an early and robust CD8+ T-cell immune response (17, 18). Such a reaction could lead to tumor eradication or impede tumor progression from early stages. Of note, the presence of CD8+ T cells did not associate with the lymph node status of tumors, which highlights the role of the CD45+/CD8−/CD56−/GZMB+ cell population. The understanding of their role in counteracting tumor metastases could prove essential for the development of novel immunotherapeutic approaches targeting advanced tumors.

METHODS

Patient material

A cohort of 90 colorectal carcinomas, derived from 86 patients with Lynch syndrome, was compiled. Corresponding formalin-fixed, paraffin-embedded tissues were collected throughout The Netherlands. All patients were carriers of genetically proven, pathogenic, germline mutations in *MLH1* (n = 31), *MSH2* (n = 25), *MSH6* (n = 24), or *PMS2* (n = 6) as determined by the Leiden Diagnostic Genome Centre of the Leiden University Medical Centre (LUMC), Leiden, The Netherlands. In addition, all except one patient, for whom extended clinical information was not available, fulfilled the revised Bethesda criteria for Lynch syndrome (42). Pathologic tumor- (lymph) node–metastasis (TNM) staging was retrieved from 79 tumors of which 5 tumors were staged as T1, whereas 19, 43, and 12 cases were staged as T2, T3, and T4, respectively. Twenty-four cases presented with lymph node metastases. Metastases in distant organs were only observed in 2 cases. The study was approved by the Medical Ethical Committee of the LUMC (protocol P01-019). Patient samples were handled according to the medical ethical guidelines described in the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies.

Immunohistochemistry

Expression of HLA class I and β2m were assessed by means of a 2-step indirect immunohistochemistry procedure on 4-μm tissue sections. Following deparaffinization and rehydration, the tissue sections underwent heat-mediated antigen retrieval in a 10 mmol/L citrate buffer solution (pH 6). After cooling, endogenous peroxidase activity blockage was carried out with a 0.3% hydrogen peroxide/methanol solution. Thereafter, the sections were incubated overnight with one of the following primary antibodies: the monoclonal antibody (mAb) HCA2, which recognizes β2m-free HLA-A (except -A24), -B7301, and -G heavy chains (32, 33); the mAb HC10, which recognizes a determinant expressed on all β2m-free HLA-B and -C heavy chains and on β2m-free HLA-A10, -A28, -A29, -A30, -A31, -A32, and -A33 heavy chains [refs. 33, 34; supernatants kindly provided by Prof. J. Neefjes, Netherlands Cancer Institute (NKI), Amsterdam, The Netherlands, and Prof. H.L. Ploegh, MIT, Boston, MA]; and the rabbit anti-β2m polyclonal antibody A0072 (Dako). The following day, primary antibody binding was detected with the BrightVision Poly-HRP (horeradish peroxidase) Detection System (Immunologic). Scoring of HLA class I and β2m expression in tumor cells was always carried out against an internal positive control, provided by the staining of stromal cells. Negative controls were generated by replacing the primary antibodies by a 1% bovine serum albumin (BSA)/PBS solution during the procedure.

A double-staining immunohistochemistry procedure was carried out to study the morphology of CD3−/CD8−/GZMB+ cells. Tissue sections were treated as described for the HLA class I and β2m staining, but heat-mediated antigen retrieval was done in a 1 mmol/L EDTA solution (pH 9) instead. Tissue sections were incubated overnight with a mixture of anti-CD8 [4B11, immunoglobulin G2b (IgG2b)], and anti-Granzyme B (GZMB; GrB-7, IgG2a) antibodies. Further detail on the primary antibodies is provided in Table 2. The following day, tissue sections were incubated with an anti-mouse IgG2a–HRP and goat anti-mouse IgG2b–AP solution (1:200; Southern Biotech). Staining development was carried out with 3,3'-diaminobenzidine (Dako) and Fast Red (Roche Applied Science) according to the manufacturer's instructions.

Immunofluorescence

Characterization of lymphocytic infiltration in Lynch carcinomas was carried out with triple immunofluorescence. After deparaffinization and rehydration of the 4-μm tissue sections, heat-mediated antigen retrieval with a 1 mmol/L EDTA solution (pH 9) and blockage of nonspecific antibody binding with 10% normal goat serum (Dako) were carried out. A solution containing 3 primary antibodies was applied to the tissue sections overnight. The next day, a mixture

of fluorescently labeled secondary antibodies was used to detect primary antibody binding. A detailed list of primary and secondary antibodies used is provided in Table 2. Initial characterization of the lymphocytic infiltrate was done with a combination of anti-CD3, anti-CD8, and anti-GZMB antibodies. The CD3+/CD8− cell population was considered to be composed of CD4+ and γδ T cells. The discovery of an elusive CD3−/CD8−/GZMB+ cell population prompted us to screen additional tissue sections with primary antibodies directed against various hematopoietic markers. Anti-CD2, -CD16, -CD45, -CD56, -CD57, -CD68, -CD117, -NKp46, and anti-TCR-γ antibodies were used in combination with anti-CD8 and anti-GZMB antibodies (antigens and dilutions provided in Supplementary Table S1). Immunofluorescence was detected with a LSM700 confocal laser microscope (Carl Zeiss), equipped with a ZEISS LCI Plan-NEOFLUAR ×25/0, 8 DIC Imm Korr objective. Approximately 4 mm2 of tumor tissue was scanned. Density of lymphocytic infiltration was determined by dividing the number of intraepithelial (or immediately adjacent to the epithelium) lymphocytes by the tumor area. Both lymphocyte counting and measurement of the tumor area were conducted with the ZEN2009 LE software (Carl Zeiss). Negative controls were generated by replacing primary antibodies by a 1% BSA/PBS solution.

Statistical analyses

All statistical tests and graph construction were carried out with GraphPad PRISM (version 5.04). The Mann–Whitney U test was used when assessing the differences in the amount of lymphocytic infiltrate between HLA class I phenotypes and lymph node-negative and -positive tumors. ANOVA was conducted for comparing lymphocyte density among the different T tumor stages (TNM classification).

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Frameshift truncating mutations in TGFBR2 are reverted by transcriptional slippage in colorectal cancer

5

Chapter 5

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ABSTRACT

The TGF-β type 2 receptor gene (*TGFBR2*) is mutated in the majority of colorectal cancers that display a high degree of microsatellite instability (MSI-H). *TGFBR2* contains a ten adenine ((A)10) microsatellite that is a hotspot for the occurrence of nucleotide deletions and insertions in MSI-H cancers. As these translate to truncated protein forms it has generally been believed that the majority of MSI-H colorectal cancers lack TGFβR2-mediated signaling. Nevertheless, previous works have shown that TGFβR2-deficient cell lines retain sensitivity to TGF-β.

Here we demonstrate that TGF-β signaling is active in MSI-H colon tumors and that TGF-β activation in colorectal cancer cells occurs in a TGFβR2-dependent manner, despite the presence of truncating biallelic mutations in its gene. The ability of *TGFBR2*-mutated cells to respond to TGF-β was dependent on the preservation of *TGFBR2*'s microsatellite sequence. The replacement of the latter by synonymous, non-microsatellite sequences ablated the capacity of *TGFBR2*-mutated cells to respond to TGF-β.

We postulate that the restoration of the reading-frame in *TGFBR2*-mutants occurs by transcriptional slippage. Notably, there is an apparent selective advantage for MSI-H colorectal cancers to retain responsiveness to TGF-β stimulation, which might support an important role for TGF-β signaling during carcinogenesis of these tumors.

INTRODUCTION

The transforming growth factor-β (TGF-β) signaling pathway regulates cellular processes such as proliferation, differentiation and apoptosis. It constitutes an important mediator of tissue homeostasis, being crucial during tissue remodeling and inflammation (1-3). TGF-β signaling is found altered in a variety of tumors, including colorectal cancers (4, 5). It is believed that tumor cells acquire deleterious alterations in TGF-β signaling components in order to selectively escape its cytostatic effects (6, 7). Paradoxically, activation of TGF-β has also been associated with increased malignant behavior and metastatic capacity of cancer cells (8-10).

TGF-β signaling initiates its cellular responses by binding a TGF-β type 2 serine/threonine kinase transmembrane receptor, termed TGFβR2 (11). This interaction is facilitated by TGF-β type 3 receptor (TGFβRIII), a co-receptor lacking kinase activity (12). Upon ligand binding, TGFβR2 attracts and phosphorylates an additional serine/threonine kinase receptor, TGFβR1, which in turn phosphorylates the transcription factors Smad2 and Smad3 (13, 14). When activated, Smad2 and Smad3 associate with the co-Smad, Smad4, and this complex accumulates in the cell nucleus where it activates or represses expression of gene targets in conjunction with additional transcription factors (15). Although alternative, homologous receptors exist, TGFβR1 and TGFβR2 are considered fundamental for the transduction of TGF-β signaling responses in epithelial cells.

The manner by which colorectal cancers acquire alterations in the TGF-β pathway is related to the type of genetic instability observed in tumors. The majority of colorectal cancers display chromosomal instability (CIN), where gains or losses of entire chromosomal regions are frequent (16). Loss of genetic material affecting the chromosome region 18q21.1, where *SMAD2* and *SMAD4* are located, is found in the majority of CIN colorectal cancers (17, 18). Mutations in any of these genes are almost exclusively found in *SMAD4* (19), and when genetically inherited, explain a subset of cases of juvenile polyposis syndrome (20). Furthermore, Smad4 expression is lost in approximately half of

CIN tumors and reduced in a significant proportion of cases (21, 22). The remaining fraction of colorectal cancers presents a microsatellite instability-high (MSI-H) phenotype, derived from a defective DNA mismatch repair (MMR) system (23). MSI-H colorectal cancers are characterized by the genome-wide accumulation of insertions and deletions at microsatellite sequences, peri-diploid DNA content and lack of gross chromosomal aberrations (24-26). Smad4 defects are uncommon in MSI-H colorectal tumors that instead target the TGF-β pathway at the receptor level (22). The third exon of the *TGFBR2* gene contains a ten adenine microsatellite sequence that is found mutated in the majority of MSI-H colorectal cancers (26, 27). Deletions and insertions at this microsatellite produce frameshifts that lead to truncated forms of TGFβR2. Mutations are also frequent in *ACVR2A*, a gene that encodes an alternative type 2 TGF-β receptor and possesses two microsatellites of eight adenines at its third and tenth exons (28). Since the availability of either receptor is considered to be essential for TGF-β transduction, the current dogma of colorectal carcinogenesis assumes that this pathway is inactive in the majority of MSI-H cancers. Nevertheless, two independent reports have shown that cells carrying biallelic, truncating mutations in *TGFBR2* and *ACVR2A* remained sensitive to TGF-β stimulation in vitro (29, 30). Alterations in other components of the TGF-β pathway include: mutations in the 3' untranslated region of the *BMPR2* gene in the majority of MSI-H colorectal cancers (31), and mutations in the *SMAD3* gene in approximately 4% of all colorectal cancers (32).

In this work, we sought to explain the basis of retained TGF-β sensitivity in *TGFBR2*-mutated MSI-H colorectal cancer cells. We provide ex vivo and in vitro evidence that TGFβR2- and ACVR2A-deficient MSI-H colorectal cancer cells remain sensitive to TGF-β. Furthermore, we suggest that mutated *TGFBR2* genes can still generate functional TGFβR2 proteins, most likely by reverting to their wild-type sequences by transcriptional slippage. Such form of "transcriptional instability" might be essential for the regulation TGF-β activity in a malignant context.

RESULTS

The TGF-β pathway is active in MSI-H colon cancers

TGF-β signal transduction through TGFβR2 results in the phosphorylation of Smad2 at residues Ser465/467 (13). Thereafter, Smad2 associates with Smad4 and translocates to the nucleus where it activates or represses TGF-β transcription targets (14, 15). Immunohistochemical detection of the phosphorylated form of Smad2 (P-Smad2) was used to assess whether TGF-β signaling was active in a cohort comprised of 76 right-sided colon cancers (Figure 1). Approximately half of samples showed strong nuclear accumulation of P-Smad2 while the remaining cases presented either weak (34%) or absent (16%) expression of P-Smad2 in the nucleus. A similar distribution was observed for MSI-H tumors specifically, suggesting that TGF-β signaling is active in right-sided colon cancers, independent of their background of genetic instability. P-Smad2 nuclear accumulation was positively correlated with nuclear Smad4 expression (correlation coefficient = 0.266, $P = 0.021$, Spearman's rank), with the latter being most frequently lost in microsatellite stable (MSS) tumors.

Smad2 phosphorylation in MSI-H colon cancers does not relate to *TGFBR2* mutation status

The *TGFBR2* gene contains a ten adenine ((A)10) microsatellite sequence in its third exon that is targeted by deletions and

insertions in the majority of MSI-H colon cancers. We screened *TGFBR2*'s (A)10 microsatellite for mutations in 32 MSI-H colon cancers by means of fragment analysis (Figure 2A). Prior to that, tumors were flow-sorted on the basis of cell's keratin and vimentin expression in order to separate epithelial tumor cells from stromal cells. By removing stromal contaminants from the tumor samples we were able to determine the allelic state of *TGFBR2* mutations (Figure 2A). Mutations in *TGFBR2*'s (A)10 microsatellite were found in 94% of MSI-H colon cancers. One-nucleotide deletions were most frequent but two-nucleotide deletions and one nucleotide insertions were also observed (data not shown). Two-thirds of mutated cases presented *TGFBR2* mutations in both alleles (Figure 2B). In a third of mutated cases, a wild-type allele could still be detected (Figure 2B). P-Smad2 levels were not higher in tumor samples that conserved a wild-type *TGFBR2* allele.

In order to exclude that Smad2 phosphorylation in MSI-H tumors could be due to activation of the TGF-β pathway

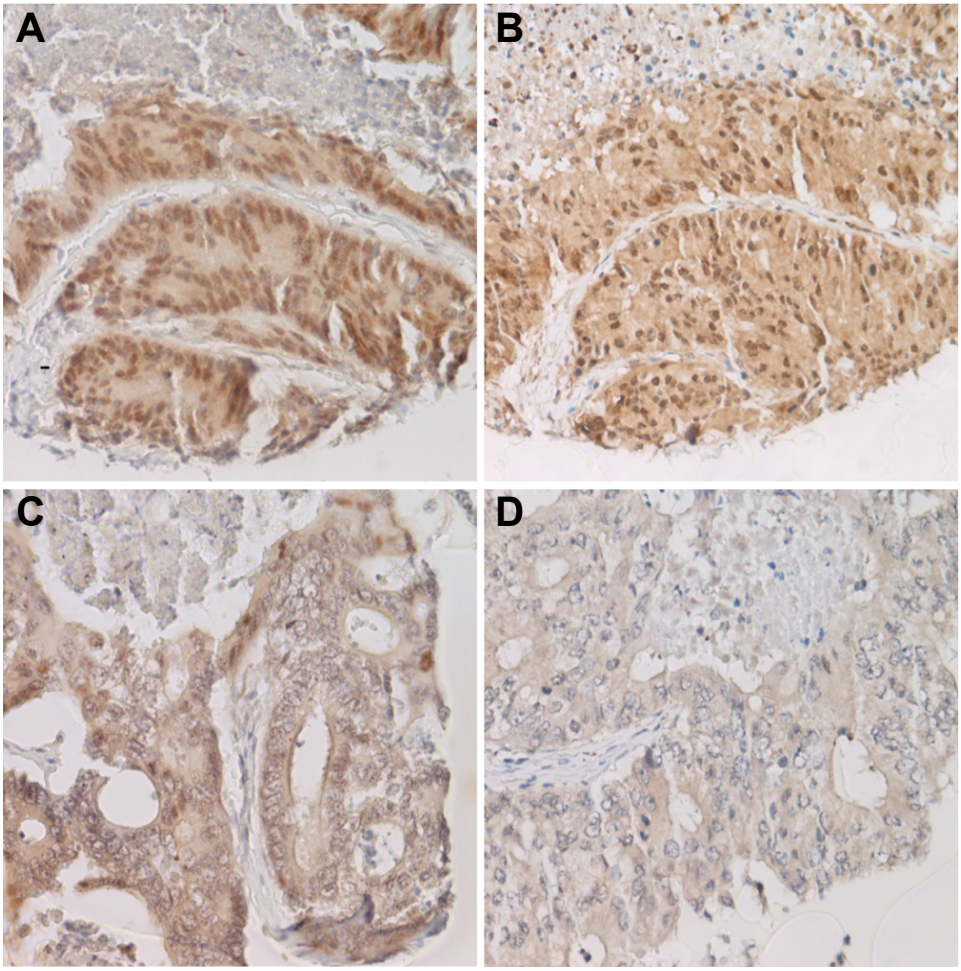

Figure 1. P-Smad2 and Smad4 expression in colon cancers. P-Smad2 expression was detected either in the nucleus (A), suggesting an active TGF-β signaling, or at the cytoplasm (B). Nuclear localization of P-Smad2 was generally dependent on Smad4 expression (C). Downregulation of Smad4 expression was characteristic for microsatellite stable colon cancers.

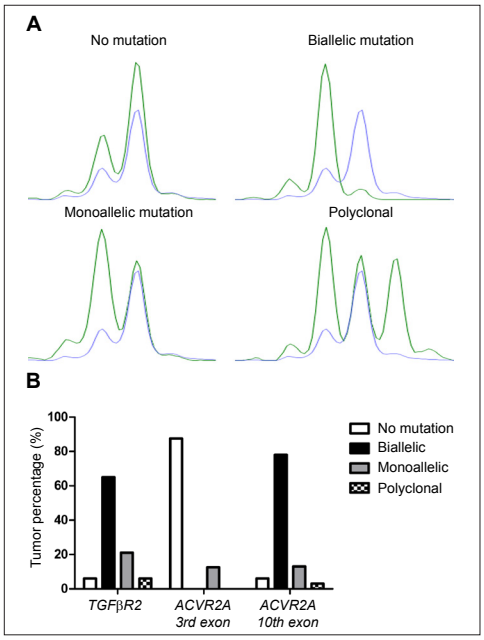

Figure 2. Mutations at *TGFBR2* and *ACVR2A* microsatellite repeats. Analysis of *TGFBR2* and *ACVR2A* microsatellites was performed by means of fragment analysis (A). Tumor alleles (green) were overlaid with their respective normal samples (blue). Four different allelic states were recognized after fragment analysis (B). Mutations in *TGFBR2* and the tenth exon of *ACVR2A* most often affected both alleles.

Table 1. *TGFBR2*'s microsatellite length in colorectal cancer cell lines.

Cells	Allele 1	Allele 2
LS411N	(A)8	(A)8
RKO	(A)8	(A)10
HCT-116	(A)9	(A)9
SW48	(A)9	(A)9
LS180	(A)9	(A)9
SW837	(A)10	(A)10

through another type 2 receptor we investigated the presence of mutations in the *ACVR2A* gene. *ACVR2A* possesses two 8 adenine ((A)8) microsatellite sequences in its third and tenth exons. Less than a third of MSI-H tumors still conserved a wild-type allele for *ACVR2A* (Figure 2B) and, similar to *TGFBR2*, no relation was found between the allelic state of *ACVR2A* mutations and P-Smad2 expression. In sum, the retention of wild type alleles in type 2 TGF-β receptor genes does not explain TGF-β activation in MSI-H colon cancers.

TGFBR2-mutated colorectal cancer cell lines remain sensitive to TGF-β stimulation

Several MSI-H colorectal cancer cell lines, with distinct mutation backgrounds for *TGFBR2* (Table 1), and one microsatellite stable cell line were tested for their ability to respond to TGF-β stimulation. This was achieved by transiently transfecting cells with a (CAGA)12-luciferase-reporter construct that contains binding sites for Smad3 and Smad4 and is effectively induced by cellular stimulation with TGF-β (33). Transcriptional reporter activity was greatly induced in LS180 and SW48 cells when stimulated with TGF-β, whereas it was only slightly induced in HCT116 and RKO cells (Figure 3A). LS411N cells remained insensitive to TGF-β stimulation (Figure 3A). The microsatellite stable cell line SW837, which is not known to carry mutations in components of the TGF-β pathway, demonstrated high sensitivity to TGF-β stimulation (Figure 3A). The two MSI-H cell lines in which TGF-β pathway stimulation was more pronounced (LS180 and SW48), possess a biallelic one-base pair deletion in *TGFBR2* (Table 1). Strikingly, TGF-β stimulation, perceived in the form of Smad2 phosphorylation at Ser465/467, could be suppressed selectively by targeting *TGFBR2* transcripts by short-hairpin RNA's in MSI-H colorectal cells, as demonstrated for the LS180 cell line (Figure 3B). Additionally, binding of a radio-labeled form of TGF-β to TGFβR2 could be detected in LS180 cells, although to a lesser extent than in RKO and SW837 cells, which possess at least one wild-type *TGFBR2* allele (Figure 4).

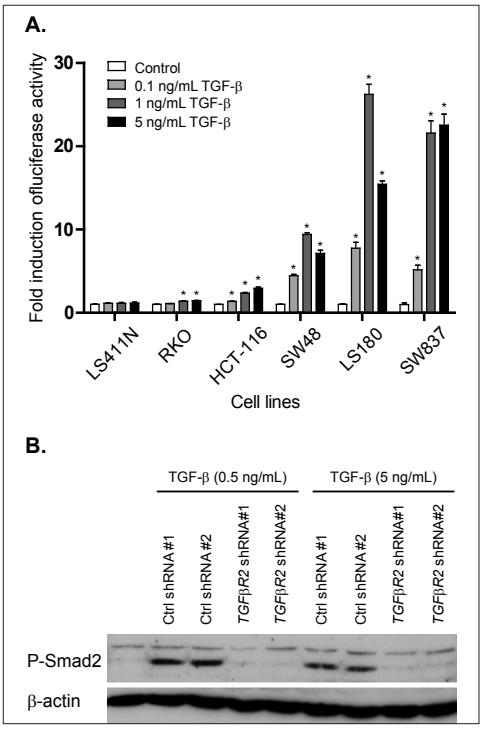

Figure 3. Induction of TGF-β pathway activity in a panel of colorectal cancer cell lines. TGF-β activation was possible in all colorectal cancer cell lines except LS411N (A), the only cell line carrying a biallelic two nucleotide deletion in *TGFBR2* microsatellite (Table 1). Means and standard deviations are depicted (* - $P < 0.05$, Bonferroni post test). Smad2 phosphorylation could be suppressed by employing shRNA's directed against *TGFBR2* transcripts, as demonstrated for LS180 (B), a cell line carrying biallelic mutations in that gene.

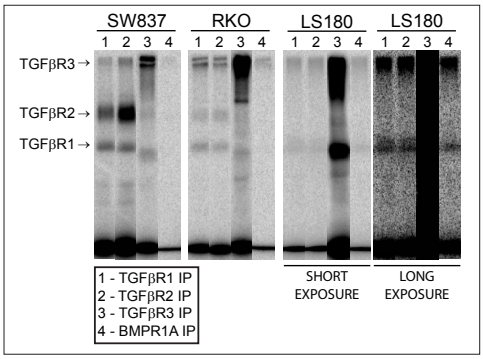

Figure 4. Cross-linking of TGF-β ligand to TGF-β receptors in colorectal cancer cells. Following stimulation of cells with a radio-labeled TGF-β ligand, cross-linking was induced an immunoprecipitations carried out with anti-TGFβR1, TGFβR2 and TGFβR3 antibodies. A clear binding of the TGF-β ligand to TGFβR2 and TGFβR1 (ALK5) could be observed for the microsatellite stable cell line SW837 and for RKO, a microsatellite unstable cell line with a monoallelic mutation in *TGFBR2*. Very weak binding to *TGFBR2* could be detected in LS180 cells. Immunoprecipitations with an anti-BMPR1A antibody were carried-out as negative controls.

TGF-β pathway activity in LS411 cells can be rescued by *TGFBR2* constructs carrying deletions in (A)10 microsatellite

LS411N was the only colorectal cancer cell line that was unresponsive to TGF-β stimulation in the current study. Curiously, this cell line was also the only to carry a homozygous two-nucleotide deletion in *TGFBR2*'s (A)10 microsatellite (Table 1). Of note, no MSI-H colon cancer was identified with biallelic two-nucleotide deletions in *TGFBR2*. We hypothesized that, depending on the type of mutation present in *TGFBR2*, TGF-β pathway activation was still possible without the presence of a wild type *TGFBR2* gene. We produced wild-type and *TGFBR2* mutant constructs with either one, two or three base-pair deletions in the (A)10 microsatellite (Figure 5A). Both (A)9 and (A)8 *TGFBR2* constructs lead to the production of frame-shifted, early truncated TGFβR2 proteins, while the (A)7 form originates an in-frame, one amino acid deletion. TGF-β pathway activity could be rescued by transfecting the (A)10 (wild-type), (A)9 and (A)7 but not (A)8 constructs into LS411N cells (Figure 5A). Notably, transfection of LS411N cells with the (A)9 *TGFBR2* construct rescued TGF-β response to similar levels achieved by the construct representing the wild-type *TGFBR2* form, as demonstrated by the (CAGA)12-luciferase-reporter assay (Figure 5A).

We considered that the slippage of RNA polymerases at *TGFBR2*'s (A)9 mutated sequence could generate in-frame transcripts derived from one nucleotide insertions at the RNA level. We also speculated that the (A)8 microsatellite present in LS411N cells is more stable and less prone to generate in-

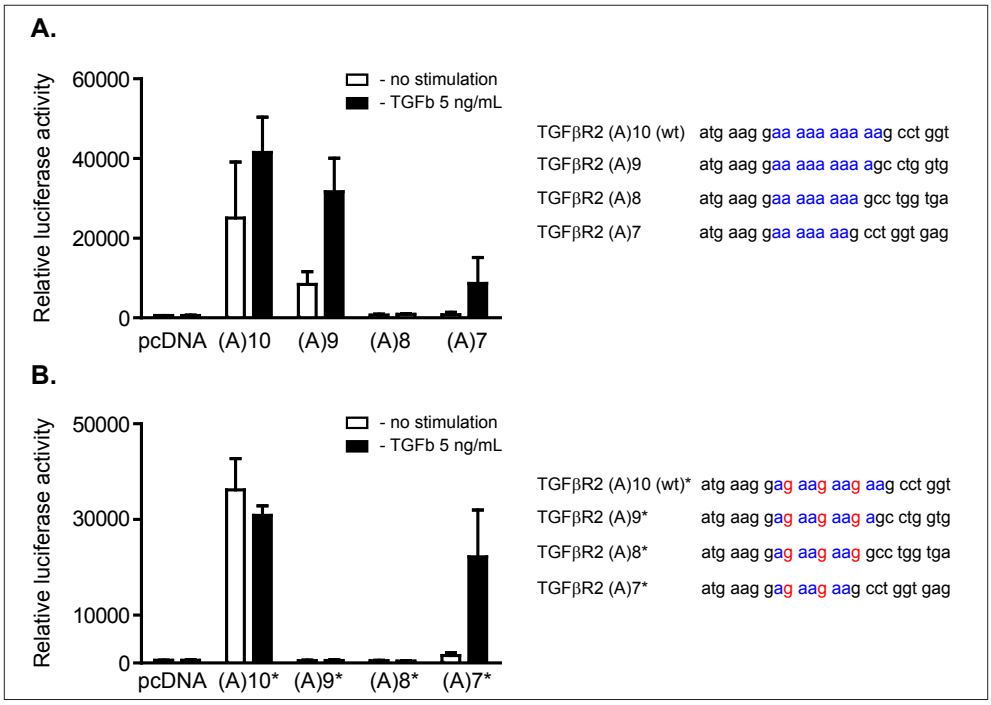

Figure 5. Rescue of TGF-b sensitivity in LS411N colorectal cancer cells. Constructs containing different lengths of the *TGFBR2*'s microsatellite were produced in order to test their ability to rescue TGF-β activity in LS411N cells, which were shown to be insensitive to TGF-β stimulation (A). Transfection with the *TGFBR2* constructs containing a microsatellite with 9 or 7 adenines rescued TGF-β sensitivity in these cells, while the *TGFBR2* construct containing 8 adenines failed to do so. Constructs containing synonymous substitutions at *TGFBR2*'s microsatellite were produced in order to remove the mononucleotide sequence pattern and consequently suppress genomic instability at this region (B). In contrast to what was previously observed, the (A)9 length construct carrying silent mutations at its microsatellite repeat failed to re-sensitize LS411N cells to TGF-β signaling.

frame transcripts. In order to demonstrate that the retained TGF-β sensitivity in *TGFBR2*-mutated cells was indeed due to transcriptional slippage, we produced *TGFBR2* constructs carrying synonymous mutations at the adenine stretches (Figure 5B), thereby abrogating the microsatellite sequence. After transfecting LS411N cells with *TGFBR2* constructs lacking a microsatellite sequence, TGF-β activation could not be rescued by the construct equivalent to the (A)9 microsatellite form (Figure 5B). These results support that *TGFBR2* mutants presenting a (A)9 microsatellite sequence most likely revert to the wild-type sequence at the transcriptional level.

DISCUSSION

Genetic inactivation of *TGFBR2* is a hallmark of MSI-H colorectal cancers (26, 27). By becoming insensitive to TGF-β, tumors cells are thought to acquire a selective advantage by circumventing its growth inhibitory signals (6, 7). Furthermore, TGF-β is particularly important during inflammatory processes, being involved in the recruitment of lymphocytes and inflammatory cells to damaged tissues (2). MSI-H colorectal cancers are notable for presenting a marked intraepithelial infiltration by activated CD8 T cells but also elevated counts of inflammatory cells at the tumor sites (34-37). Accordingly, immune escape phenotypes that include the

loss of HLA class I expression are frequent in MSI-H tumors (38-40). The abrogation of TGF-β signaling by MSI-H colorectal cancer cells could also constitute an immune escape mechanism in itself as leukocytes and macrophages produce this cytokine (41), (42). Conversely, while becoming insensitive to TGF-β, cancer cells may increase its production in order to modulate their microenvironment and cripple anti-tumor immune responses (43-45).

MSI-H colorectal cancers develop in the context of MMR deficiency that predisposes to the accumulation of nucleotide deletions and insertions at *TGFBR2*'s (A)10 microsatellite sequence. As expected, we found such alterations in the majority of MSI-H cancers analyzed and, most often, mutations affected both *TGFBR2* alleles. Paradoxically, TGF-β signaling was found active in several MSI-H tumors, indicating that the presence of frameshift, truncating mutations in *TGFBR2* did not relate to the activation status of this pathway. We also excluded that the retained sensitivity to TGF-β could be due to signal transduction through ACVR2A, an alternative type 2 receptor for activins and BMPs, as we found biallelic mutations in ACVR2A in the majority of MSI-H cancers. Strikingly, we demonstrated, in vitro, that TGF-β activation in MSI-H colorectal cancer cells was dependent on *TGFBR2* expression, despite the presence of biallelic inactivating mutations in this gene. Retained TGF-β sensitivity in MSI-H colorectal cancer cells that carried inactivating mutations in *TGFBR2* was previously reported by two independent groups but the precise mechanisms of TGF-β activation were not clarified (29, 30). We propose that the restoration of the reading-frame of *TGFBR2* mutants occurs by transcriptional slippage, where nucleotide insertions or deletions at the RNA level allow the production of in-frame transcripts. To our knowledge, this is the first report describing such mechanism in cancer, while the restoration of reading frames or frameshifting due to transcriptional slippage has been reported for several organisms (46-49). A similar observation to the one presented in the current report was made for a mutant human *apolipoprotein B* (*APOB*) allele that causes hypobetalipoproteinemia (50). This allele possesses a deletion of a single cytosine that creates a novel microsatellite of eight adenines that is susceptible to produce in-frame transcripts containing nine adenines. Furthermore, as demonstrated here for *TGFBR2*, the replacement of the *APOB*'s-mutant microsatellite, abolished the capacity of this allele to generate productive transcripts (51). We considered the amplification of cDNA from *TGFBR2* mutants to prove the presence of wild-type transcripts but we could not exclude that the presence of wild-type transcripts was derived from PCR amplification artifacts.

TGF-β signaling has for long been considered to be ablated in the majority of MSI-H tumors due to the high frequency of inactivating mutations in *TGFBR2*. With this work we demonstrate that MSI-H colorectal cancer cells still depend on TGFβR2 to respond to exogenous TGF-β signals despite the presence of biallelic inactivating mutations in its gene. Interestingly, a selective advantage might be in place for cells that conserve some responsiveness to TGF-β. The most frequent *TGFBR2* mutations in MSI-H colorectal cancer cells are one base pair deletions that result in a microsatellite sequence of nine adenines. Such sequence remains prone to instability and if no selection would be imposed on this mutation, one would expect that further DNA replication errors would result in tumor clones carrying eight adenines. The latter sequence is considerably more stable and produces few artifacts in vitro but does not confer sensitivity to TGF-β signaling as demonstrated here. It is tempting to speculate that MSI-H colorectal cancers are driven to preserve some responsiveness to TGF-β signaling, although down-modulated, that

could perhaps avoid its tumor suppressive effects while retaining its oncogenic properties.

METHODS

Patient material:

A tissue microarray (TMA) containing 76 formalin-fixed, paraffin embedded right-sided colon cancer samples was utilized for the immunohistochemical detection of Smad4 and of the phosphorylated form of Smad2 (P-Smad2). Thirty-two cases were classified as MSI-H, according to the recommendations of the National Cancer Institute/ICG-HNPCC (52). Those MSI-H cancers were further processed for cell sorting of tumor and stromal cells by flow cytometry. The study was approved by the Medical Ethical Committee of the LUMC (protocol P01-019). Patient samples were handled according to the medical ethical guidelines described in the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies.

Cell lines:

Six colorectal cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA): five MSI-H (HCT-116, LS180, LS411, RKO and SW48) and one microsatellite stable cell line (SW837). Cells were cultured in RPMI1640 medium supplemented with 10% Foetal Bovine Serum and 100 units/ml of Penicillin/Streptomycin (Gibco Invitrogen, Paisley, UK). 293T cells were used for the production of lentiviral particles.

Immunohistochemistry:

A standard two-step immunohistochemistry protocol was employed for the detection of P-Smad2 and Smad4 in the colon cancer TMA. Four micrometer tissue sections were cut from the TMA and transferred onto glass slides using a tape-transfer system (Instrumedics Inc., Madison, IL, USA) and dried overnight at 37oC. Following deparaffinization and rehydration, tissue sections underwent heat-mediated antigen retrieval in 10 mM citrate buffer solution (pH 6) for P-Smad2 or in 10 mM Tris, 1 mM EDTA solution (pH 9) for Smad4 staining. After cooling, endogenous peroxidase blockage was carried out with a 0.3% hydrogen peroxide/methanol solution. An additional blocking step with 10% normal goat serum (DAKO, Golstrup, Denmark) in phosphate buffered saline (PBS, pH 7.4) was performed. Thereafter, tissue sections were incubated over-night with the following primary antibodies: clone 138D4 (1:200, Cell Signaling Technology, Danvers MA, USA) which recognizes Smad2 only when phosphorylated at Ser 465/467 and clone B-8 (1:400, Santa Cruz Biotechnology, Santa Cruz, CA, USA) directed against Smad4, diluted in 1% bovine serum albumin/PBS solution. The following day, after washing, tissue sections were incubated for thirty minutes with Powervision Poly-HRP solution (Immunologic, Duiven, The Netherlands) and immunhistochemical staining was developed with DAB+ chromagen system (DAKO) for 5 minutes. Negative controls were generated by replacing the use of primary antibody with a 1% BSA/PBS solution. Three scoring categories were utilized: negative, weak, and strongly positive to which the numerical values of 0, 1 and 2 were assigned for statistical analysis. Scorings were always performed in comparison to an internal positive control provided by stromal cells and/or leukocytic infiltrate.

FACS-sorting:

A flow cytometric sorting procedure was employed to allow the detection of *TGFBR2* and *ACVR2A* mutations in separate fractions of tumor and stromal cells. Tissue preparation, staining and flow cytometry analysis was performed as described previously (53). Briefly, 2 mm diameter tissue punches from selected areas of 32 formalin-fixed, paraffin embedded MSI-H colon carcinomas were digested enzymatically in a mixture of 0.1% collagenase I-A (Sigma-Aldrich, St Louis, MO, USA) and 0.1% dispase (Gibco Invitrogen). After determination of cell concentration, one million cells were incubated with 100 µl of mAb mixture directed against keratin and vimentin containing clones MNF116 (anti-keratin; IgG1; DAKO), AE1/AE3 (anti-keratin; IgG1; Chemicon International Inc, Temecula, CA, USA), and V9-2b (anti-vimentin; IgG2b; Department of Pathology, LUMC (54)). Next day, cells were incubated with 100 µl of premixed FITC and RPE-labeled goat F(ab')2 anti-mouse subclass-specific secondary reagents (Southern Biotechnology Associates, Birmingham, AL, USA). After washing, cells were incubated with 10 µM propidium iodide (PI) and 0.1% DNase-free RNase (Sigma). The next day cells were analyzed by flow cytometry. A standard FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) was used for the simultaneous measurement of FITC, RPE, and PI. Tumor and normal cell populations were flow-sorted using a FACSVantage flow-sorter (BD Biosciences) using the FACSCalibur filter settings. DNA from flow-sorted material was isolated with protease K (0.5 mg/ml) (Roche Diagnostics, Basel, Switzerland) in PK-1 buffer solution (50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM MgCl2, 0.45% NP40, 0.45% Tween 20, and 0.1 mg/ml gelatin) with subsequent purification with the Wizard Genomic DNA Purification Kit according to manufacturer's instructions (Promega, Madison, WI, USA).

TGFBR2 and *ACVR2A* fragment analysis in flow-sorted tumor cells:

In order to detect instability in *TGFBR2* and

ACVR2A microsatellite sequences, primers around *TGFBR2*'s 3rd exon (fw-CCTCGCTTCCAATGAATC, rv-TCTGAGAAGATGATGTTGTC), *ACVR2A*'s 3rd exon (fw - GCTTATTTATAGGACTGATTGTG, rv - AATCTACAGTTGAGCAAACC) and *ACVR2A*'s 10th exon (fw - TTGAAAGTCAGGAGGATTTTAATG, rv - CTAACTGGATAACTTACAGCATG) were designed. Forward primers were 5' labeled with 6-HEX and a "linker" sequence (5'-GTTTCTT) was added to the reverse primer (55). PCR amplifications were carried out with 10 ng of DNA isolated from tumor or stromal flow-sorted fractions and 5 pmol of each primer in iQ Supermix (Bio-Rad, Hercules CA, USA). Thirty-three cycles of amplification with an annealing temperature of 60 degrees were performed. PCR products were diluted appropriately and mixed in a formamide solution containing GeneScan™ 500 ROX™ Size Standard (Applied Biosystems, Foster City, CA, USA). Thereafter samples were loaded in a 4-capillary 3130 DNA Analyzer and results interpreted with the aid of GeneMapper 4.1 software (Applied Biosystems).

TGF-β pathway reporter transfection and activity measurement:

Colorectal cancer cell lines were seeded in 24-well plates so that the density at the day of transfection was approximately 80%. The cells were transiently transfected with 700 ng of the CAGA-luciferase reporter construct and 300 ng of the CMV-LacZ expression plasmid. For TGFβR2 overexpression in the LS411N cells, 300 ng of the CAGA-luciferase reporter construct, 200 ng of CMV-LacZ and 10ng of *TGFBR2* construct was used. The cells were transfected using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. After 2 days, cells were serum starved for eight hours and stimulated for sixteen hours with TGF-β3. Lysis and determination of luciferase activity were carried out with the Luciferase Reporter Assay System according to manufacturer's instructions (Promega, Madison, WI, USA) using β-galactosidase activity as an internal control. The results are representative of at least three independent experiments, and reporter activity is shown as the mean and standard error of triplicates.

Generation of TGFβR2 stable knockdowns:

Two TGFβR2 shRNA clones (CCGGGCAGAACACTTCAGAGCAGTTCTCGAGAACTGCTCTGAAGTGTTCTGCTTTTTG and CCGGGCCTGGTGAGACTTTCTTCATCTCGAGATGAAGAAAGTCTCACCAGGCTTTTTG) and a control non-targeting shRNA (CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTTG) from MISSION shRNA library (Sigma-Aldrich) inserted in the pLKO.1-puro lentiviral vector were co-transfected together with VSV, GAG/POL and REV lentiviral packaging plasmids (kindly provided by Prof. Rob Hoeben, Leiden University Medical Centre) in 293T cells using PEI (MW 25.000 - Polysciences, Warrington, PA, USA). Forty-eight hours after transfection the virus particles were harvested and filtered. Colorectal cancer cell lines were seeded at a density of 250.000 cells per well in 12-well plates. The following day cells were transduced with the lentiviruses in a total volume of 1 mL of medium containing DEAE and approximately 625.000 IU of virus. Following overnight incubation the medium was refreshed and two days after transduction, selection was performed with 5 μg/mL of puromycin. From day 14, puromycin concentration was lowered to 2.5 μg/mL.

Detection of TGF-β ligand binding to type 1, 2 and 3 TGF-β receptors:

TGF-β ligand was iodinated according to the chloramine-T method (56). Affinity cross-linking and immunoprecipitation protocols were adapted from a previous work (57). Briefly, cells were cultured in 145 mm dishes to confluency and washed three times with a PBS solution containing 0.9 mM of CaCl2, 0.49 mM of MgCl2, and 0.1% BSA (PBS+/BSA). Iodinated ligand was added to the cells and incubated for three hours on ice in PBS+/BSA. Following two washes with PBS+/BSA and one with PBS+, crosslinking was induced in the presence of 0.27 μM of disuccinimidyl suberate (DSS) and 0.07 μM of bis (sulfosuccinimidyl) suberate (BS3) in PBS+ during 15 minutes on ice with constant shaking. The cross-linking was quenched with detachment buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% glycerol, and 0.3 mM phenylmethylsulfonyl fluoride (PMSF) and cells were scraped. Following removal of detachment buffer by centrifugation cells were lysed with a solubilization buffer containing 125 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% Triton X-100, 1mM PMSF, 2.5 μg/mL Aprotinin and 5 μg/mL Leupeptin for 30 minutes on ice. After centrifugation and transfer of the supernatants, the cell lysates were incubated 2.5 hours at 4oC with primary antibodies targeting different TGF-β receptors (produced at the Ludwig Institute, Uppsala, Sweden). Immunocomplexes were isolated by incubating samples with protein A sepharose beads for 45 minutes on a rotator. After washing beads four times in solubilization buffer the immunocomplexes were eluted in SDS sample buffer (125 mM TRIS, 10% SDS, 20% Glycerol, 10% 2-Mercaptoethanol and Bromophenol blue (20 μg/100 mL)), boiled for five minutes at 100oC and subjected to SDS-PAGE. Gels were dried and scanned with the STORM imaging system (Amersham Biosciences).

***TGFBR2* mutant construct production**

TGFBR2 constructs replicating the mutations observed in MSI-H colorectal cancers were produced and transfected into the TGF-β unresponsive LS411N cell line and into COS-1 cells. Constructs containing

a (A)9, (A)8 and (A)7 repeat, instead of the wild-type (A)10 repeat, were obtained by site-directed mutagenesis using the pcDNA3-Myc-*TGFBR2* plasmid as template. Additional constructs replicating the previous mutations at the amino acid level but removing the microsatellite repeat by substituting the GAA and AAA codons by synonymous GAG and AAG codons respectively were also produced. Clone purity was verified by Sanger sequencing and mutant fragments were inserted in the pcDNA3-Myc-*TGFBR2* plasmid by making use of the BamH I and Pml I restriction sites.

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

6

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).

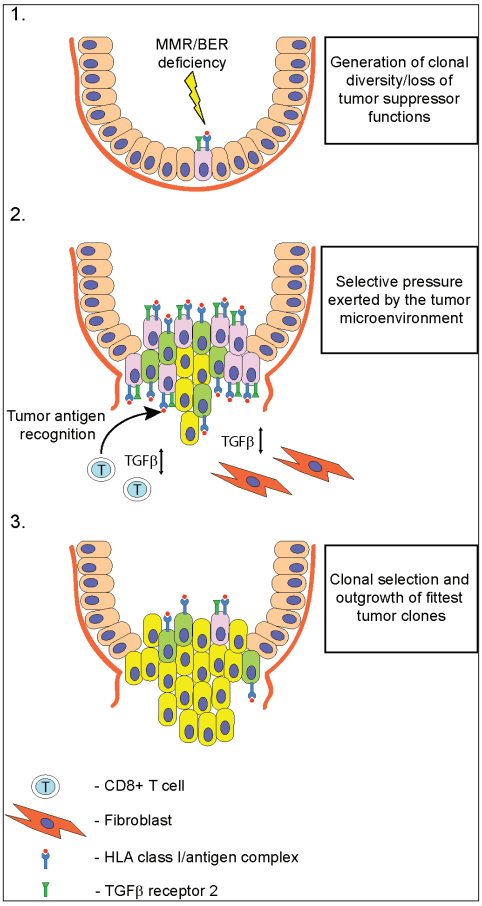

Figure 1. Diagram representing the clonal evolution occurring during MSI-H and MAP colorectal tumourigenesis. Clonal diversity is provided by the inactivation of the MMR and BER systems (1). A genetically heterogeneous population of tumour cells is then subjected to selective pressure, exerted by the 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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Concluding remarks and future perspectives

7

Chapter 7

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

MSI-H and MAP cancers are now recognized as well defined entities within the spectrum of colorectal malignancies. The high prevalence of immune escape mechanisms observed in those, particularly the widespread loss of HLA class I expression, conceals important clinical implications. Defects in the HLA class I/ antigen presenting pathway will most likely frustrate immunotherapeutic interventions that require T cell recognition of tumor antigens. Although it has been shown that HLA class I expression can be induced in cancer cells (1), this is unlikely to occur when genetic defects such as mutations in the β2m molecule are present. Nevertheless, passive immunotherapeutic approaches based on the production of antibodies against tumor antigens remain valid (2).

The outgrowth of tumor cell clones with HLA class I defects is secondary to the elimination of cancer cells that effectively presented HLA class I/antigen complexes to T cells. Thus, theoretically, the complete eradication of tumors might occur in the presence of a robust immune reaction at early stages of malignancy. Two working hypothesis can be derived from this proposition:

1. Prophylactic approaches based on the vaccination of individuals with common tumor antigens may be developed, especially in the context of cancer syndromes such as Lynch or MAP. These could elicit potent secondary anti-tumor immune responses shortly after tumor initiation and prior to the generation of immune escape phenotypes. In support of this, Schwitalle and colleagues have detected T cell-mediated responses in healthy Lynch syndrome carriers against mutated tumor-antigens that are commonly generated in MSI-H colorectal cancers (3). Such observation suggests a previous interaction between the host immune system and malignant cells in spite of the lack of a cancer diagnosis. Of note, the generation of mutated antigens in tumor cells must occur before the loss of HLA class I expression for the successfulness of this approach. Therefore, the elucidation of the sequential ordering of oncogenic events in Lynch or MAP cancers is of great importance.

2. Considering the specificity exhibited by different HLA class I molecules to different peptide motifs, one expects that certain alleles are more efficient than others in presenting certain tumor antigens. Several logical inferences can be derived from this premise: a) In a carrier of HLA class I alleles with poor tumor antigen presentation capability, the immune system might be more permissive to the development of MSI-H and MAP tumors; b) In the same context, tumors can conserve HLA class I expression due to the lack of selective pressure against HLA class I positive tumors; c) conversely, the immune system of carriers of HLA class I alleles that effectively present tumor antigens might be less tolerant to tumor development; d) accordingly, HLA class I-related immune escape phenotypes would be more likely in the latter. To establish whether HLA class I genotypes influence the risk for colorectal cancer development, particularly for MSI-H and MAP cancers, comprehensive large-scale association studies, focused on HLA class I, are required. The incomplete penetrance observed in Lynch syndrome (4) could also be related to the capacity of the host's immune system to deal with tumor cells.

It is now well recognized that the presence of a conspicuous adaptive immune response can influence tumor development and clinical outcomes. The infiltration of tumor tissues by CD8+ cytotoxic T cells (CTLs) has been consistently correlated with improved patient prognosis in a variety of cancers, including colorectal cancer (5, 6). In this

thesis, we present a similar observation made in Lynch colorectal cancers where higher densities of CTLs were found in early-staged tumors. Remarkably, the presence of an elusive immune cell population (CD45+/CD3-/CD56-/GZMB+) was highly predictive of a tumor HLA class I negative phenotype and of the absence of lymph node metastases. Unfortunately, despite the employment of numerous markers, we did not succeed in gaining further insight into the phenotype of these cells. The fact the latter were almost exclusively found in HLA class I negative tumors and that they might be involved in counteracting metastatic processes suggests a mode of action similar to the one of NK cells. Their characterization might open new possibilities for the management of tumors that have lost HLA class I expression. Our findings demand for additional investigation in MSI-H sporadic and MAP colorectal cancers, as well as in other cancer types, to establish the role of these cells in tumor progression. The improved clinical prognosis observed for MSI-H colorectal patients is likely to be associated with the immunogenic character of their tumors. Although the capacity of MSI-H and MAP cancers to evade immune responses is well demonstrated by their HLA class I phenotypes, this form of "local adaptation" might not be optimal once tumor cells enter circulation and encounter other components of the immune system. Nevertheless, a considerable proportion of MAP and MSI-H tumors do metastasize and the complexity of tumor/immune cell interaction transcends HLA class I-dependent immune responses.

The mismatch repair deficiency observed in MSI-H cancers determines that a number of gene targets are consistently mutated in these tumors. Until now, it was believed that the occurrence of microsatellite instability within coding genetic sequences led to abrogation of expression of their respective proteins. We discovered that frameshift, truncating mutations in *TGFBR2* gene did not hinder the generation of functional, in-frame proteins. We hypothesized that the reversal of the *TGFBR2* mutated sequence to a wild type form may occur at the transcriptional level, by similar mechanisms that degenerate in DNA microsatellite instability. A similar observation was previously made for a mutant human *apolipoprotein B* (*APOB*) allele that causes hypobetalipoproteinemia (7). Although colorectal cancer cells can theoretically mutate *TGFBR2* to a form that is not prone to transcriptional instability, a positive selection appears to be in place for a genotype that permits the production of functional TGFβR2. This might reflect some TGF-β dependence of tumor cells that could be exploited from a therapeutic perspective. With the current knowledge in hand, the effects of TGF-β activation and inhibition in *TGFBR2*-mutated cells should be analyzed in depth. Additionally, the "leakiness" of frameshift mutations in other mutation targets should also be investigated in MSI-H cancers.

The reversal of mutated microsatellite sequences to wild-type sequences at the transcriptional level suggests that the generation of "frameshifted" transcripts from wild-type DNA sequences also occurs. This raises a fundamental question that transcends the context of cancer research: why were microsatellite sequences located in coding DNA sequences conserved during evolution? The generation of more stable, synonymous sequences could easily be achieved by the random substitution of the last nucleotide of a codon that composes a microsatellite. Curiously, for *TGFBR2*, the opposite is observed by comparative genomics: only few primates, including humans, display a microsatellite sequence of 10 adenines in *TGFBR2* while other mammals have an 8 or 7 adenines microsatellite, resulting in a considerably more stable sequence. In bacteria, it has been established that the conservation of these homopolymeric tracts is positively selected during evolution.

However, those are normally located in frameshifted genes that generate in-frame sequences during transcription (8). The role of microsatellite sequences located in genes that are already in-frame is less clear, but might relate to intrinsic gene regulation mechanisms that impede the generation of elevated amounts of functional transcripts in a particular context.

Mismatch and base excision repair deficient colorectal cancers constitute fascinating models of cancer evolution. Their intrinsic DNA repair defects provide a source of genetic diversity that, in the case of MSI-H cancers, dispenses major chromosomal alterations. Their mutagenic character, however, comes with drawbacks: their extensive mutation landscape implicates an abundance of tumor antigens that elicit anti-tumor immune responses. The latter actively participate in the process of tumor clonal selection that favors the outgrowth of cancer clones with immune evasive phenotypes. The important role of the immune system in shaping the evolution of MSI-H and MAP colorectal cancers should not be ignored and could be explored for the development of prophylactic and therapeutic approaches.

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Chapter 8

SUMMARY

The works presented in this thesis have focused on the study of specific subtypes of colorectal cancer that arise in a context of deficiency of one of the following DNA repair systems: mismatch repair or base excision repair. Colorectal cancer is one of the most common and deadliest cancers in the world. It is a heterogeneous disease that comprises a range of malignancies that develop according to various genetic/oncogenic pathways. DNA mismatch repair deficient cancers comprise up to 20% of all colorectal cancers and are characterized for exhibiting a microsatellite instability-high (MSI-H) phenotype. The latter refers to the widespread accumulation of small insertions and deletions at microsatellite sequences, throughout the genome. MSI-H colorectal cancers can develop sporadically or due to hereditary mutations in one of the mismatch repair genes (Lynch syndrome). Base excision repair deficient colorectal cancers are caused by hereditary mutations in the *MUTYH* gene. Carriers of biallelic mutations in *MUTYH* are at increased risk of developing colorectal polyps and cancer and this syndrome is referred to as MUTYH-associated polyposis (MAP). MAP cancers constitute less than 1% of all diagnosed colorectal cancers.

MSI-H colorectal cancers often carry more mutations in their coding genome than the remaining subtypes of colorectal cancer, a consequence of their constitutive DNA repair deficiency. As mutations eventually translate to the protein level, abnormal proteins are generated that can be recognized by the immune system. Effector immune cells, such as cytotoxic CD8+ T cells, have the capacity of eliminating cancer cells that present such proteins and, therefore, tumors must adopt mechanisms that allow them to escape destruction. Among those mechanisms, one of the most important is the loss of expression of HLA class I, the molecule responsible for presenting endogenous peptides to cytotoxic CD8+ T cells. Although the loss of HLA class I expression was previously described as a common event during colorectal carcinogenesis, we and others hypothesized that it was more likely to occur in MSI-H tumors due to their hypermutated genomes. Additionally, MSI-H cancers are notable for presenting a conspicuous immune cell infiltrate in their tissues which reflects a strong anti-tumor immune reaction. Accordingly, in **chapter 2**, we describe that HLA class I alterations are clearly more frequent in MSI-H tumors than in the remaining spectrum of colorectal malignancies.

Loss of expression of HLA class I can occur through a number of mechanisms that include mutations in the HLA class I genes themselves, mutations in the gene encoding for the β chain (β2m), and mutations in molecules that are involved in the processing of the proteins to be loaded on HLA class I, denominated by antigen processing machinery. Also in the work included in **chapter 2** we discovered that HLA class I loss was more likely to be associated with deficiencies in members of the antigen processing machinery in sporadic MSI-H cancers than in their hereditary counterpart. Conversely, MSI-H tumors from Lynch syndrome patients were most often affected by deficiencies in the β2m molecule. It is known that deficiencies in β2m or in antigen processing machinery members have different consequences but the reason why sporadic and hereditary MSI-H cancers target one or the other are still elusive.

Although it was not yet established whether MAP colorectal cancers carry a higher number of mutations than non-MSI-H colorectal tumors, we considered it to be a logical assumption due to their background of base excision repair deficiency. MAP cancers also present evidence for an anti-tumor immune reaction which suggests that, similar to MSI-H cancers, these tumors are also strongly immunogenic. In such case, a strong selective pressure for adaptation mechanisms that allow them to escape immune surveillance would also be in

place. In the work presented in **chapter 3**, we describe that HLA class I loss of expression is indeed a frequent event in MAP colorectal cancers. Interestingly, and as observed for Lynch syndrome cancers, loss of HLA class I expression was most often associated with β2m defects, which appears to be a common mechanism in MAP and Lynch colorectal syndromes. With this work we completed the characterization of HLA class I phenotypes in colorectal cancers that arise in a context of DNA repair deficiency. We concluded that such deficiency is likely to be responsible for the generation of a surplus of mutated proteins in tumors, which in turn activates an immune response that favors the outgrowth of tumor clones with immune escape phenotypes.

Assuming that the quality and intensity of an anti-tumor immune response influences the HLA class I phenotype of tumors, we investigated whether this relation could be observed in Lynch colorectal cancers, in **chapter 4**. Indeed we found that cancers carrying HLA class I defects presented higher amounts of tumor-infiltrating lymphocytes, particularly CD4+ and CD8+ T cells. This observation provides a link between the agents of selective pressure (immune cells) and the positively selected tumor traits (loss of HLA class I expression). Furthermore, the magnitude of lymphocytic infiltration not only correlated with HLA class I phenotypes but also with the tumor stage at diagnosis. Infiltration by activated CD8+ T cells (cytotoxic T cells) was higher in tumors diagnosed at early stages than in advanced cancers, supporting the anti-tumorigenic role of these cells. An elusive immune cell type that expressed granzyme B, thus with cytotoxic potential, was almost exclusively present in HLA class I negative tumors that lacked metastases. These characteristics were reminiscent of NK cells but further investigation with a range of discriminative immune cell markers was inconclusive. The identification of these cells could be of great importance of the management of cancers that have lost HLA class I expression.

In **chapter 5**, we present a study on the most frequently mutated gene in MSI-H cancers, *TGFBR2*. Until now, mutations in *TGFBR2* were considered to result in loss of protein function due to the frameshift, truncating nature of the mutations found. We discovered that cells carrying biallelic, frameshift mutations in *TGFBR2* were still capable of transducing TGF-β signaling in a TGFβR2-dependent manner. We proposed that the same mechanism that leads to the generation of mutations at the DNA level is probably responsible for the restoration of *TGFBR2*'s reading frame at the transcriptional level. Thus, frameshift mutations located at microsatellite sequences have the potential to be reversed to a wild type form at the RNA level. Interestingly, we discovered that MSI-H cancers were apparently selected to retain some sensitivity to TGF-β since the occurrence of biallelic mutations that excluded the possibility of reading frame restoration was rare. We suggest that the contribution of this pathway for the tumorigenesis of MSI-H tumors should be reanalyzed in the light of these findings.

In **chapter 6**, we review our findings on MSI-H and MAP tumors in the light of others' findings and propose that the oncogenic processes in these cancers are particularly dependent on the interaction between the tumor cells and their microenvironment. In **chapter 7**, some final considerations are made and suggestions for continuing research.

NEDERLANDSE SAMENVATTING

In dit proefschrift werden twee subtypes van kanker in de dikke darm bestudeerd, die ontstaan in de context van twee DNA reparatie defecten, te weten een DNA mismatch reparatie deficientie en een DNA base excisie reparatie defect. Dikke darm kanker is een veel voorkomende en een van de dodelijkste ziekten in de wereld. Hoewel de naam eenduidig lijkt, is dikke darm kanker genetisch een heterogene ziekte. Twintig procent van alle dikke darm tumoren tonen een DNA mismatch reparatie deficiente. Ten gevolge van deze deficientie worden veel voorkomende secundaire fouten (mutaties) in het DNA, die vooral ontstaan tijdens de celdeling, niet meer goed herkend en hersteld. De aanwezigheid van de secundair ontstane mutaties in zich herhalende DNA patronen kunnen worden getest middels een "microsatelliet instabiliteits" analyse, waarbij een "MSI-High" fenotype een aanduiding is van een DNA mismatch reparatie defect. MSI-H kan sporadisch ontstaan of gevonden worden in de context van erfelijke mutaties in een van de mismatch reparatie genen, passend bij het Lynch syndroom. Mensen die zowel van hun moeder als vader een mild defect in het DNA base excisie reparatie gen *MUTYH* hebben georven, ontwikkelen meestal veel poliepen in de dikke darm en hebben dien ten gevolge een verhoogde kans op dikke darm kanker. Dikke darm kanker in de context van deze MUTYH geassocieerde polyposis (MAP) betreft minder dan 1% van alle gediagnosticeerde dikke darm kankers.

Bij microscopisch onderzoek van DNA mismatch en in mindere mate DNA base reparatie deficientie wordt vaak een opvallende influx van tumor infiltrerende lymfocyten (TIL's) gezien. Dit kan worden verklaard door een verhoogde respons van het immuunsysteem (de afweer) op gemuteerde tumor eiwit fragmenten, die ontstonden ten gevolge van de secundair ontstane DNA mutaties. De afweer reactie zou zelfs de tumor kunnen opruimen, waardoor de tumor mechanismen moet ontwikkelen om deze afweer te ontlopen. Inderdaad toonden met name de genoemde DNA reparatie deficiente tumoren een verhoogde mate van afschakeling van HLA (humane leucocyten antigeen) klasse I moleculen. HLA moleculen zijn betrokken bij het presenteren van lichaamseigen en lichaamsvreemde eiwitdelen (waaronder tumoreiwitdelen) aan de afweer. We toonden aan dat het verlies van HLA klasse I in dikke darm tumoren van Lynch en MAP patienten ontstaat middels mutaties in het beta2microglobuline gen. Daarentegen de niet erfelijke vorm van DNA reparatie deficient dikke darmkanker weet hetzelfde te bereiken door afschakeling van componenten van de antigeen presenterende machinerie (APM). Waarom er zo'n duidelijke scheiding binnen de subgroepen is onduidelijk, doch intrigerend.

Vervolgens werd in een relatief grote subgroep van Lynch syndroom dikke darm kankers onderzocht of er een relatie bestaat tussen de aanwezigheid en de hoeveelheid TIL's, hun subtypering en het HLA expressie verlies. Tevens werd de relatie onderzocht met het stadium van de ziekte. In tumoren met HLA klasse I verlies werden grotere hoeveelheden geactiveerde TIL's gevonden, alsof deze tumoren onder selectieve druk stonden tumor subklonen te genereren die de afweer reactie konden ontlopen. Een grotere mate van TIL's werd gevonden in vroegere vergeleken met latere tumor stadia, hetgeen een anti-tumorigene rol van deze TIL's doet vermoeden. Vervolgens werd ook een geactiveerde immuun cel gevonden met kenmerken van een zogenaamde "natural killer" cel. Uitgebreide marker analyse van deze cel leidde niet tot preciese karakterisering van dit celtype. De aanwezigheid van deze geactiveerde immuuncel bleek te correleren met de afwezigheid van tumor uitzaaiingen.

Daarnaast werd het meest frequent gemuteerde gen in MSI-H tumoren,TGF-β, bestudeerd. Tot nu toe werd aangenomen dat mutaties in dat gen resulteerden in het verlies van

eiwitfunctie door de frameshift mutaties die gevonden werden. Er werd ondekt dat cellen met een biallelische frameshift mutatie in *TGFBR2* nog in staat zijn om TGF-β signalen door te geven. Er werd verondersteld dat hetzelfde mechanisme dat leidt tot DNA mutaties ook verantwoordelijk is voor het herstel van veranderingen in *TGFBR2* op transcriptieniveau. Het interessante is dat MSI-H tumoren nog enige mate van TGF-β gevoeligheid bleken te hebben gezien het voorkomen van bialelische mutaties zonder frameshift reparatiemechanisme zeldzaam zijn. In het licht van de bovengenoemde bevindingen zal het heersende beeld over de rol van het TGF-β signaalroute in de tumorigenese van MSI-H tumoren herzien moeten worden.

Voorts werden de bevindingen aangaande MSI-H en MAP tumoren in het licht van andere onderzoeksresultaten geplaatst en werd voorgesteld dat de oncogene processen in deze tumoren voornamelijk afhankelijk zijn van de interactie van de tumor cellen met hun micromilieu. Tenslotte werd een aantal sugesties gedaan tot het voortzetten van dit onderzoek.

CURRICULUM VITAE

Noel Filipe da Cunha Carvalho de Miranda was born on June 20th of 1982 in Póvoa de Varzim, Portugal. In 1999 he obtained his high-school diploma at Escola Secundária Rocha Peixoto in Póvoa de Varzim. In the same year he was admitted in the University of Évora where he studied Biology for one year. The following year he transferred his studies to the University of Minho, in Braga, where he completed his degree in Applied Biology in 2004. As part of the latter he performed a six-month internship at the Leiden University Medical Centre under the supervision of Dr. J.W. Dierssen, Prof.dr. C.J. Cornelisse and Prof.dr. J. Morreau. From October 2004 to October 2005 he carried out a one-year research project under the supervision of Prof.dr. J. Morreau after which he initiated his PhD studies. Since 2011 he is a researcher at Prof. Qiang Pan-Hammarström's laboratory at the Division of Clinical Immunology of Karolinska Institutet, Stockholm, Sweden.

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