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Molecular pathology of mismatch repair deficient tumours with emphasis on immune escape mechanisms

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Molecular pathology of mismatch repair deficient tumours with emphasis on immune escape mechanisms

Jan Willem Frederik Dierssen

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General Introduction

Chapter 1

The accumulation of genetic defects is a hallmark of cancer, and its detailed characterization has illuminated potential therapeutic targets and will keep on providing those. Yet, the identification of distinct colon tumour entities makes it necessary to adjust therapeutic strategies. One promising therapeutic strategy is the employment of the adaptive immune system in eradicating host tumour cells. However, its feasibility in eradicating human cancer is not well known as tumour escape mechanisms have not been studied in detail. In this chapter, current concepts of colon tumour development and tumour immunology are discussed and an outline of this thesis is given.

1. COLON TUMOUR DEVELOPMENT

1.1. A roadmap of cancer

Despite the huge heterogeneity of cancer, even between tumours from the same histological subtype, it has been proposed that there are only six general features any cell needs to acquire in order to become a cancer cell [1]. Like a roadmap that includes inevitable roadblocks. These features or roadblocks are: insensitivity to anti-growth signals, self-sufficiency in growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

The roadmap does go with a manual. There are many different ways in disrupting cell signalling pathways in order to get to the same point; some are shortcuts, others require multiple steps just to cross one roadblock (see figure 1). Which steps may be taken will be explained in paragraph 1.2. And there is no necessary order of the roadblocks to be taken. The final course is tumour specific and probably depends on the cell's constitutive biology as well as its micro-environment. For details, see also paragraph 1.3.

For colorectal cancer, one possible route which partly contains the roadblocks mentioned above has already been mapped (see figure 1) [2]. This route parallels the development of distinct histological features of the majority of colon tumours: the progression from normal epithelium - to aberrant crypt foci - to polyps (early, intermediate and late adenoma) - to carcinoma. That unique feature makes colon cancer nearly an *in vivo* cancer model of its own, which is probably why the route of colon cancer has been studied so well.

Many of the roadblocks were originally identified by *in vitro* cell culture experiments and animal models. Meanwhile most have been confirmed in human tumour tissues. However, the roadmap still has its blind spots, for instance on micro-environment interactions, and future research likely will identify other roadblocks to be cleared by developing tumours including those of the colon. In this thesis we focus on the need for tumour cells to evade recognition and destruction by the immune system.

1.2. Genetic instability and clonal selection: how to cross roadblocks?

Tumours may be described as accumulations of cells exhibiting acquired disruptions of cell signalling pathways leading to loss of (normal) growth control. Both qualitative as well as quantitative changes in the protein components may account for the compromised functionality of signal transduction pathways in cancer cells. These changes may result from alterations at the genomic, transcriptional, translational or post-translational level. We will focus on those at the genomic and transcriptional level.

Genomic alterations comprise various types of irreversible changes in the content and organization of the genetic information of cells. For instance numerical and structural chromosome

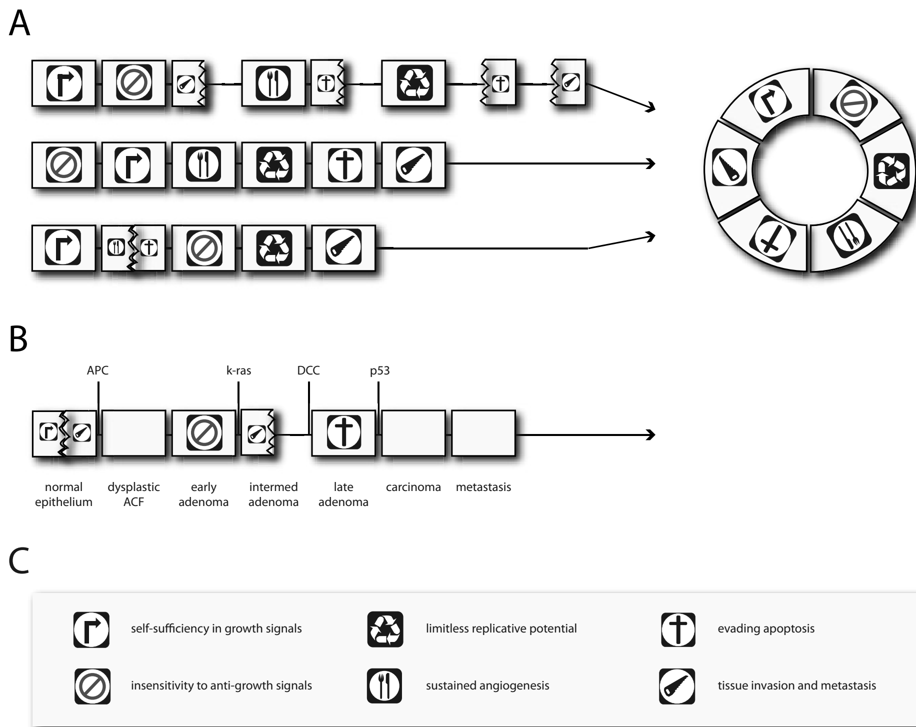

Figure 1. The roadmap to colon cancer.
The six features of cancer cell biology shown in C, need to be acquired. However, the order of acquisition may differ between different tumours, as illustrated in A. The seven-step model of colorectal cancer nicely fits to this model, as shown in B. Adapted from [1] and [2].

alterations have been describes almost a century ago. Only much later alterations at the single gene level were identified and genes altered in cancer were classified in two main categories: oncogenes and tumour suppressor genes [3, 4].

Distinct types of genetic alterations have been identified in cancers. Normally, these alterations would activate specific error detection systems that would either repair them or, if unsuccessful, arrest further cell cycle progression. Classically, they can be divided into 4 categories: subtle sequence changes, alterations in chromosome or chromosome fragment number, chromosome translocations, and gene amplification [5]. In colon cancer, the former two have been most frequently studied. Put simply, genetic instability in colon cancer may manifest itself as either subtle single gene changes or as large chromosomal aberrations. Grossly, the former case has been associated with microsatellite instability (MIN), the latter case is also denoted as chromosomal instability (CIN). MIN tumour cell DNA content usually is conserved in the peri-diploid state, whereas in CIN tumours, tumour cells' DNA content is altered leading to a state of aneuploidy [2]. Interestingly, in colon cancer, these two manifestations seem to (at least partly) exclude each other.

The level of gene transcription is influenced by quantity of transcription factors and micro RNAs, or by altered DNA accessibility for these. Accessibility is determined by the state of DNA

hyper- and hypomethylation, and histone modifications [6]. The epigenetic state can be passed on to daughter cells. The epigenetic state can be passed on to daughter cells and the accumulation of both genetic and epigenetic changes is a typical characteristic of tumour development.

As cancer is not the plan of an evil genius but rather the unfortunate outcome of chance events cells do not have a will of their own, nor do tumours wish to become destroyers of their host; they are simply the survivors of a Darwinian micro-evolution [7]. The acquisition of necessary features therefore are the result of a multistep progression process, in which – like in macro-evolution –mutations may provide a selective growth advantage leading to clonal expansion of daughter cells and to clonal divergence. Furthermore, a tumour comprises in fact a collection of heterogeneous cell populations, but may in the long run be overgrown by a population that is best adapted to its micro-environment [8].

Is has been a debate for decades whether genetic instability is the cause or effect of neoplastic transformation, and the same discussion may be repeated for epigenetic mechanisms [9]. Do tumours need increased mutability as a driving force, or is it merely an accumulation of normally occurring errors derived by clonal expansion? Is genetic instability a state or rate? Mathematical models showed that the number of mutations found in tumours is too great to be explained by basal mutation rate [10]. However these models did not take into account the effect of clonal selection and expansion, nor the fact that cell turnover exceeds tumour growth. Such models show that an increased mutation rate may merely be a side effect, not a necessity [11]. It has also been argued that the level of aneuploidy itself contributes to malignant transformation. By increasing the expression of thousands of genes at once, this might lead to the necessary qualitative changes of cell physiology and metabolism [12]. However, as mentioned above, not all neoplastic cells are aneuploid. Finally, it is stated that increasing genetic instability would be detrimental for a tumour, as the chances of deleterious mutations would exceed the chance of growth advantage. Yet, evolutionary models have shown exactly the opposite. Enlarging its diversity a cell population increases its chance of surviving micro-environmental changes and genetic instability achieves just that [13]. Besides, clones giving birth to non viable progenitors will be overgrown by more successful clones. Darwinian selection results automatically in a 'just right' rate of instability [14]. So as the state or rate question remains, we conclude that tumours have two 'vehicles' available during their roadtrip, genetic instability and clonal expansion, and they probably use both.

1.3. Tumour development and metastasis: which order of appearance?

Clinically, metastases are considered the end stage of cancer. Dissemination increases the tumour chances to survive medical therapy annihilation and therefore commonly causes cancer patients' death. However as discussed in paragraph 1.2, tumours can not anticipate to a changing environment. Therefore metastasis should be considered as a side effect of the micro-evolutionary process by which tumour cells acquire adaptations to the local micro-environment and vice versa. So what determines metastatic progression and when is it acquired? Different theories exist, which we will discuss hereinafter.

- The progression model. Ever since Nowell's theory of step-wise evolution, this classical model foresees a step by step progression driven by clonal expansion of random mutants, which was elegantly implemented

in the multi-step model of colon cancer (see paragraph 1.1), where metastasis confines the last step [2, 7]. Considering tumour heterogeneity, the subpopulation with metastatic capacity would actually not need to contain the majority of tumour cells; in fact, one may expect it not to be so, since the cells tends to disseminate and the acquisition of metastatic prone mutations would not likely lead to growth advantages at the primary site.

- Cancer stem cells. Tumours comprise heterogeneous cell populations and may contain a subpopulation of stem cells that - in contrast to other populations - has unlimited replicative potential. Only these cells would be capable of forming new tumours, even at distant locations, as they can self-renew infinitely [15].
- Alternative pathways. As addressed in paragraph 1.1, the roads toward the acquisition of proliferative benefits are many. Thus, there are multiple alternative genetic paths that lead to tumour formation [16]. According to this model, some combinations of mutations yield a high tendency to metastasize, whereas other combinations would not. This implies that metastatic potential may already be defined early during tumorigenesis and does not result from mutations or epigenetic changes involving specific genes.
- Genetic predisposition model. This model pleads for the contribution of the allelic composition of the host genome [17, 18]. Subtle changes in gene functions, already present before tumour formation, may determine the metastatic potential. Interestingly, this may also predict that the micro-environment, both at primary and distant sites, fills a principal part in tumour development.

We may conclude that we still have insufficient insight in the factors driving the metastatic process. This is illustrated by the fact that despite huge efforts so far only few 'metastasis' genes have been identified. Furthermore, it remains puzzling that disseminating tumour cells can be detected even at early stages of tumour development which does not necessarily predict distant metastasis [15, 19, 20]. Finally, it is remarkable that metastatic potential may be predicted by the gene expression profile of bulk tumour tissue including multiple heterogeneous tumour subsets and a significant share of tumour stroma [21, 22].

2. MULTIPLE COLON CANCER SYNDROMES AND ROADMAPS

The identification of hereditary cancer syndromes has contributed largely to the identification of tumour suppressor genes. Based on Knudson's two hit model, carriers of heterozygous germline mutations have such a raised risk of developing cancer that it is inherited in an autosomal dominant way [23]. Another hereditary colon cancer syndrome that has been identified, the *MUTYH*-associated polyposis, is inherited in an autosomal recessive way. Interestingly, genetic and genealogical studies have revealed that the increased risk only applies for specific tissues, or even specific tumour types. This issue remains a puzzle. The syndromes associated with increased risk of colon tumours are displayed in table 1.

Sporadically developing colon tumours, i.e. non-syndrome associated (constituting almost 95% of all colon tumours), also bear mutations of the colon cancer syndromes associated genes, and their development may parallel the hereditary counterpart in some way. Classically, they have been assigned to the tumorigenic pathway of either of the two most commonly studied colon cancer syndromes: familial adenomatous

Neoplastic lesions	Inheritance	Syndrome	OMIM	Genes responsible
colorectal cancer without polyps	AD	Lynch syndrome or Hereditaru non-polyposis colorectal cancer (HNPCC)	#120435	*hMLH1, hMLH2, hMSH6, hPMS2*
colorectal cancer with adenomatous polyps	AD	Familial adenomatous polyposis (FAP)	#175100	*APC*
colorectal cancer with hamartomatous/mixed/hyperplastic polyps	AD	Birt-Hogg-Dube syndrome*	#135150	*FLCN*
	AD	Cowden disease (CD)*	#158350	*PTEN*
	AD	Hereditary mixed polyposis syndrome 1*	%601228	*#15q15.2-q22.1*
	AD	Hyperplastic polyposis syndrome*	unassigned	
	AD	Juvenile polyposis (JPS)	#174900	*SMAD4, BMPR1A*
	AD	Juvenile polyposis/hereditary hemorrhagic telangiectasia syndrome (JPHT)*	#175050	*SMAD4*
	AD	Peutz Jegher's syndrome (PJS)	#175200	*STK11*
colorectal cancer with adenomatous, serrated adenoma and hyperplastic polyps	AR	MUTYH-associated polyposis (MAP)	#608456	*MUTYH*

Table 1. Clinical syndromes with an increased risk of colorectal cancer. Adapted from [24]. OMIM annotation as used and explained on http://www.ncbi.nlm.nih.gov/omim. * colorectal cancer risk not clear

polyposis (FAP) and Lynch syndrome (previously depicted hereditary non-polyposis colorectal cancer). The former, FAP, is characterized by mutations of *APC* [2]. Normally, APC protein captures free cytoplasmic β-catenin in order to have it destroyed. β-catenin is involved in at least two distinct cellular processes: cellular adhesion (through E-cadherin, and important in the normal crypt organization of the colon epithelium), and the Wnt signalling pathway leading to transcription of, amongst others, cell cycle promoters *c-myc* and *cyclin D1* [25, 26]. Both processes may be involved in the *APC* gatekeeper function in colon tumours. Patients develop hundreds of adenomatous polyps, of which some may develop into the carcinoma stage. The Lynch syndrome is caused by germline mutations of members of the DNA mismatch repair family *hMLH1*, *hMSH2*, *hMSH6*, and *PMS2*. Mutation carriers also have an increased risk of developing endometrial carcinoma and other lesions, but do not develop many polyps. Due to mismatch repair defects these tumours are characterized by a large accumulation of subtle DNA sequence changes, preferably of microsatellite repeats, referred to as microsatellite instability (MSI). Approximately 60% of

sporadically developed tumours develop from a polyp and bear somatic mutations of *APC*. However, in 15% of consecutive series of colorectal cancer MSI is observed, mainly due to epigenetic knock-out of the mismatch repair gene *hMLH1* [27].

During embryogenesis, the mid-gut develops into the proximal colon (cecum, ascending colon and two third of the transverse colon), whereas the hindgut develops to the distal colon (one third of transverse colon, descending colon, and rectum). These separate embryological origins have distinct blood- and lymph supply and drainage, e.g. the micro-vascular volume is greater in the proximal colon. The regions are exposed to different dietary and digestive constitutes, pH conditions and microbial colonization [28]. Therefore, one may suggest that tumours arising from the proximal (also called right-sided) colon differ from those of the distal colon. This is partly reflected in molecular tumour development. MSI tumours primarily develop in the proximal colon. Furthermore, especially within the proximal colon distinct subsets of carcinomas have been identified based on differences in morphology, microsatellite instability, (underlying germline) mismatch repair gene mutations, high or low frequent CpG island hypermethylation, and *KRAS* and *BRAF* mutations [29].

The identification of multiple pathways of development pleas for distinct tumour entities even though they arise from the same organ and cell type. Indeed, subsets have been associated with distinct clinical features such as dissemination and chemotherapy response [30]. Unravelling these differences is necessary to direct future therapeutic strategies of colon cancer effectively.

3. TUMOUR IMMUNOLOGY

3.1. Immunosurveillance

Neoplasms develop from autologous cells and therefore intuitively one may think these would not evoke an immune response. Yet, the accumulation of genetic aberrations during tumour development leads to numerous tumour specific antigens, which are potential targets of the adaptive immune system [31]. In fact, tumour immune control has already been proposed over a century ago [32] and has later been introduced as the immunosurveillance hypothesis [33]. Initially, the hypothesis could only be supported by studies on virally induced or allograft tumour development in immune compromised organisms. It was not until the 1990s that increased risk ratios of developing sporadic tumours in immune compromised patients were shown to exist, although colorectal cancer is not typically frequent in such patients [34, 35]. Meanwhile, evidence for immunosurveillance has been backed up by the numerous tumour escape mechanisms that have been identified [36] as more knowledge is gained about the mechanisms leading to proper adaptive (anti-tumour) immune responses, which will be discussed in the following paragraphs.

3.2. Colon cancer immunity

A current model of colon cancer immunity is depicted in figure 2. It shows the key steps in the generation of a functional or non-functional immune responses to tumours. We will briefly discuss it here, and then explore several steps in more detail in the following paragraphs.

Tumour antigens are picked up from dying or dead tumour cells by dendritic cells. These sentinels (as one might consider them) will sequentially bring the antigens to nearby lymph nodes were they can activate cytotoxic T cells assisted by helper T cells through a process

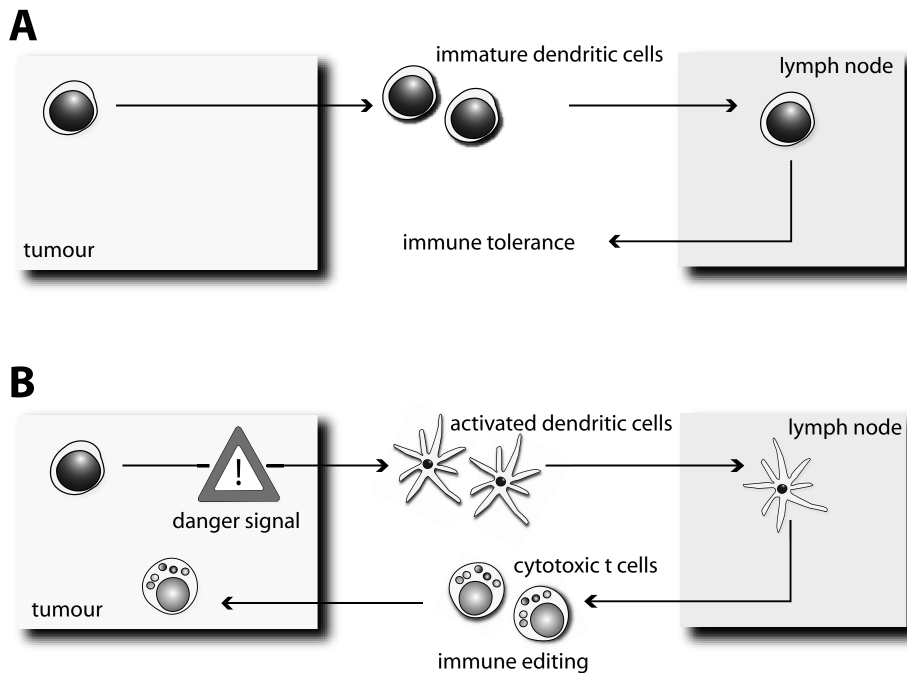

Figure 2. Model of cancer immunity. A. Tumour antigen pick up in the absence of danger signal leads to immune tolerance. B. In the presence of danger signal, activated dendritic cells will home to draining lymph nodes and there lead to activation of cytotoxic T cells, which will invade and attack the tumour. Adapted from [42].

called cross-presentation [37]. However, in order to do so, the sentinels must be awakened to an active state first, while searching the debris. Such awakening is caused by the local inflammatory environment, which is effectuated by the innate immune system; this is called the danger signal [38-40]. Without it, dendritic cells will only educate T cells to tolerate tumour antigens, leading to immune tolerance [41]. When tumour specific killer T cells are activated, they will migrate to, infiltrate and eventually kill tumour cells. They thereby represent clear selective forces of tumour development, a process referred to as immune editing [34]. The outcome of such is either tumour rejection or immune escape.

It is noteworthy to mention that consecutively disseminating tumour cells may encounter quite different immune responses as they enter a completely different arena: the lymph and blood. However, this is beyond the scope of this introduction.

3.3. Innate immune system and danger signal: an inflammation battlefield

Inflammation is the early physiological response to tissue damage. It is the first action of war. Furthermore, it may determine following attack modes, viz. the nature of adaptive immune responses. Therefore it is also referred to as the danger signal. If performed effectively inflammation leads to tissue repair, growth and remodelling. Therefore a complex interplay of multiple units of the innate immune system army is needed. The units are directed towards the battlefield by signal molecules, including

cytokines, chemokines, growth factors and proteases [43, 44].

At the front line are the neutrophils (who get there by regulating the expression of consecutively selectins, integrins, cell-adhesion molecules, and, finally, proteases). Once arrived they will start 'firing' a considerable set of cytokines and chemokines. Eventually, this will recruit downstream effector cells and dictate the nature of an immune reaction.

The second movement involves the mononuclear phagocytes. Entering battle as monocytes they differentiate into either mature macrophages or immature dendritic cells (DCs). Mature macrophages produce the main body of growth factors and cytokines (the acute phase) and remodel the extracellular matrix by cleaning up (phagocytosis of dead cells and production of proteolytic enzymes) and by rebuilding (production of matrix components and promoting angiogenesis and lymphangiogensis). Dendritic cells pick up antigens from the debris through various mechanisms and are essential in directing adaptive immune responses as discussed in paragraph 3.4.

Then the mast cells arrive which have stored various inflammatory mediators including histamine, cytokines and lipid mediators such as prostaglandins and leukotrien. Once the mast cells are activated through the complement system or by binding to immunoglobin E-antigen complexes these factors are secreted leading to clinical inflammation features, e.g. the increase of plasma into the tissue and consequent drainage of tissue fluids into lymph nodes.

Finally, fibroblasts take care of collagen deposition, epithelial cells reepithelialise while anti-inflammatory cytokines slowly evaporate the fog of war.

Normally, the intricately linked processes eventually fade out once the damage is repaired. Tumours however can be considered as chronic tissue damage like wounds that do not heal [45]. Paradoxically, as already known for over a century, chronic inflammation, f.e. inflammatory bowel disease, can also lead to neoplastic conversion [46, 47]. Growth factors resulting from inflammation actually stimulate tumour growth (and even sometimes suppress immunity). Furthermore, tumours also produce chemokines and cytokines, thereby directing inflammation themselves [43, 47]. Thus, inflammation actually serves a dual role in cancer: signalling danger and activating the immune system on the one hand, on the other hand growth stimulation and tissue remodeling [43, 48].

3.4. Cross-presentation: intelligence at the battlefield

The regional lymph nodes are 'command centres' of the adaptive immune response. It is here that molecular information about the 'enemy', antigen, is delivered by antigen presenting cells. This is called cross-presentation. Occasionally a 'deserter' of the enemy troops may enter the 'camp' by itself which could also supply the lymph node with information. However, it has been shown that this process in highly inefficient and does not lead to proper immune response [38, 49]. There are actually three cell types that are able to pick up and show foreign antigens to effector cells, macrophages, B cells and DCs, but only the latter are able to pick up intracellular antigens and are able to activate naïve T cells.

As they are summoned at the site of inflammation, immature DCs start collecting antigens through various mechanisms: through opsonisation of apoptotic bodies, through receptors of (tumour) heat shock proteins that bound to (tumour) intracellular antigens, through receptors of immunoglobins bound to antigens, or by pinocytosis [50]. During the collection of

antigens, dendritic cells develop into mature dendritic cells. This educational process, directed by earlier mentioned danger signals and possibly other factors, is important for the outcome of cross-presentation later on.

Once mature and forced by the increasing lymph flow, DCs migrate to local lymph nodes where they spread throughout the cortex. There they will encounter naïve T cells who continuously pass by through high endothelial venules (HEVs). The first encounter is managed by transient binding of cell adhesion molecules. Now, T cells have time to sample a large number of antigens as they are presented on the DC surface by human leukocyte antigen family molecules (HLA, which are responsible for antigen presentation at every immune cell encounter, and will be discussed extensively in the next paragraphs). If a T cell recognizes an antigen presented (by binding of its unique T cell receptor to the HLA:antigen complex), it leads to stabilisation of the cell adhesion up to days, which gives the DC an opportunity to educate the naive T cell about the nature of the antigen, as itself was taught during maturisation. This is called co-stimulation and numerous factors are involved in this co-stimulatory dialogue, including CD4/CD8, B7:CD28, CD40:CD40L, 4-1BBL:CD137 and ICOS:ICOSL [44]. If successful it leads to clonal expansion (proliferation and differentiation) of the T cell, called cross-priming. If co-stimulation is poor or absent, it leads to T cell anergy (inactivation), hence to immune tolerance of the specific antigen which is known as cross-tolerance.

3.5. Adaptive cell-mediated immunity: the T cell army

The T cell arm of adaptive immunity is specialised in antigen specificity, which is a key feature in distinguishing tumour cells from normal cells. It basically encompasses two distinct 'soldiers': CD8 and CD4. Both are activated by DC cross-priming. They differ in communicating antigen specificity through a different class of HLA molecules, being HLA class I for CD8, and HLA class II for CD4. HLA class I molecules present intracellular antigens, HLA class II molecules present extracellular antigens; CD8 and CD4 are the T cell receptor co-receptors for HLA class I and class II respectively. Since virtually all somatic cells express HLA class I molecules, the CD8 T cells are able to target these, and hence become - once activated – armed cytotoxic effector cells (CTL). To support the 'search and destroy' missions CD4 T cells are required. These provide regulatory signals to prime and orchestrate the immune response, and become, once activated, so-called helper cells (Th). Basically, T helper cells do so by either of two strategies, Th1 or Th2. During co-stimulation naive CD4 T cells either develop into Th1 or Th2 cells. Th1 cells enforce man-to-man battle, cell-mediated immunity by priming CTLs and macrophages. Th2 cells command the 'artillery', viz. humoral immunity by activating plasma cells and granulocytes. Th1 and Th2 cells enforce their strategy by producing a variety of cytokines. Simultaneously, they inhibit one another. Hence, once again, an adequate, full scale adaptive cell mediated immune attack is dependent on proper DC 'intelligence': cross-presentation, which on its turn is dependent on the danger signal.

3.6. Immune editing: tumour attack and escape

Once a proper cell-mediated immune response is generated, armed effector T cells will engage the enemy back at the battlefield. First, they initiate attack by antigen-nonspecific cell-adhesion of LFA-1: ICAM-1/ICAM-2 [44]. If specific tumour-antigen is sensed, this adhesion is enforced and remained long enough for the T cell to reorient its cytoskeleton around in order to release induced effector molecules by exocytosis of

lytic granules. The CD8 CTL effector molecules encompass perforin (perforating the target cell membrane), granzymes (proteases that trigger apoptosis), and Fas ligand (targeting the 'death receptor', Fas)[51, 52]. Furthermore, CTLs secrete cytokines IFN-γ, TNF-α, and TNF-β which among other things activate macrophages and can even be cytotoxic directly. This scenario described above appears to apply to colon tumours as colon tumour-antigen-specific CD8 CTLs have been identified [53, 54], as well as CTLs infiltrating colon tumours [55], which have been found to produce effector molecules [56].

So now that we know how tumour cells are besieged, we can also figure out the ways of tumours to try to escape this: 1) tumour cells may shield by down regulation of the receptors for effector molecules, including Fas and IFNγ receptor [57, 58] or block, e.g. expression of PI-9 which subsequently inactivates granzyme B [59], 2) tumour cells may fight back: expressing Fas ligand themselves [60], produce immunosuppressive cytokines [61], or even manipulate the tumour stroma to make it impenetrable for CTLs [62, 63], 3) tumour cells may hide: they may shed (tumour) antigen presentation, by altering expression of HLA:antigen complexes. This would seem an effective strategy, for it guarantees escape from any CTL damage. There are different ways to do this, which is explained in the next chapter.

3.7. Manipulating HLA class I expression: the art of hiding

Human leukocyte antigens are the human counterpart of the major histocompatibility complex, which was originally discovered by tumour allograft studies in mice. Antigen presentation, as executed by these molecules, is now considered to play a key role in the formation and execution of adaptive immunity as it makes up the immunologic language to communicate self from non self. As mentioned in paragraph 3.5, two classes of HLA molecules are distinguished: HLA class I antigens are expressed on nearly all body cells, HLA class II antigens primarily on lymphocytes and mononuclear phagocytes. HLA class I molecules are made up of a highly polymorphic heavy or α chain and a conserved light or β chain called β2-microglobulin (β2m, encoded on chromosome 15q21). The heavy chain contains two peptide binding domains enabling it to present a nonamer peptide which constitutes the actual antigen [64]. The repertoire of peptides potentially presented by HLA complexes is limited and is determined by the organisms set of class I heavy chains, which is encoded by a cluster of 6 genes all located within the gene-dense chromosome region 6p21.3, denoted as *HLA A* to *G*. Of these, *HLA A,B,* and *C* are called the classical HLA class I antigens, as they are the most polymorphic with 124, 258, and 74 different alleles distinguished [65]. *HLA E, F*, and *G*, the non-classical HLA class I antigens, vary much less with 5,1, and 14 different alleles respectively, and are considered less important in antigen presentation as they mainly present antigens derived from HLA class I heavy chains themselves.

Before the HLA:antigen complex is transported to the cell surface, antigen – primarily derived from endogenous, intracellular proteins - needs to be processed, and loaded on HLA molecule, which is assembled in the endoplasmic reticulum (see figure 3). Antigen processing is part of cellular 'housekeeping', i.e. removal of redundant proteins, which is executed by the proteasome, a multi-enzyme complex, composed of a 20S complex, which is sandwiched by two 19S regulatory complexes. As characterized in yeast, the 20S comprises 28 subunits organized into 4 stacked rings. The outer rings are formed by seven homologous polypeptides termed α subunits, the inner rings are formed by 7 β subunits, 3 of which perform the actual proteolytic activities [66, 67]. A subset of catalytic β

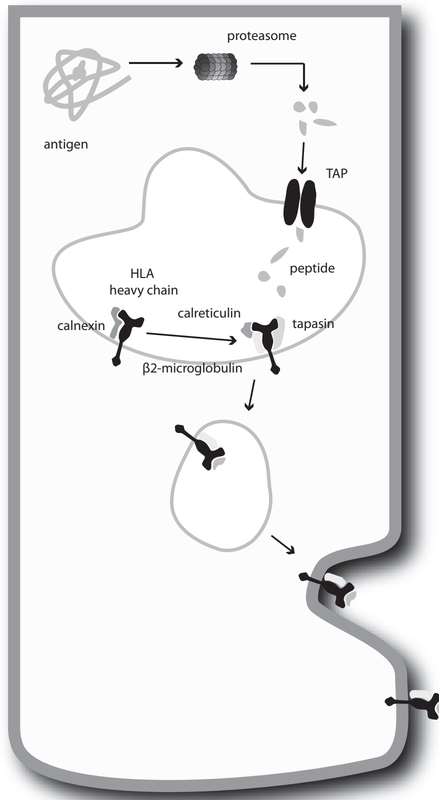

Figure 3. Antigen processing and HLA class I assembly. Adapted from [68].

subunits called the large multifunctional peptidases LMP2, LMP7, and LMP10 (MECL1) are encoded in the same chromosome region as the genes encoding for *HLA*. They replace the constitutive subunits X (MB1), Y (delta), and Z after exposure to IFNγ, which enhances production of peptides capable of associating with HLA class I molecules or, alternatively, increases the variety of peptides that can be produced within a cell [67]. The peptides are then transported into the endoplasmic reticulum by TAP1-TAP2 heterodimers, loaded on class I molecules. Consecutively, the secondary and tertiary structure of this HLA:antigen complex is obtained with help from chaperones calnexin, calreticulin, tapasin, and ERp57. Through the Golgi complex, the newly assembled HLA:antigen complexes are then transported to the cell surface [64].

If we restrict ourselves to the classical HLA I antigens, every individual carries two copies of the *HLA* genes leading to a phenotype of maximally six different alleles. In order to escape the presentation of a certain set of antigens, many phenotypic alterations are possible, but they have been classified into 5 types of altered *HLA class I* expression [69, 70]. Alterations of HLA phenotype in colorectal cancer have been reported in up to 87% [71], but because detection of the type of alteration strongly depends on laboratory techniques used, reports on frequencies of alterations detected seem to be incomplete (see chapter 5). Here, we will solely discuss the molecular mechanisms leading to these alterations.

- Loss of all HLA class I antigens. Multiple mechanisms may lead to a total loss of HLA I molecules on the surface, although they appear to be rather complex. Theoretically, it may be the result of the accumulation of mutations in all single HLA alleles or the deletion of the entire chromosomal HLA region of both chromosomes. More efficient would it be to get rid of the β2-m light chain by mutation or deletion, but this would probably still require mutation of both copies. Transcriptional silencing of the β2-m promoter or the entire *HLA* region might be an alternative mechanism, although this has not been observed yet.
- Haplotype loss. Loss of a single copy of the *HLA* region on 6p21.3 leads to loss of a haplotype. Chromosomal aberrations causing loss of heterozygosity are frequent in tumours and lead to such an altered phenotype.
- Locus down regulation. Down regulation of for instance both *HLA A* copies would need disturbance of transcriptional regulation, by manipulation of either genetic promoter

elements, or the transcription factors. There are some differences in regulatory elements and factors between the different *HLA* loci [72].

- Allelic loss. Mutation of a single *HLA* allele.
- Composite phenotypes. Although originally designated as the waste bin of unexplained phenotypic alterations not matching one of above, composite phenotypes can now be considered as the dominant type of alteration in colon cancer (see chapter 5 and 6). Immune editing may require loss of some alleles but retention of others, complex phenotypes may be evolved during tumour development and appear not to be as random as expected (see chapter 5 and 6). Furthermore, disturbances in antigen processing and HLA assembly may lead to down regulation of some, but not all *HLA* alleles (see chapter 6).

4. OUTLINE OF THIS THESIS

In this thesis, we present our studies on the nature and distribution of HLA class I aberrations in colon cancer in relation to underlying genetic background.

First, we studied the feasibility of the use of both immunohistochemistry of mismatch repair genes and microsatellite instability analysis in Lynch syndrome-associated endometrial and colon tumours. As described in **chapter 2**, this is an effective approach in order to predict germline mutations in Lynch syndrome patients. We applied the same strategy this time for large series of hereditary colon tumours in **chapter 3**. Furthermore, we extended the approach on a large series of hereditary colon tumours by constructing tissue micro-arrays and analysed its reliance. Now, we acquired a powerful tool to study large series of colon tumour with preservation of essential information of molecular subtype.

Focussing on mismatch repair deficient colon tumours, we identified frequent mutations within the untranslated part of the *IFNGR1* gene, coding for the IFNγ receptor. This cytokine is important for CTLs in order to effectively kill target cells, in part by boosting tumour cell HLA expression. In the current literature, conclusions regarding the importance of such mutations is often based on the frequency of its observation. However, in **chapter 4** we show that this is not always the case. Studying functional consequences remains necessary. Mismatch repair deficient colon tumours retain their sensitivity to IFNγ.

In **chapter 5**, we focus on HLA expression in colon tumours. Using an elaborate flow cytometry technique enabled us to study allele specific quantitative expression of freshly isolated single tumour cells. Doing so, we gained intriguing insights regarding the frequency and nature of HLA class I alterations: a) our study showed that complete eradication of HLA antigens is less common than previously assumed, but b) we identified two different patterns of HLA alterations, c) the patterns are related to distinct tumour subsets: mismatch repair deficient tumours and tumours from the proximal colon (i.e. proximal to the splenic flexure). Both entities showed frequent HLA alterations, which may cause them insensitive to immunotherapy approaches. Subsequently, we constructed tissue arrays to study large series of both sporadic and hereditary mismatch repair deficient tumours and tumours from the proximal colon, which, as explained in **chapter 6**, confirmed our findings above. Furthermore, we comprehensively screened for genetic mutations contributing to the alterations of HLA phenotype which we identified in half of the cases. Interestingly, different mechanisms were identified and related to the three subtypes, underlining the difference in tumour development and behaviour of colon tumour subsets.

In **chapter 7**, we conclude with a few general remarks concerning the topics covered in this thesis.

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Prediction of a mismatch repair gene defect by microsatellite instability and immunohistochemical analysis in endometrial tumours from HNPCC patients

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1. ABSTRACT

Instability of microsatellite repeat sequences has been observed in colorectal carcinomas and in extracolonic malignancies, predominantly endometrial tumours, occurring in the context of hereditary non-polyposis colorectal cancer (HNPCC). Microsatellite instability (MSI) as a feature of human DNA mismatch repair (MMR)-driven tumourigenesis of the uterine mucosa has been studied primarily in sporadic tumours showing predominantly somatic hypermethylation of *MLH1*. The present study shows that all endometrial carcinomas (n=12) from carriers of *MLH1* and *MSH2* germline mutations demonstrate an MSI-high phenotype involving all types of repeat markers, while in endometrial carcinomas from MSH6 mutation carriers, only 36% (4 out of 11) demonstrate an MSI-high phenotype. Interestingly, an MSI-high phenotype was found in endometrial hyperplasias from *MSH2* mutation carriers, in contrast to hyperplasias from *MLH1* mutation carriers, which exhibited an MSI-stable phenotype. Instability of only mononucleotide repeat markers was found in both endometrial carcinomas and hyperplasias from *MSH6* mutation carriers. In 29 out of 31 (94%) endometrial tumour foci, combined MSI and immunohistochemical analysis of MLH1, MSH2, and MSH6 could predict the identified germline mutation. The observation of MSI in endometrial hyperplasia and of altered protein staining for the MMR genes supports the idea that inactivation of MMR genes is an early event in endometrial tumourigenesis. A correlation was found between the variation in the extent and level of MSI and the age of onset of carcinoma, suggesting differences in the rate of tumour progression. A high frequency of MSI in hyperplasias, found only in *MSH2* mutation carriers, might indicate a more rapid tumour progression, correlating with an earlier age of onset of carcinoma. The present study indicates that assessment of altered protein staining combined with MSI analysis of endometrial tumours might direct the mutational analysis of MMR genes.

2. INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC), or Lynch syndrome, is an autosomal dominant disease characterized by an excess of colorectal cancer, endometrial cancer, and/or a variety of other cancers [1,2]. The disease is caused by a defect in the DNA mismatch repair system. Germline mutations in five genes involved in the human DNA mismatch repair (MMR) mechanism have been identified: *MSH2*, *MLH1*, *PMS1*, *PMS2*, and *MSH6* [3]. Three of these genes, MSH2, MLH1, and MSH6, account for the MMR defect in the majority of HNPCC families. Defects in (one of) these genes lead to genetic instability characterized by expansion or contraction of simple repeat sequences (microsatellite instability, MSI). Genetic instability is responsible for a rapid accumulation of somatic mutations in different oncogenes and tumour suppressor genes which play a crucial role in tumour initiation and progression [4].

Among the extracolonic malignancies that occur in HNPCC, endometrial cancer is the most common and was recently included in the clinical criteria for HNPCC (Amsterdam II criteria)[5]. A high frequency of microsatellite instability (MSI-high phenotype) in endometrial tumours has been reported in 25-30% of sporadic endometrial carcinomas, particularly of the endometrioid subtype [6,7]. Such tumours, usually referred to as MSI-high endometrial tumours, were shown to be diploid [6], indicating an absence of chromosomal instability; this is in contrast with MSI-stable non-endometrioid endometrial carcinomas, which are generally aneuploid [8,9].

No advanced MSI analysis of HNPCC-associated endometrial carcinomas has been described yet.

Classical HNPCC is characterized by an early age of onset of colorectal carcinoma (mean age of 44 years) [4]. Recently, *MSH6* germline mutations have been identified in patients from atypical HNPCC families with a later age of onset of carcinomas and a high frequency of extracolonic malignancies, in contrast to usual HNPCC families with germline mutations in *MLH1* or *MSH2* [10,11]. In a study by our group, we observed that endometrial carcinoma was the main clinical manifestation of (atypical) HNPCC among females from families with *MSH6* germline mutations [12]. We found that the endometrial carcinomas from these families demonstrated only instability of mononucleotide repeat markers.

Previous authors have recommended MSI analysis in families with a low probability of detecting a mutation in MMR genes as a first screening test for MMR defects [13,14], but the detection of MSI in tumours does not define which one of the MMR genes is involved. A more direct assessment of the specific MMR gene involved might be possible by immunohistochemical analysis of MMR genes, as was shown for MLH1 and MSH2 in colorectal carcinomas [15].

The aims of the present investigation were, firstly, to study microsatellite instability (MSI phenotype) in a series of HNPCC-associated endometrial tumours from patients with germline mutations in different MMR genes and, secondly, to correlate MSI phenotype, mutation status, and immunohistochemical protein staining for MLH1, MSH2, and MSH6 in endometrial carcinoma and hyperplasia. It was of particular interest to study MSI and MMR protein staining in endometrial hyperplasia, to assess whether defective DNA mismatch repair by inactivation of MMR genes is an early event in the tumourigenesis of the uterine mucosa.

3. MATERIALS AND METHODS

3.1. Patient selection

We analysed in total 31 endometrial and six colorectal tumour foci in HNPCC patients from 15 families complying with the Amsterdam I criteria [16], in which a germline mutation in one of the MMR genes *MLH1*, *MSH2* or *MSH6* had been identified. For endometrial tumour foci, we investigated 23 carcinomas and eight hyperplasias. From four HNPCC patients (patient ID: 102-1, 5-12, 5-15, and 88-2), both endometrial carcinoma and associated hyperplasia were analysed.

Histological review was based on the corresponding haematoxylin and eosin (H&E)-stained tissue sections for determination of tumour cell type and grade, using the criteria of AFIP [17] and FIGO [18]. The group of 31 endometrial tumour foci consisted of 14 endometrioid adenocarcinomas (B1), two endometrioid adenocarcinomas with squamous differentiation (B2), one serous papillary adenocarcinoma (B3), one squamous cell carcinoma (B6), one mixed carcinoma (B7/B9), four endometrioid adenocarcinomas with complex hyperplasia with atypia (A4/B1), six complex hyperplasias with atypia (A4), one complex hyperplasia without atypia (A3), and one simple hyperplasia with atypia (A2) (Table 1).

According to the FIGO criteria [18], 12 carcinomas were classified as grade 1, eight carcinomas as grade 2 (patient IDs: 9796-1, 91-1, 7-96, 5-11, 867-6, 686-3, 686-4, and 22-52), and three carcinomas as grade 3 tumours (patient IDs: 686-5, 22-6, and 50176-3); low grade denotes a predominance of gland formation and high grade its relative absence. FIGO stage was assigned on the basis of histological reports and findings. Seventeen carcinomas were classified as stage IA, IB or IC; one carcinoma as stage IIA; and one carcinoma as stage IIIA.

Tumour-type	Patient ID	Histology-AFIP	MSI		Immunohistochemistry		
			Total	NCI	MLH1	MSH2	MSH6
MLH1 germline mutation							
E	102-1	A3	6/24	0/3	+	+	0
E	5-12	A4	2/40	0/4	+	+++	+
E	5-15	A4	3/25	0/3	+	+++	+++
E	9769-2	A4/B1	25/30	5/5	0	+++	++
E	9769-1	B1	22/28	5/5	++	+++	++
E	91-1	B1	11/28	3/5	+	+++	++
E	7-96	B1	22/29	3/3	+	++	+
E	102-1	B1	12/27	3/5	+	+	0
E	5-11	B1	18/28	3/4	+	++	0
E	5-12	B1	22/40	4/5	+	+++	+
E	5-15	B2	25/28	4/5	+	+++	+++
C	5-11	-	22/32	2/3	+	++	+++
C	9769-1	-	18/25	2/3	0	++	+
MSH2 germline mutation							
E	139-1	A2	20/32	4/5	+	0	0
E	88-2	A4	12/34	2/4	+	0	0
E	99-1	A4	11/34	3/5	++	0	0
E	139-1	A4	18/33	3/5	+	0	0
E	57-33	A4/B1	11/38	3/5	+++	0	0
E	77-2	B1	6/9	2/3	++	0	0
E	88-2	B1	14/26	2/3	+	0	0
E	90-1	B1	16/30	2/4	++	0	0
C	77-2	-	19/24	4/4	++	0	0
C	99-1	-	22/33	5/5	+	0	0
MSH6 germline mutation							
E	50176-5	A4	3/35	2/5	+	++	0
E	867-1	A4/B1	3/36	2/5	+++	+	0
E	867-6	B1	3/36	2/5	+++	+	0
E	22-2	B1	1/25	0/5	na	na	0
E	50176-2	B1	2/36	1/5	++	+	0
E	50176-4	B1	2/36	0/5	+++	++	0
E	686-3	B1	1/36	2/5	+++	+	0
E	686-4	B1	2/36	1/5	+++	+	0
E	686-5	B2	3/36	0/5	+++	+	0
E	22-6	B3	4/27	2/4	++	+	0
E	22-52	B6	0/31	0/4	0	++	0
E	50176-3	B7	3/36	1/5	+	+	0
R	50176-2	-	19/23	4/5	+	0	0
R	867-1	-	10/36	4/5	+	++	0

Table 1. Clinicopathological, microsatellite instability (MSI), and immunohistochemical analysis of HNPCC-associated tumours.

Tumour type: E, endometrial; C, colorectal; R, rectum; patient ID, family-patient number; clinicopathology: AFIP [17]: A2, simple hyperplasia with atypia; A3, complex hyperplasia without atypia; A4, complex hyperplasia with atypia; B1, endometrioid adenocarcinoma; B2, endometrioid adenocarcinoma with squamous differentiation; B3, serous papillary adenocarcinoma; B6, squamous cell carcinoma; B7, mixed carcinoma; microsatellite instability: total, set of 40 microsatellite markers; NCI, international set of five microsatellite markers [19]; +, instability; 0, no instability; immunohistochemistry: +++, strong; ++, moderate; +, weak; 0, no staining; na, not analysed.

3.2. DNA isolation

Genomic DNA of normal and tumour tissue was isolated from formalin-fixed, paraffin-embedded material by microdissection. In all cases except one (patient ID: 867-6), normal epithelial and tumour cells were microdissected from tissue embedded in the same paraffin block. From five cases (patient IDs: 91-1, 9769-1, 50176-2, 686-3, and 686-5), tissue was obtained by curettage, but from all other cases, including 686-5, resection material was available. Using a Chelex extraction method, DNA was isolated from five consecutive 10 µm sections, resuspended in 250 µl of PK-1 lysis buffer [50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM MgCl2, 0.45% NP40, 0.45% Tween 20, 0.1 mg/ml gelatine] containing 5% Chelex beads (Biorad, Hercules, USA) and 10 µl of proteinase K (10 mg/ml), and incubated for 12 h at 56°C. The suspension was incubated 10 min at 100°C, centrifuged, and the supernatant was carefully decanted.

3.3. Microsatellite instability analysis

We analysed 40 microsatellite markers, including five markers (two mononucleotide repeats: BAT25, BAT26; and three dinucleotide repeats: D2S123, D5S346, D17S250) recommended by the 1997 National Cancer Institute (NCI) workshop on Microsatellite Instability for Cancer Detection and Familial Predisposition held in Bethesda, December 1997 (referred to as the NCI set) [19]. Also analysed were one mononucleotide repeat: BAT40; 16 dinucleotide (CA) n repeats: TP53, D1S158, D3S1029, D3S1611, D7S255, D8S133, NEFL, D10S197, D11S901, D11S35, D11S968, D13S175, D13S153, D17S588, D18S58, D18S61; four trinucleotide repeats: FABP2, DRPLA, D4S243, D17S1288; and nine tetranucleotide repeats: D3S1768, D3S2456, D4S1629, D8S1130, D11S1998, D13S321, D15S1232, D16S752, D17S1537. In addition, five mononucleotide repeats in the coding regions of *transforming growth factor receptor type II (TGFRII)*, *insulin-like growth factor receptor type II (IGFRII)*, and *BAX* [20-22], as well as the mismatch repair genes *MSH3* and *MSH6*, were analysed [23]. All primer sequences for microsatellite repeat markers can be attained from the Genome Database (http://www.gdb.org).

One microlitre of DNA was loaded on a polyvinyl-chloride microtitre plate (Dynatech Laboratories, Chantilly, USA). Polymerase chain reaction (PCR) was performed in a total reaction volume of 12 µl, containing 10 pmol of forward and reverse primer, 1% BSA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.0 mM MgCl2, 200 µM dATP, 200 µM dGTP, 200 µM dCTP, 200 µM dTTP, 0.2 µM fluorescent dCTP (R110 or R6G) or 0.8 µM (Tamra) (PE Applied Biosystems Inc., Foster City, USA), and 2.5 U of AmpliTaq gold polymerase (PE Applied Biosystems Inc., Foster City, USA) for 12 min at 96°C and 33 cycles of 1 min at 96°C, 2 min at 55°C, 1 min at 72°C, and a delay extension step of 7 min at 72°C in a GeneAmp 9700 thermocycler (PE Applied Biosystems Inc., Foster City, USA). 3.5 µl of loading mix (2.5 µl formamide, 0.5 µl of xylene-cyanol loading dye, 0.5 µl of Rox-500 size standard) (PE Applied Biosystems Inc., Foster City, USA) was added to 1.5 µl of sample (0.5 µl of R110, 0.5 µl of R6G, and 0.5 µl of Tamra labelled PCR product). After denaturation for 5 min at 96°C, 3.0 µl was subjected to electrophoresis on a 0.1 mm thick, 6% polyacrylamide gel containing 7 M urea and 1xTBE [0.09 M Tris- borate, 20 mM EDTA (pH 8.0)] on an ABI 377 sequencer (PE Applied Biosystems Inc., Foster City, USA) for 3 h with run profile GS-2400A and analysed with GeneScan 2.0.

Instability of a microsatellite marker is characterized by additional PCR products from the tumour DNA, compared with the electrophoresis profile of the matching normal DNA. This is facilitated by loading the differently fluorescent-

labelled PCR products from tumour and normal DNA in the same lane.

According to the recently established international criteria for MSI at the NCI workshop [19], tumours were classified as (i) tumours with instability for two or more markers (MSI-high); (ii) tumours with instability for one marker (MSI-low); and (iii) no instability (MSI-stable). Since these criteria for MSI analysis are based on studies of colorectal carcinomas and have not been tested for endometrial carcinomas, the MSI results in this report were presented based on the set of 40 markers and the NCI set (Table 1).

3.4. Immunohistochemical analysis of MLH1, MSH2, and MSH6

Immunohistochemical staining was performed on 3 µm sections of formalin-fixed, paraffin-embedded tissues using standard procedures. After antigen retrieval by boiling for 10 min in 1 mM EDTA (pH 8.0) for MLH1 and MSH2, and in 10 mM citrate buffer (pH 6.0) for MSH6, tissue sections were incubated overnight at room temperature with antibodies against human MLH1 (clone 14; Calbiogen, Cambridge, USA), dilution 1:75; human MSH2 (clone GB-12; Calbiogen, Cambridge, USA), dilution 1:75; or human MSH6 (clone 44; Transduction Laboratories/Becton Dickinson, Hamburg, Germany), dilution 1:800. Immunoreactivity was detected using an Envision two-step peroxidase detection system (DAKO, Denmark). Staining was evaluated semi-quantitatively, using normal epithelial cells or the centres of lymphoid follicles as an internal control. Both the intensity of nuclear staining, presented in Table 1 as strong (+++), moderate (++), weak (+), or absent (0), and the percentage of tumour cells showing positive nuclear staining were scored in the area which was microdissected for MSI analysis. The latter scoring did not, however, alter the overall interpretation of the individual cases and is therefore not shown.

4. RESULTS

4.1. Clinicopathological features of endometrial carcinoma

Seventy-eight per cent (18/23) of endometrial carcinomas were of the endometrioid subtype (histology AFIP) [17]. According to the FIGO criteria [18], 12 of 23 (52%) carcinomas were grade 1 tumours, eight of 23 (35%) were grade 2 tumours and there were three grade 3 tumours. The majority of carcinomas (89%) were classified as FIGO stage I. The mean age of onset of all endometrial carcinomas was 48.7 years. A significantly earlier age of onset of endometrial carcinoma was found in patients with a germline mutation in *MSH2* than in *MLH1* mutation carriers (41.0 vs. 49.5 years, p=0.032). The mean age of onset of endometrial carcinoma in *MSH6* mutation carriers (55.5 years) was significantly later than in *MLH1* and *MSH2* mutation carriers (p=0.005 and p=0.006, respectively).

4.2. Microsatellite instability analysis

In the group of eight MLH1 mutation carriers, a MSI-high phenotype (according to the NCI criteria [19]) was observed in all endometrial carcinomas. Instability of all types of repeat markers was found by analysing the set of 40 markers (Table 1). Remarkably, in two foci of endometrial complex hyperplasia with nuclear atypia, we found instability of predominantly tri- and tetra-nucleotide repeat markers (i.e. FABP2, D3S2456, D8S1130, D15S1232, and D16S752). In one focus of endometrial complex hyperplasia without nuclear atypia (patient ID: 102-1), instability was found in 25% (6/24) of the markers investigated, involving all types of repeat markers (D15S1232, D8S1130, FABP2, D13S175, D18S58, and BAT40). No instability was found in the NCI set of markers in the foci of endometrial hyperplasia. Tumours were therefore classified as MSI-stable (Table 1).

Endometrial hyperplasias and carcinomas from six *MSH2* mutation carriers demonstrated an MSI-high phenotype involving all types of repeat markers. Instability was also found in two or more markers from the NCI set (Table 1).

In the group of 12 *MSH6* mutation carriers, a low frequency of instability was observed in all endometrial tumours by using the set of 40 markers. Instability was only found in the mononucleotide repeat markers (Table 1). According to the NCI criteria, four endometrial carcinomas were classified as MSI-high since BAT25 and BAT26, included in the NCI set, exhibited instability. In three endometrial carcinomas, instability was observed of only one of these two mononucleotide repeat markers, and in four endometrial carcinomas no instability was found of BAT25 or BAT26. These tumours were classified as MSI-low and MSI-stable, respectively (Table 1). One endometrial complex hyperplasia with nuclear atypia demonstrating instability of BAT25 and BAT26 was classified as MSI-high.

We also investigated the instability for five intragenic mononucleotide repeat sequences in *TGFRII*, *IGFRII*, *BAX*, *MSH6*, and *MSH3*, respectively, which frequently show instability in colorectal tumours [20-23]. Contraction of the A(10) repeat sequence (deletions) in *TGFRII* was found in all six colorectal carcinomas tested, but in none of the endometrial tumours. Contraction of the (A)8 repeat in *IGFRII* and the (G)8 repeat in *BAX* was found in respectively four and five of 23 endometrial carcinomas, but in none of the colorectal carcinomas. Interestingly, no instability was found of the (G)8 in *BAX* in an endometrial complex hyperplasia without atypia from a patient (patient ID: 102-1) with a germline mutation in *MLH1*, whereas contraction of this repeat was present in the endometrial carcinoma from the same patient. Contraction of the (C)8 in *MSH6* and the (A)8 in *MSH3* were found in respectively two and one of 12 endometrial carcinomas from *MLH1* and *MSH2* mutation carriers. As we have shown previously [12], in MSH6 mutation carriers, alterations in the (C)8 in *MSH6* were found in all endometrial and colorectal carcinomas, whereas alterations in the (A)8 in *MSH3* were only found in the colorectal carcinomas.

4.3. Immunohistochemical analysis of MLH1, MSH2, and MSH6

Loss of or markedly reduced nuclear staining for MLH1, MSH2 or MSH6, respectively, was found in all tumours from HNPCC patients (Table 1 and Figure 1). In all specimens, pre-existing glands or germinal centres staining for MLH1, MSH2, and MSH6 were retained, although we observed variations in the staining intensity, probably related to differences in the fixation standards and intervals of tissue storage.

In the majority of endometrial carcinomas and hyperplasias from *MLH1* mutation carriers a reduced or complete loss of MLH1 nuclear staining was found. In 5/11 of these tumours, both MSH2 and MSH6 staining was retained. Furthermore, in both the endometrial hyperplasia and carcinoma from one patient (patient ID: 102-1), markedly reduced staining of MSH2 and loss of MSH6 staining were also found. In three endometrial carcinomas, additional reduced (patient IDs: 5-12 and 7-96) or absent (patient ID: 5-11) MSH6 staining was found. All tumours from *MSH2* mutation carriers showed loss of MSH2 and complete loss of MSH6 staining. Conversely, in tumours from *MSH6* mutation carriers, loss of MSH6 staining and primarily reduced MSH2 staining were found. In two endometrial carcinomas and four foci of hyperplasia from *MSH2* and *MSH6* mutation carriers, reduced MLH1 staining was also observed.

It is remarkable that in an area of complex hyperplasia without atypia (patient ID: 102-1, Table 1 and Figure 1B) and in an area of simple hyperplasia without atypia (patient ID: 57-33,

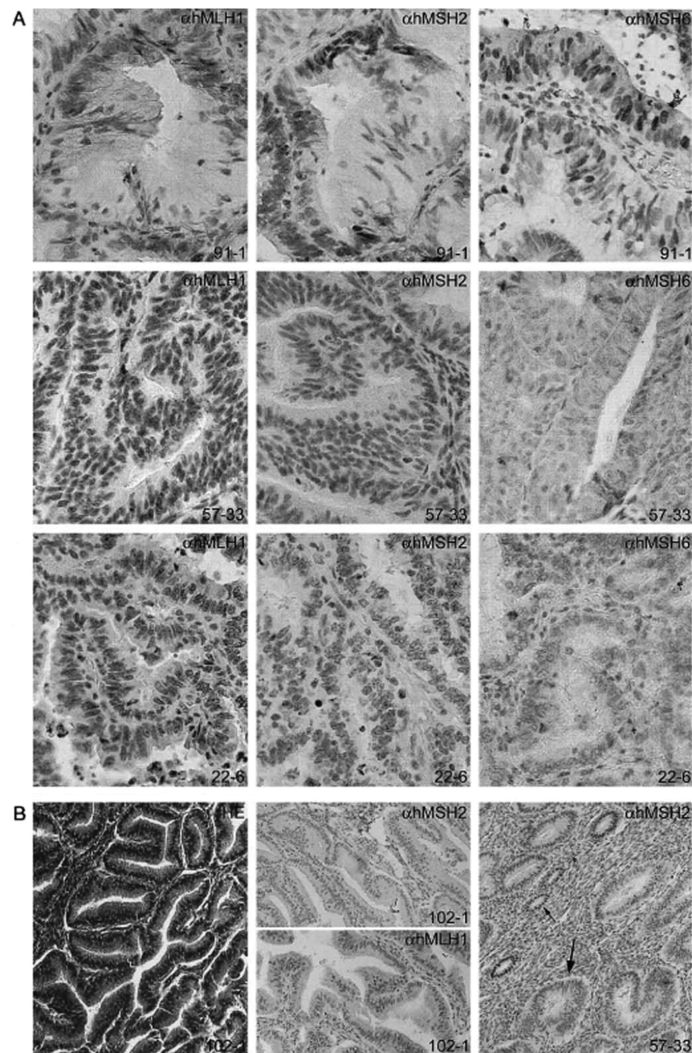

Figure 1. (A) Immunoreactivity for MLH1, MSH2, and MSH6 in three endometrial tumours from Table 1. First row: endometrial carcinoma of patient 91-1 (MLH1 mutation carrier) stained with anti-human MLH1, MSH2, and MSH6 antibodies, respectively. MLH1 nuclear staining is reduced. MSH2 and MSH6 staining is retained. Second row: endometrial carcinoma of patient 57-33 (MSH2 mutation carrier) stained with anti-human MLH1, MSH2, and MSH6 antibodies, respectively. Nuclear staining for both MSH2 and MSH6 is absent (abrogation MutS/). MLH1 staining is retained. Third row: endometrial carcinoma from patient 22-6 (MSH6 mutation carrier) stained with anti-human MLH1, MSH2, and MSH6 antibodies, respectively. MLH1 staining is retained. Nuclear staining for MSH6 is absent. MSH2 staining is reduced (abrogation MutS). (B) Areas of complex and simple endometrial hyperplasia. Left: H&E staining of the complex hyperplasia without atypia from patient 102-1 (MLH1 mutation carrier, Table 1). Middle: same tumour area stained with anti-human MSH2 and MLH1 antibodies, respectively. With both antibodies, nuclear staining is markedly reduced (MSH6 staining was absent, not shown). Right: area of simple hyperplasia without atypia from patient 57-33 (MSH2 mutation carrier). In hyperplastic glands, MSH2 staining (large arrow) is absent, whereas in normal glands MSH2 nuclear staining (small arrow) is retained.

Figure 1B) diminished or absent nuclear staining of MLH1 or MSH2, respectively, can already be observed.

5. DISCUSSION

In the present study we performed MSI and immunohistochemical analysis in endometrial carcinomas and hyperplasias from patients with identified gene mutations in *MLH1*, *MSH2* or *MSH6*. All endometrial carcinomas from carriers of *MLH1* and *MSH2* germline mutations showed, according to the NCI citeria [19], an MSI-high phenotype involving all types of repeat markers, while endometrial carcinomas from *MSH6* mutation carriers exhibited an MSI-high phenotype in only 36% (4 out of 11) of the tumours. An overall low frequency of MSI was found by analysing the set of 40 markers, but in all *MSH6*-associated tumours, instability was observed only of the mononucleotide repeat markers. Interestingly, an MSI-high phenotype involving all types of repeat markers was found in endometrial hyperplasias from MSH2 mutation carriers, but not in hyperplasias from MLH1 mutation carriers exhibiting an MSI-stable phenotype. In these hyperplasias, extensive analysis with the set of 40 markers detected instability predominantly of tri- and tetranucleotide repeat markers. In conclusion, in our study we found a mutated MMR gene-specific phenotype of MSI in endometrial hyperplasia from HNPCC patients.

The immunohistochemical analysis using antibodies directed against the MMR gene proteins MSH2 and MSH6 showed a complete loss of staining for these proteins in both endometrial carcinomas and hyperplasias from HNPCC patients, concordant with identified germline mutation in these genes. Reduced staining for MLH1 was found in endometrial carcinomas and hyperplasias from *MLH1* mutation carriers. In tumours from *MSH2* mutation carriers, absent staining for the MSH6 protein was also observed, while conversely, in tumours from *MSH6* mutation carriers, reduced staining for MSH2 was found. These reduced nuclear staining patterns are most likely the result of abrogation of the MutS complex [24,25]. Residual MSH2 staining in tumour cells might be due to MSH2 protein within the MutS complex. The observation of MSI in endometrial hyperplasia, and altered protein staining for MLH1, MSH2, and MSH6, respectively, concordant with identified germline mutation in one of these MMR genes, supports the hypothesis that inactivation of MMR genes is an early event in endometrial tumourigenesis. The finding of MSI and altered MMR protein staining in one endometrial complex hyperplasia without atypia is remarkable. Endometrial hyperplasia without atypia is a manifestation of unopposed oestrogenic stimulation, but by itself is not thought to be a precursor of endometrial carcinoma.

The observation of an MSI-high phenotype in both endometrial hyperplasias and carcinomas from *MSH2* mutation carriers, the MSI-high phenotype in carcinomas but not in hyperplasias from *MLH1* mutation carriers, and the predominantly MSI-low or -stable phenotype in carcinomas from *MSH6* mutation carriers, may suggest differences in tumour progression. The variation in the extent and level of MSI may account for the differences in the age of onset of endometrial cancer between carriers of germline mutations in different MMR genes. The series of HNPCC-associated endometrial carcinomas in the present study has a relatively early age of onset (mean 48.7 years) compared with sporadic cases (mean 59 years) [26]. Interestingly, we observed a significant difference in the age of onset of endometrial carcinoma between patients with a germline mutation in the MMR genes *MLH1*, *MSH2*, and *MSH6* (49.5, 41.0, and

55.5 years, respectively). The high frequency of MSI in endometrial hyperplasias found only in *MSH2* mutation carriers, correlated with an early age of onset of carcinoma, might indicate rapid tumour progression. The predominantly MSI-low or stable phenotypes in carcinomas from *MSH6* mutation carriers correlate with a later age of onset of carcinoma, indicating a slower tumour progression rate.

Previous studies have evaluated genes containing intragenic mononucleotide repeats. A high frequency of instability of *BAX*, *TGFRII*, *IGFIIR*, *MSH3*, and *MSH6* has been reported in colorectal carcinomas [20-23]. We identified instability of *BAX*, *IGFRII*, and *TGFRII* in respectively 22%, 17%, and 0% of endometrial carcinomas, similar to previous studies of MSI in sporadic endometrial carcinomas [6,7,27-30]. On the other hand, in all endometrial carcinomas from *MSH6* mutation carriers, there was instability of the (C)8 repeat in *MSH6* [12]. In contrast, only 17% of carcinomas from *MLH1* and *MSH2* mutation carriers demonstrate instability of *MSH6*. In sporadic endometrial carcinomas, instability of *MSH6* was reported in 7% of tumours [7]. The high frequency of instability in our series of HNPCC-associated endometrial tumours might indicate that *MSH6* represents a preferential target for somatic mutations in *MSH6* mutation carriers, which is in agreement with earlier observations [10-12]. Although inactivation of *MSH6* might be important for tumour progression in *MSH6* mutation carriers, other molecular targets of MSI in endometrial tumourigenesis remain to be elucidated.

The search for MMR gene mutations is time-consuming and thus expensive. Wijnen et al. described that if the probability of detecting a mutation in MMR genes based on clinical features in HNPCC families is low, MSI analysis should be considered as an initial screening test for MMR defects [13]. The present study has shown that it is possible to predict which MMR gene is mutated by using MSI and immunohistochemical analysis of MLH1, MSH2 and MSH6 in endometrial tumours. In 29 out of 31 (94%) endometrial tumour foci, both carcinoma and hyperplasia, combined MSI and immunohistochemical analysis could predict the identified germline mutation. The distinction between *MSH2* and *MSH6* defects, however, is very subtle; instability of only mononucleotide markers indicates a *MSH6* defect.

In endometrial carcinomas, MSI analysis alone could not predict the identified germline mutations in the MMR genes *MLH1* and *MSH2*, since a high frequency of instability is found in all types of microsatellite markers in both groups. However, a focus of hyperplasia with instability in all types of microsatellite markers (MSI-high) might indicate an *MSH2* defect. Selective instability in predominantly mononucleotide repeat markers in both endometrial carcinomas and hyperplasias from *MSH6* mutation carriers can predict *MSH6* germline mutations in 8/12 tumours, using the NCI set, and in 11/12, using the extended marker set.

Only in 3/11 endometrial tumour foci of *MLH1* mutation carriers could immunohistochemical analysis alone predict the germline mutation in *MLH1*. In the group of *MSH2* and *MSH6* in 8/8 and 9/12 tumours, respectively, immunohistochemistry could predict a potential *MSH2* or *MSH6* mutation. However, although the immunohistochemical data seem promising, we do not favour the use of immunohistochemistry alone to direct mutation analysis. Distinction between *MSH2* and *MSH6* defects by immunohistochemistry is very subtle and the staining pattern predicting an *MLH1* defect is unreliable in our hands. In families with two or more endometrial cancers, with an endometrial cancer patient diagnosed at an unusually young age, or with colorectal and endometrial cancer,

in which colorectal cancer tissue is not available, we recommend MSI analysis and immunohistochemistry as a first investigative step.

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Conventional and tissue microarray immunohistochemical expression analysis of mismatch repair in hereditary colorectal tumors

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Chapter 3

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1. ABSTRACT

Immunohistochemistry (IHC) of mismatch repair (MMR) proteins in colorectal tumors together with microsatellite analysis (MSI) can be helpful in identifying families eligible for mutation analysis. The aims were to determine sensitivity of IHC for MLH1, MSH2, and MSH6 and MSI analysis in tumors from known MMR gene mutation carriers; and to evaluate the use of tissue microarrays for IHC (IHC-TMA) of colon tumors in its ability to identify potential carriers of MMR gene mutations, and compare it with IHC on whole slides. IHC on whole slides was performed in colorectal tumors from 45 carriers of a germline mutation in one of the MMR genes. The TMA cohort consisted of 129 colon tumors from (suspected) hereditary nonpolyposis colorectal cancer (HNPCC) patients. Whole slide IHC analysis had a sensitivity of 89% in detecting MMR deficiency in carriers of a pathogenic MMR mutation. Sensitivity by MSI analysis was 93%. IHC can also be used to predict which gene is expected to harbor the mutation: for *MLH1*, *MSH2*, and *MSH6*, IHC on whole slides would have correctly predicted the mutation in 48%, 92%, and 75% of the cases, respectively. We propose a scheme for the diagnostic approach of families with (suspected) HNPCC. Comparison of the IHC results based on whole slides versus TMA, showed a concordance of 85%, 95%, and 75% for MLH, MSH2, and MSH6, respectively. This study therefore shows that IHC-TMA can be reliably used to simultaneously screen a large number of tumors from (suspected) HNPCC patients, at first in a research setting.

2. INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of death because of malignancy in the Western world. The cause of CRC is multifactorial, involving hereditary and environmental factors and somatic genetic changes during tumor progression [1]. A family history of CRC is a clinically significant risk factor and may be found in up to 15% of all patients with CRC [2]. The most common hereditary CRC syndromes are familial adenomatous polyposis coli (FAP), accounting for <1% of CRC cases and HNPCC (hereditary nonpolyposis colorectal cancer), accounting for 1 to 6% of the cases [3]. HNPCC is an autosomal dominantly inherited disorder that is clinically defined by the Amsterdam Criteria [4,5]. In HNPCC, germline mutations have been identified in four DNA mismatch repair (MMR) genes, *MSH2* [6], *MLH1* [7], *PMS2* [8], and *MSH6* [9-14]. In 50 to 70% of the families fulfilling the Amsterdam criteria a germline mutation is detected in *MLH1* or *MSH2* [15,16]. Germline mutations have been found in *MSH6* in families with atypical HNPCC, ie not entirely fulfilling the Amsterdam criteria [11-14].

Microsatellite instability (MSI) in colorectal tumors, first reported in 1993 [17-19], is caused by a failure of the DNA MMR machinery to repair errors occurring during DNA replication and leading to length alterations in simple, repetitive microsatellite sequences distributed throughout the genome [20]. According to international guidelines, a panel of five specific microsatellite markers has been recommended for MSI evaluation [20]. If at least two markers show instability, the tumor is referred to as MSI-high (MSI-H), if only one marker is unstable, the tumor is considered MSI-low (MSI-L). MSI is reported in 85 to 92% of CRC associated with HNPCC and in 10 to 15% of sporadic CRC [17,21-23].

In 1996, Leach and colleagues [24] and Thibodeau and colleagues [25] reported the use of monoclonal antibodies directed against

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MSH2 and MLH1 in the immunohistochemical analysis of CRCs. Subsequent reports described immunohistochemistry (IHC) of MLH1, MSH2, and MSH6 in sporadic and HNPCC tumors with varying results [26-35]. Some authors suggested that IHC can be used as a prescreening method for the actual mutation analysis of the MMR genes [32,36,37]. Others concluded, however, that IHC cannot replace MSI analysis as a prescreening method, because of a lower sensitivity [35,38,39]. The studies on the value of IHC published so far are hampered by small numbers of tumors associated with a known MMR gene mutation. In addition, most studies focused on tumors associated with *MLH1* and *MSH2* mutations and not *MSH6* mutations.

IHC for diagnostic purposes is usually performed on whole slides. A novel approach that allows high-throughput IHC is provided by the so-called tissue microarrays (TMAs) composed by large numbers of small punched-out tissue cores from different tumors [40]. With this technique up to 1000 different samples can be analyzed in a single immunohistochemical staining experiment [41]. Previous reports concluded that binary immunophenotypes can be reliably investigated on TMAs using two to three representative cores per tumor sample [42]. However, validation of data generated by TMA is needed to determine the minimal amount of tissue cores/tumor required in one TMA and to inventory possible problems of TMA that might influence staining results including technical artifacts such as differences in fixation of archived material or loss of tissue [43].

The aim of the present study was to evaluate whether IHC analysis of colorectal tumors could predict the presence of a MMR mutation in tumors in a large series of HNPCC patients with a known mutation, and to compare these results with the outcome of MSI. In addition, we compared the results of IHC performed on whole slides with the TMA technique. Validation of TMA for IHC has not yet been performed for colorectal tumors.

3. PATIENTS AND METHODS

3.1 Patients

A total of 45 patients (25 males and 20 females) with a known germline mutation in *MLH1*, *MSH2*, or *MSH6*, were selected from 35 HNPCC families. Information on cancer site, age at diagnosis, and location of the colon tumors were collected for all patients (Table 1). The paraffin-embedded tissue blocks from these patients dated back from 1976 until 1999. One tumor from each patient was used for the analysis. In total, 44 CRCs and a single duodenal carcinoma were analyzed.

Among the 35 families with a known MMR defect, 27 different germline mutations have been identified by denaturing gradient-gel electrophoresis or Southern blotting [44-47]: 14 in *MLH1*, 11 of which were pathogenic (nonsense, frameshift, or splice site mutants) and 3 unspecified variants; 8 in *MSH2* (seven pathogenic, one unspecified variant); five in *MSH6* (three pathogenic, two unspecified variants) (Table 1). Twenty-four of the 45 patients in our cohort, originating from 20 families, carry an *MLH1* mutation. The same mutation was identified in six different families (1852_1854del, K618del). Thirteen of the 45 patients, originating from 10 families, were carriers of a mutation in *MSH2*. Two common mutations have been identified in two different families. Seven patients from five families were carriers of five different *MSH6* mutations.

The average age at cancer diagnosis of the carriers of *MLH1* (n = 21), *MSH2* (n = 12), and *MSH6* (n = 4) pathogenic mutations was 44 years (range, 28 to 68 years), 41 years (range, 23 to 61 years), and 54 years (range, 26 to 84 years), respectively.

Gene	Mutation	Exon	Patho-genic?	Number of families	Number of patients	Sex	Age of diagnosis	Site of tumor	IHC MLH1	IHC MSH2	IHC MSH6	MSI	Family diagnosis
a. *MLH1*	18_34del17, G6fsX25	1	Yes	1	2	M	35	Colon	0	0	0	H	1
						M	39	Ascendens	0	+	0	na	
	102_103delGA, E34fsX36	1	Yes	1	1	F	30	Coecum	0	+	+	H 2/2+	1
	445C>A, Q149X	5	Yes	1	1	F	43	Coecum	0	+	+	H	1
	545+3A>G (splice donor)	6	Yes	1	1	M	31	Transversum	0	+	0	H 4/4+	1
	677+1delG (splice donor)	8	Yes	1	1	F	28	Colon	+	+	+	na	1
	677G>A, R226Q (splice donor)	8	Yes	1	3	M	55	Coecum	0	+	+	S	1
						F	65	Ascendens	0	+	+	H 4/5	
						M	46	Ascendens	0	+	0	H 4/5+	
	806C>G, S269X	10	Yes	2	1	F	52	Coecum	0	+	+	H 2/3+	1
					1	M	45	Coecum	0	+	0	H 5/5+	1
	1731+15G>A (splicedonor)	15	Yes	1	1	M	36	Colon	0	+	+	na	1
	1852_1854del, K618del	16	Yes	6	2	F	57	Flexura hepatica	0	+	+	L 1/4++	1
						M	39	Transversum	+	+	+	H 3/4	
					1	F	45	Colon	+	+	+	H 3/4+	1
					1	M	57	Colon	0	+	+	na	1
					1	F	29	Transversum	0	0	0	H 2/2+	1
					1	M	50	Descendens	0	+	0	H 5/5+	1
					1	F	68	Coecum	0	+	+	H 5/5+	1
	EX16del	16	Yes	1	1	F	44	Colon	0	+	+	na	1
	2103+1G>A (splice donor)	18	Yes	1	1	M	31	Coecum	0	0	0	na	1
	277A>G, S93G	3	?	1	2	F	90	Transversum	+	+	+	H 2/3+	3

						M	53	Transversum	0	+	0	H 4/4+	
	793C>T, R265C	10	?	1	1	M	39	Coecum	+	+	0	L 1/1+	1
	1744C>T, L582F	16	?	1	1	M	37	Flexura hepatica	0	+	+	H 3/4+	4
b. *MSH2*	Ex 3del (in frame)	3	Yes	2	2	F	29	Flexura lienalis	+	+	0	H 3/5	2
						F	23	Duodenum	+	0	0	H 3/4++	
					1	M	34	Colon	+	0	0	H 2/4+	1
	862C>T, Q288X	5	Yes	1	1	M	45	Sigmoid	+	0	0	na	1
	R308fsX333	5	Yes	1	1	M	46	Colon	+	0	0	na	1
	EX6del	6	Yes	1	1	F	37	Colon	+	0	0	na	1
	EX1_6del	1–6	Yes	2	1	M	31	Coecum	+	0	0	na	1
					2	M	28	Colon	+	0	0	na	1
						M	57	Colon	+	0	0	na	
	1139delT, L380fsX411	7	Yes	1	1	F	54	Transversum	+	0	0	H 2/3+	1
	2038C>T, R680X	13	Yes	1	2	F	44	Colon	+	0	0	H 4/5+	1
						M	61	Transversum	+	0	0	H	
	1666T>C, L556L	11	?	1	1	F	36	Sigmoid	+	+	0	L 1/3+	2
c. *MSH6*	742C>T, R248X	4	Yes	1	1	M	26	Coecum	+	+	0	H 2/4+	1
	1784delT, L595fsX609	4	Yes	1	2	M	84	Coecum	+	+	0	H 4/5+	2
						F	49	Transversum	+	+	0	S 0/5+	
	4001G>A (splice donor)	9	Yes	1	1	F	65	Coecum	+	+	+	H 5/5+++	1
	2008G>A, G670R	4	?	1	1	F	55	Sigmoid	+	+	0	H 2/4+	3
	642C>T, Y214Y	4	?	1	2	M	79	Sigmoid	+	+	na	H 2/4+	3
						M	73	Rectum	+	0	na	H 2/4+	

Table 1. MLH1, MSH2, and MSH6 Mutations
M, Male; F, female. IHC: 0, no nuclear staining; +, nuclear staining; na, not analysed. MSI: H, MSI-H; L, MSI-L; S, MSS; eg, 3/5: 3 of the 5 Bethesda markers tested instable, +, ++, +++: respectively 1, 2, or 3 of the 3 additional (BAT40, MSH3, and MSH6) markers are instable; na, no MSI analysis performed. Family diagnosis 1, Amsterdam II+; 2, suspected HNPCC; 3, late onset; 4, sporadic young age.

We defined four categories of clinical diagnoses (Table 1). The first category includes families that fulfilled the revised Amsterdam criteria (AII+) [5]. The second category includes suspected HNPCC families, ie, familial cases fulfilling the Bethesda criteria (B+) [48]. The third category encompasses late onset families consisting of three CRC patients within two or three generations, with no diagnosis made at younger than the age of 50 years. The fourth category includes sporadic patients diagnosed at younger than the age of 40 years.

3.2. MSI

MSI analysis was performed on paired tumor-normal tissue DNA samples using the Bethesda panel of microsatellite markers (D2S123, D5S346, D17S250, BAT25, and BAT26) [20]. This panel was extended with the additional markers BAT 40, *MSH3*, and *MSH6*, as previously reported [49]. Tumors were scored as MSI-H (high) if at least two of the five Bethesda markers showed instability, MSI-L (low) if only one of these markers showed instability, or MSS (stable) if none of the Bethesda markers showed any shift in mobility.

The annotations +, ++, or +++ were used to indicate if, respectively, one, two, or three of the additional (BAT40, *MSH3*, and *MSH6*) mononucleotide markers showed instability.

3.3. Tissue Microarray (TMA)

TMAs were assembled from formalin-fixed, paraffin-embedded tissues as previously described [40] using a 0.6-mm-diameter punch (Beecher Instruments, Silver Spring, MD). The arrays encompass 362 tissue cores from colorectal tumors derived from 129 (suspected) HNPCC patients, including the 45 tumors from MMR gene mutation carriers. These tumor samples dated back from 1974 until 2000. Also, we included three tissue cores from normal colonic mucosa and one core of lung tissue (for orientation purposes).

Using a tape-transfer system (Instrumedics, Hackensack, NJ), 4 µm sections were transferred to glass slides. We were unable to analyze, because of tissue loss during processing, 52 (14%), 41 (11%), and 56 (15%) of the punches for MLH1, MSH2, and MSH6, respectively. Because two to three punches were taken per tumor this meant that for six (5%), five (4%), and five (4%) of the tumors, respectively, we were unable to analyze the staining of these proteins.

3.4. IHC

Conventional IHC on whole tumor sections was performed for all tumor samples. Immunohistochemical staining was performed on 4 µm sections of formalin-fixed, paraffin-embedded tissues. Whole tissue slides and TMA slides were stained with antibodies against MLH1 (clone 14; Calbiochem, Cambridge, MA), MSH2 (clone GB12) and MSH6 (clone 44; Transduction Laboratories/Becton Dickinson, Lexington, KY) in a DAKO Techmate 500+ (Glostrup, Denmark) automated tissue stainer using standard protocols [49] and procedures as indicated by the manufacturer. We initially tested the influence of different fixation intervals on the results of IHC for MLH1, MSH2, and MSH6. Therefore tumor parts of two control cases (one colon carcinoma and one rectal carcinoma) were fixated in buffered formalin for 1, 7, and 40 days, respectively. Overall the results were comparable. When fixation was performed with ethanol (for 1 and 4 days, respectively) in comparison with fixation in buffered formalin (also for 1 and 4 days) staining results were extremely poor after fixation with ethanol. For two initially frozen tissues, subsequent fixation in buffered formalin for 1 and 4 days seems to give less strong staining than what we normally experience after immediate fixation in formalin. Furthermore, when testing

tissue blocks from laboratories that use a pretreatment step with acetone for tissues such as colon resections we seem to encounter a negative influence on the quality of the stainings of the MMR proteins.

Staining patterns of MMR proteins were evaluated using normal epithelial, stromal, or inflammatory cells, or the centers of lymphoid follicles as internal controls. The pathologist and technician who reviewed the immunostaining of the tissue samples were blinded to the germline mutation status of the patients.

Stained slides and individual cores were scored as either positive (showing nuclear staining in at least some tumor cells) or negative. To validate TMA, patients were considered positive if at least one tissue core showed nuclear staining and negative if none of the tissue cores showed nuclear staining of the protein.

4. RESULTS

4.1. MSI Analysis

MSI analysis was performed in 33 of the 45 tumors derived from HNPCC mutation carriers (Table 1). Although the majority of the cases showed a high frequency of instability (28 MSI-H), 3 MSI-L and 2 MSS tumors (one of which was MSS+), were found. One of the MSI-L tumors was found in a carrier of a pathogenic *MLH1* mutation (1852_1854del, K618del, exon 16). In this tumor, too, the BAT40 and MSH6 markers showed instability. IHC showed positive staining for MSH2 and MSH6 and absent staining for MLH1. Moreover, a CRC from an additional family member was MSI-H. The other two MSI-L tumors occurred in carriers of an unclassified variant. The MSS tumor occurred in a carrier of a pathogenic *MLH1* mutation (677G>A, R266Q, splice donor, exon 8) and could not be tested for the three additional markers BAT40, *MSH3*, and *MSH6*. IHC indicated a mutation in *MLH1*. Furthermore, two tumors from additional carriers of the same mutation were scored as MSI-H. In the MSS+ tumor, found in a carrier of a pathogenic *MSH6* mutation (1784delT, L595fsX609, exon 4), the BAT40 marker additionally showed instability. IHC showed absent staining for MSH6 while staining was positive for MLH1 and MSH2. Again, another tumor from an affected family member was MSI-H.

The BAT40 marker showed instability in 26 of the 29 tumors in which it was tested. MSI analysis, if both patients with MSI-H and with MSI-L tumors are considered candidates for mutation analysis, gives a sensitivity of 93% (25 of 27) in predicting an MMR pathogenic mutation if the five standard Bethesda markers are used and a sensitivity of 96% (26 of 27) using the BAT40, *MSH3*, and *MSH6* markers in addition to the standard markers. The increase of sensitivity is mainly because of the use of the BAT40 marker.

In addition, 71 of 84 colorectal lesions from (suspected) HNPCC patients without known mutations were tested for MSI. The vast majority (53 of 71, 74%) of these tumors were classified as MSI-H, whereas 6% (4 of 71) were MSI-L and 20% (14 of 71) MSS.

4.2. Whole Slide Immunohistochemical Analysis of Tumors from Mutation Carriers

4.2.1 Individual Staining of the MMR Proteins

Twenty of the 25 (80%) tumors derived from *MLH1* mutation carriers did not stain for the MLH1 protein. When unspecified variants are excluded, this figure rises to 18 of 21 (86%) (Table 2). The remaining three (14%) MLH1-positive tumors were found in carriers of small in-frame deletions or splice mutations. Notably, two of the MLH1-positive tumors were part of a series of seven tumors from carriers of the 1852_1854del

IHC	MMR	*MLH1*	*MSH2*	*MSH6*	Total
No nuclear staining		18 (86%)	11 (92%)	3 (75%)	
Nuclear staining		3 (14%)	1 (8%)	1 (25%)	
Total		21	12	4	37

Table 2. IHC in Carriers of a Pathogenic Mutation

mutation in exon 16. The remaining five stained negative. Contrasting MLH1-staining patterns were also obtained with tumor samples from different carriers of the 277A>G, S93G missense mutation. The third MLH1-positive case was found in a carrier of a splice donor (677 + 1delG) mutation (according to the splice site prediction program, BDGP splice site prediction by Neural Network, the value decreased from 0.98 to 0.14).

Eleven of 13 tumors (85%) from *MSH2* mutation carriers show no MSH2 staining. Again, this percentage increases when unspecified variants are excluded (11 of 12, 92%) (Table 2). MSH2-positive staining was observed in only one of three tumors from carriers of an in-frame exon 3 deletion.

Seventy-five percent (three of four) of the tumors from carriers of a pathogenic *MSH6* mutation show no staining for the corresponding protein. Of the three tumors from patients with an *MSH6* unspecified variant, only one (G670R) tumor could be analyzed: no MSH6 staining was found, thus indicating, but not proving, pathogenicity of this mutation. When unspecified variants are included 80% (four of five) of the tumors from *MSH6* mutation carriers show absent staining of the corresponding protein.

To determine sensitivity of IHC in detecting MSI in general we considered all tumor samples that showed abrogation of at least one of the three proteins tested to be positive for MMR deficiency. In 86% (18 of 21), 100% (12 of 12), and 75% (3 of 4) of tumors from carriers of a *MLH1*, *MSH2*, or *MSH6* pathogenic mutation, respectively, absent staining for at least one of the three proteins was shown. MMR deficiency would thus have been detected in 89% (33 of 37) of the cases.

4.2.2 Staining Patterns

MMR-IHC analysis in carriers of pathogenic *MLH1* mutations revealed that in only 48% of the cases was an MLH1-negative staining accompanied by normal MSH2 and MSH6 staining patterns (Table 3). In these cases, the IHC results clearly direct the mutation analysis to a single gene, namely *MLH1*. However, in another subset (24%) of the *MLH1*-mutant tumors, an MSH6-negative staining pattern accompanied the loss of MLH1

IHC	MMR	*MLH1*	*MSH2*	*MSH6*
1+/2+/6+		3 (14%)		1 (25%)
1-/2+/6+		10 (48%)		
1+/2-/6+				
1+/2+/6-			1 (8%)	3 (75%)
1-/2+/6-		5 (24%)		
1+/2-/6-			11 (92%)	
1-/2-/6+				
1-/2-/6-		3 (14%)		
Total		21	12	4

Table 3. IHC Staining Pattern in Carriers of a Pathogenic Mutation
1, MLH1; 2, MSH2; 6, MSH6; +, nuclear staining; -, no nuclear staining.

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signal, thus providing a more ambiguous indication for the subsequent mutation analysis. In three tumors (14%) positive staining for all three proteins was found. All three were scored as MSI-H. Therefore, these patients would not have been considered candidates for mutation analysis if IHC alone had been performed. Another three tumors (14%) showed no staining for all three proteins. Notably, negative staining patterns for all three MMR proteins were found exclusively in combination with a germline *MLH1* mutation (Table 1 and 3).

In the vast majority of tumors from pathogenic *MSH2* mutation carriers (11 of 12, 92%), loss of the MSH2 signal is accompanied by MSH6-negative and MLH1-positive staining patterns. Only in one case (exon 3 deletion) were the corresponding MSH2 and MLH1 signals positive while MSH6 staining was lost. The latter would have unjustly indicated mutation analysis of the *MSH6* gene. However, IHC analysis of a tumor from an additional patient from the same family clearly showed both MSH2- and MSH6-negative staining patterns.

In the three of four cases with a pathogenic *MSH6* mutation, the expected MSH6-negative staining is accompanied by normal MLH1 and MSH2 patterns. In the fourth case, in which it was predicted that a missense mutation would affect RNA splicing (4001G>A, splice donor), positive staining for all three MMR proteins was found. The tumor was MSI-H (Table 1c).

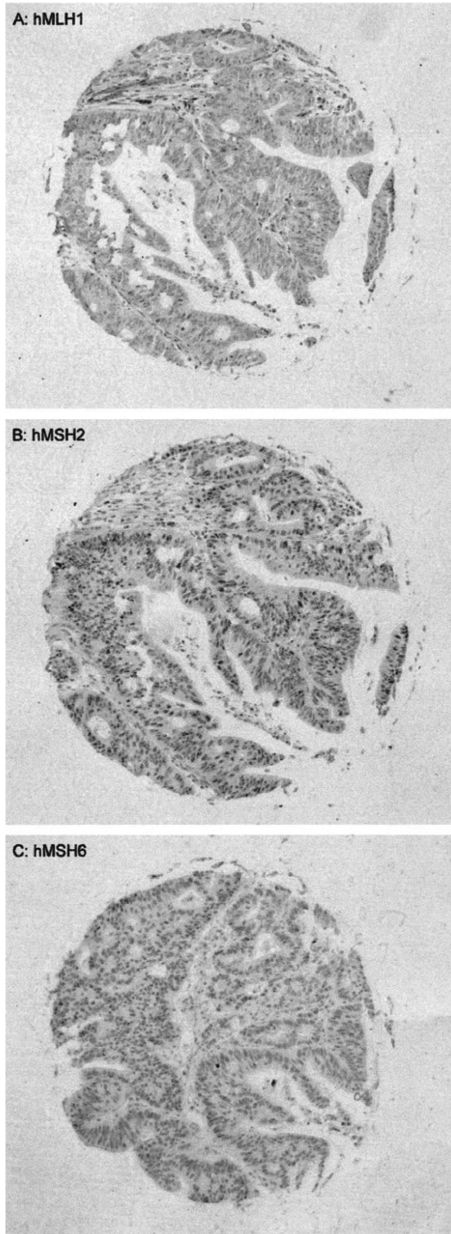
4.3. Tissue Microarray Immunohistochemical Analysis (TMA-IHC)

A TMA encompassing the total cohort of 129 colorectal tumors was generated. We evaluated TMA-IHC staining for the presence or absence of the three main MMR proteins in the (suspected) HNPCC tumors and compared these with the results obtained by whole tumor section IHC when available (Table 4). An example of the staining pattern in a tumor from an *MLH1* mutation carrier (1744 C>T, L582F; Table 1a) is shown in Figure 1 for the MLH1 (Figure 1A), MSH2 (Figure 1B), and MSH6 (Figure 1C) protein, respectively; MLH1 is abrogated, whereas MSH2 and MSH6 are present in the nuclei of the tumor cells.

Staining was concordant in 71 of 84 (85%) cases tested for MLH1, and in 77 of the 81 (95%) for MSH2. A somewhat lower level of concordance was found for MSH6: only 49 of 65 (75%) tumors showed similar results, mainly because of a high number [13] of positive staining results in TMA, scored as negative on whole slides. Of the latter samples six belonged to *MLH1* mutation carriers (all AII+), two to *MSH2* mutation carriers (all AII+), one to an *MSH6* mutation carrier and four samples belonged to individuals in whom no mutation was identified (2 times AII+, 2 times AII-, B+).

	ws +, tma +	ws +, tma -	ws -, tma -	ws -, tma +	Total	Concordance	Sensitivity	Specificity
MLH1	52	10	19	3	84	85%	84%	86%
MSH2	55	2	22	2	81	95%	96%	92%
MSH6	23	3	26	13	65	75%	88%	67%

Table 4. Validation of TMA for Mismatch Repair Proteins
Staining results of the array compared to results of staining of whole slides from the same patients for MLH1, MSH2, and MSH6. Staining was scored as either positive or negative, as described above. Tma, tissue microarray; ws, whole slide; +, positive nuclear staining; -, negative nuclear staining; conc, percentage concordance; sensitivity (percentage of true positives), specificity (percentage of true negatives).



5. DISCUSSION

The identification of MMR gene mutations in suspected HNPCC families is of great relevance for allowing the identification of mutation carriers for whom surveillance of the colon is required and has been proven to lower the risk to develop and to die of colorectal carcinoma [50]. A potential problem in the everyday clinical practice is that MMR genetic testing is expensive and time-consuming. In this study, we first evaluated the sensitivity of conventional whole section IHC analysis of MLH1, MSH2, and MSH6 in colon tumors from 45 established carriers of a MMR gene mutation and compared it with MSI analysis.

The sensitivity of IHC in predicting a pathogenic mutation was 89% (33 of 37), only slightly lower than that of MSI analysis using the Bethesda panel of five markers (93%, 25 of 27), or using the additional three markers (96%, 26 of 27). For IHC these results are remarkable because the paraffin blocks dated back from 1976 until 1999, with 35% of the samples older than 10 years, and fixation until now not fully standardized. We argue that intratumor heterogeneity will not be a problem in hereditary cases because of the fact that loss of MMR, and consequently often abrogation of MMR protein expression, is such an early event that it is present in all tumor cells. Should IHC become a standard of care in unselected cases heterogeneity is an issue that still needs further investigation, although in colorectal tumors with a MMR defect because of somatic abrogation of *MLH1*, this feature seems to be a dominant characteristic, as

Figure 1. Immunoreactivity in a TMA of mainly (suspected) HNPCC patients. A tissue core with a colon carcinoma from a patient with a germline hMLH1 missense mutation (1744C>T, L582F) is shown, stained for MLH1 (A), MSH2 (B), and MSH6 (C). MLH1 is abrogated, whereas MSH2 and MSH6 are present in the nuclei of the tumor cells. Slides were stained with antibodies against MLH1 (clone 14, Calbiochem, Cambridge, MA), MSH2 (clone GB12, Calbiochem), and MSH6 (clone 44, Transduction Laboratories/Becton Dickinson, Lexington, KY). Original magnifications, x100.

can be interpreted from a study on such heterogeneity [51].

An important advantage of IHC compared to MSI analysis is represented by the prediction of the specific MMR gene mutated in the germline of the corresponding patient. In tumors from most *MLH1* mutation carriers (80%), staining of the MLH1 protein was absent, as expected. However, in only half (48%) of the tumors associated with a MLH1 mutation, the staining pattern (MLH1-, MSH2+, MSH6+) would have predicted unequivocally a pathogenic mutation in the *MLH1* gene. In the future, the inclusion of PMS2 staining, which is often negative in tumors associated with *MLH1* germline mutations [52], will most likely lead to a further increase of IHC sensitivity.

Negative staining for both MSH2 and MSH6 was found in tumors from *MSH2* mutation carriers. Tumors from *MSH6* mutation carriers, showed a lack of MSH6 staining only. These findings are most likely because of the failure of MSH6 to form a stable heterodimer in the absence of MSH2 [53]. On the other hand, if MSH6 is absent, a heterodimer can still be formed between MSH2 and MSH3, thus resulting in stabilization and positive staining of the MSH2 protein [49,54]. In our study, the specific staining pattern (MLH1+, MSH2-, MSH6-)of *MSH2* mutated tumors would have correctly predicted the mutated MMR gene in all but one (92%) of the cases, while the specific staining pattern of tumors from *MSH6* mutation carriers (MLH1+, MSH2+, MSH6-) would have predicted the presence of a *MSH6* mutation in 75% of the cases.

In previous smaller studies, the sensitivity of IHC and MSI analysis has been evaluated in colorectal tumors of carriers of specified *MLH1*, *MSH2* [25,30,32,35,55], and *MSH6* [27,54,56] mutations. A problem we cannot solve is the possibility that variable outcomes of IHC analyses might be because of differences in staining protocols and antibodies used. Furthermore, a considerable number of mutations included in these studies are unclassified variants in which pathogenicity is by definition uncertain. In the present study, only pathogenic mutations were included in the determination of the sensitivity of IHC on whole slides.

The results of our study show that both IHC and MSI are sensitive prescreening methods to identify patients for mutation analysis. At present, IHC cannot completely replace MSI analysis until the sensitivity of MLH1 staining is improved, as recently discussed by de la Chapelle [57], and as long as the role of other putative MMR genes in hereditary CRC has not been elucidated. Because of its gene predictive value, and because of its speed and low cost, we would recommend IHC as a first diagnostic step in families fulfilling the revised Amsterdam criteria in which the probability of detecting a MMR gene mutation is relatively high [58] and MSI analysis is likely to give superfluous information. If a negative staining pattern is found, mutation analysis of the respective gene(s) is the next step. In case of doubtful interpretation or positive staining of all MMR proteins, MSI analysis should be performed In the case of the absence of microsatellite instability (MSS), the analysis of a second tumor from the same family is recommended, to exclude intrafamilial variability in MSI analysis and IHC results, as shown in this study, and/or the presence of phenocopies.

In families not fulfilling the Amsterdam criteria, we would recommend MSI analysis as the first step. In these cases, the probability of detecting a *MLH1* or *MSH2* mutation is low [58] and IHC is less likely to be informative. *MSH6* families, predominantly found not to comply with the Amsterdam criteria, represent exceptions. In the total group of Amsterdam-negative families MSI analysis is expected to provide

global information on loss of MMR function, including pathogenic missense mutations and alterations in MMR genes other than the known ones. In MSI-H or MSI-L (if the unstable marker is a mononucleotide marker) cases, IHC of all four MMR proteins should be performed as second step. In the case of MSS, IHC for MSH6 is recommended as it was shown that tumors from MSH6 mutation carriers are characterized by a variable MSI phenotype [54,59]. If no IHC abnormality is found, examination of a second tumor could be considered depending on the family history and age of the patient already tested. A scheme for clinical use, summarizing our current approach to patients from families with suspected HNPCC, is given in Figure 2.

The generation of a tissue array encompassing microsatellite unstable tumors has provided us with a powerful tool to quickly characterize the immunohistochemical staining patterns of MMR proteins in hereditary colorectal tumors for research purposes. We found a high level of concordance for MLH1 and MSH2 (85% and 95%, respectively). A somewhat lower concordance level was found for MSH6 (75%), primarily because of positive staining within the TMA and negative staining with the whole slide IHC. Six of the 13 tumors with discordant results for MSH6 originated from patients in whom an *MLH1* mutation has been identified, where positive staining for MSH6 is expected. Two and one samples originated from carriers of a MSH2 and MSH6 mutation, respectively, where negative staining for MSH6 is expected. The other four samples originate from individuals in whom to date no mutation is identified and therefore no golden standard is available. Our first goal is to rapidly characterize the staining of other candidate MMR proteins particularly in tumors from (suspected) HNPCC patients in whom to date

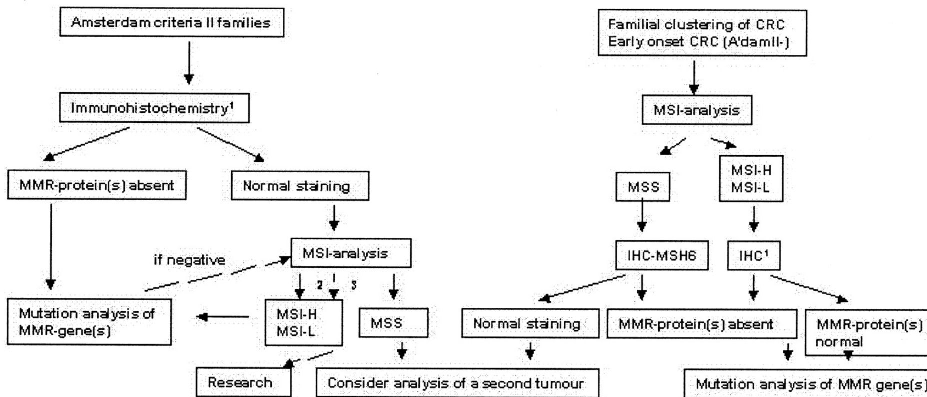


Figure 2. Approach of patients with familial clustering of CRC. 1: IHC for MLH1, MSH2, MSH6, PMS2. 2: If the tumor is MSI-H, mutation analysis is the next step. 3: If the tumor is MSI-H and mutation has already been performed, research is the next step.

no mutation was detected, to direct mutation analysis. Problems relative to differences in fixation standardization, age of tissues, punching outside the tumor area, and loss of tissue still represent serious obstacles. For fixation standardization we tend to suggest fixation for 1 day in buffered formalin. Use of ethanol fixation and acetone pretreatment should be avoided (see Patients and Methods). However, our study shows the general validity of this approach in the molecular diagnosis of familial CRC. Accordingly, recent IHC-TMA analysis has enabled us to identify a number of patients with abrogated staining of PMS2 with or without MLH1 staining, thus providing direction to mutation analysis of PMS2.

In conclusion, we have demonstrated the value of IHC using both whole slides and TMA as prescreening tools in selecting patients eligible for mutation analysis of MMR genes, in diagnostic and research settings respectively.

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Frequent mutations in the 3' untranslated region of *IFNGR1* lack functional impairment in microsatellite-unstable colorectal tumours

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1. ABSTRACT

Microsatellite repeats are frequently found to be mutated in microsatellite-instable colorectal tumours. This suggests that these mutations are important events during tumour development. We have observed frequent mutations in microsatellite-instable (MSI-H) tumours and cell lines of a conserved A14 repeat within the 30-untranslated region of the *interferon γ receptor 1 gene (IFNGR1)*. The repeat was mutated in 59% (41 of 70) of colon carcinomas and in all four MSI-H colon cancer cell lines tested. In-vitro analysis of these cell lines did not show a decreased responsiveness to standard IFNγ concentrations when compared to microsatellite-stable colon cancer cell lines. A functional consequence of the frequently found microsatellite instability in *IFNGR1* is therefore not evident.

2. INTRODUCTION

DNA mismatch repair deficiency in tumours is characterized by a high frequency of microsatellite instability (MSI-H)[1]. Microsatellite instability is associated with the hereditary non-polyposis colorectal cancer syndrome and also appears in approximately 15% of sporadic colorectal tumours [2]. A subset of genes that encompass coding microsatellites may be specifically targeted in MSI-H tumours. Systemic sequence database searches have identified in the human genome up to 17.000 intra-exon coding repeat sequences with a length of six or more nucleotides [3,4].

Frameshift mutations in MSI-H colorectal tumours have been demonstrated for genes involved in signal transduction (*TGFBRII*, *IGFIIR*, *PTEN*), apoptosis (*BAX*, *CASPASE 5*), DNA repair (*hMSH3*, *hMSH6*, *MBD4*), transcriptional regulation (*TCF-4*) and immune surveillance (*B2M*), with mutation frequencies ranging from 4 to 80% [5]. Non-coding microsatellite repeats, however, may just as well be mutated. Of those with potential functional significance are those that lie within the untranslated regions (UTR) of genes. Elements in such regions can regulate mRNA degradation, translation and localization [6–8]. However, such elements have not been extensively studied for and their identification is complicated by the fact that their activity often depends on specific secondary RNA structures [9]. Suraweera et al [10] characterized frequent mutations in a number of conserved mononucleotide repeats within UTRs and high levels of mutations were found in a novel T25 mononucleotide marker in the 3' UTR of the *CASP2* gene [11].

Interferon γ (IFNγ) is important in regulating cell mediated immune responses. It directly increases the sensitivity of a target cell to CD8+ cytotoxic lymphocyte attack by upregulating the expression of a variety of genes, including the *human leukocyte antigen (HLA) class I genes* and *Fas/CD95* [12]. Mismatch repair-deficient tumours potentially exhibit a large number of tumour-specific 'frameshift' antigens due to instability of coding microsatellite sequences. This has been suggested to render them more susceptible to both native and therapeutically induced antitumour immune responses [13]. Indeed, these tumours are associated with an elevated CD8+ intra-epithelial infiltrate [14,15]. Tumour IFNγ responsiveness is important in regulating anti-tumour immune response in vivo, partly depending on antigen presentation [16,17]. Downregulation of the IFNγ receptor has been shown in hepatocellular carcinoma [18], basal cell carcinoma [19] and virus-associated tumours [20,21], and was correlated with larger

tumour size and a higher frequency of metastases [18]. IFNγ also increases the sensitivity to chemotherapy-induced Fas-mediated apoptosis [22]. Although the use of IFNγ solely has yielded somewhat disappointing results in clinical anticancer treatment trials [23], its use in combination with different chemotherapeutics, such as 5-fluorouracil, indomethacin, phenyl butyrate, mitomycin C, cyclosporine A or clarithromycin has shown synergetic anti-tumour effects [24 – 30]. Thus, loss of IFNγ responsiveness would denote a loss of potential for future immuno- and chemo-based adjuvant therapies.

In this study, we describe frequent mutation of the A14 repeat within the 3' UTR of *IFNGR1*. As will be discussed, it is difficult to draw conclusions regarding its importance on basis of frequencies alone. Therefore, we also analysed IFNγ responsiveness of colon cancer cells bearing these mutations.

3. MATERIALS AND METHODS

3.1. Colon tumour samples and cell culture

Tumour samples were derived from formalin-fixed, paraffin embedded material sent to our department for MSI analysis between 1999 and 2002. Cases were analysed following the medical ethnical guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences. The present study falls under approval by the Medical Ethical Committee of the LUMC (protocol P01–019).

Cell lines HCT 15, LoVo, LS180, LS411N, SW480 and SW837 were obtained from the American Type Culture Collection (Atlanta, GA, USA) and cultured and harvested according to standard methods.

3.2. Microsatellite instability and sequence analysis

MSI and sequence analyses were performed as described previously [31]. MSI analysis was performed using the NCI markers BAT25, BAT26, D2S123, D5S346, D17S250 [32], supplemented with BAT40, *MSH3* and *MSH6*. Tumours were classified as MSI-H (instability of at least 30% of the markers) or MSS (no instability). IFNGR1 was analysed using forward primer 50-GAGGATGTGTGGCATTTTCA-30 and reverse primer 50-TGCTATACCAAGGCAGAGAAAAG-30.

3.3. IFNGR1 mRNA expression

Total RNA was isolated using TriZol (Ambion, Austin, TX, USA) following the manufacturer's recommendations. RNA (2 mg) was used for cDNA synthesis using Oligo dT primers and AMV Reverse Transcriptase (Roche Applied Science, Penzberg, Germany). We performed quantitative real-time PCR using SYBR Green (Eurogentec, Seraing, Belgium) on an iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Ct values were calibrated to an amount of Human Total Colon RNA (Clontech, BD Biosciences, Palo Alto, CA, USA) and normalized to the level of expression of three stably expressed housekeeping genes *HNRPM*, *CPSF6* and *TBP* [33]. Calibrated Ct values were divided by Ct values of 2 mg Human Total Colon RNA. Primers for amplification of IFNGR1 mRNA used were 50-TCCTCAGTGCCTACACCAACTAA-30 (nucleotides 79–101, exon 1–2), and 50-CTCGTCACAATCATCTTCCTTCTG-30 (nucleotides 568–591, exon 5).

3.4. Flow cytometry of IFNγ-stimulated cells

HLA I and Fas expression was analysed by flow cytometry of viable cells as described previously [34,35] using the primary antibodies W6/32 (supernatant), 2R2 (Alexis Biochemicals,

Carlsbad, CA, USA), and mouse isotype controls (Dako Cytomation, Glostrup, Denmark). Samples containing 1 mM propidium iodide (Calbiochem, San Diego, CA, USA) were collected on an FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Linearized mean fluorescence intensity values were determined by WinList (Verity Software House Inc., Topsham, ME, USA) and subtracted by control values. Means derived from duplicate experiments were used; single experiments encompassed one-session analyses of all cell lines using the same instrument settings. We constructed a dose–response curve using 8, 40, 200 and 1000 U/ml IFNγ (PeproTech Inc., Rocky Hill, NJ, USA) for 48 h. All cell lines were already maximally responsive to a concentration of 8 U/ml. The extent of upregulation was further analysed using 200 U/ml IFNγ.

3.5. Fas-induced apoptosis

To quantify Fas-induced apoptosis using 1 mg/ml 2R2 (Alexis Biochemicals) and 1 mg/ml protein A for 6 h, percentages of apoptotic cells were determined using the Nicoletti assay as described previously [22,36]. For analysis we used a modified ModFit algorithm previously described [37] and kindly provided by Verity Software House Inc. Means derived from duplicate experiments were used.

4. RESULTS AND DISCUSSION

Frequently found MSI suggests a clonally selective advantage, although passenger mutations due to a possible lack of selection pressure of innocent bystander genes, ie genes whose function is irrelevant during tumourigenesis, cannot be excluded [38]. For *TCF-4*, it has been shown that frameshift mutations due to instability of a coding A9 repeat lack functional consequences [39]. Therefore, comprehensive criteria have been proposed at the Bethesda meeting to define valid target genes in MSI-H tumours, including (1) a high frequency of mutation, (2) bi-allelic inactivation, (3) involvement in a tumour suppressor pathway, which is (4) also involved in MSS tumours and (5) functional consequences of the mutation [2]. However, few reports have taken these criteria into account [40,41] and statistical methods have been proposed to identify valid target genes by statistical analysis of mutation frequencies only [42,43].

To examine the (in)stability of the *IFNGR1* repeat we first analysed 17 MSS colon cancer samples and 2 MSS colon cancer cell lines (SW480 and SW837). In all cases the repeat was conserved, ie monomorphic [10]. Subsequently, 70 MSI-H colon tumour specimens were tested from patients complying with the clinical Bethesda criteria [1], 38 of whom a germ line mutation was identified in one of the MMR genes. MSI in the repeat was found in tumours from 12 of 20 (60%) *hMLH1* patients, 6 of 12 (50%) *hMSH2* patients, 2 of 6 (33%) *hMSH6* patients, in 21 of 32 (66%) of the residual MSI-H tumours and in 4 of 4 (100%) MSI-H colon cancer cell lines.

The frequency of mutations in microsatellite tracts is associated with the number and type of repeats [32,44]. Previous studies reported a mutation frequency up to 54% in 29 mononucleotide repeats (8–10 bp) within intronic sequences;38 in conserved repeats instability was found in up to 28% of tumours only [42]. Furthermore, G-repeats are considerably more prone to mutations than A-repeats [38]. In 19 conserved UTR repeats studied [10] a mutation frequency was found up to 95%, but the repeat lengths ranged from 15 up to 32 nucleotides. Led by the Bethesda criteria [2] we decided to study whether 59% MSI in the A14 repeat is truly indicative of a valid target.

First, we studied bi-allelicity of the mutations. The microsatellite analysis used cannot discriminate between mono- or bi-allelic mutations of tumour tissue due to contaminating normal cells. Therefore, we analysed the cell lines described above and performed sequence analysis for confirmation of the observed deletions. A bi-allelic A3 deletion was detected in LoVo and LS411N, in LS180 both an A4 and an A5 deletions were seen and in HCT 15 only a mono-allelic deletion of a single A was observed. No mutations in the MSS cell lines SW480 and SW837 were observed in line with the MSI results.

To detect a possible decline in IFNGR1 mRNA expression upon 3′ UTR mutations we applied quantitative RT-PCR. The receptor is normally expressed on colon epithelium [45] and no decreased IFNGR1 mRNA expression of the MSI-H colon cancer cell lines was observed when compared to the MSS cell lines (Table 1).

To rule out other regulatory effects of the 3′ UTR mutation we investigated the extent of IFNγ-mediated HLA class I and Fas/CD95 upregulation in MSI-H compared to MSS colon cancer cell lines. HLA class I molecules are not present on the cell surface of LoVo and HCT 15 cells, due to mutations of the light chain *β2-microglobulin* [46]. We analysed dose–response curves to make sure the experiments were performed under conditions leading to maximum responsiveness. No decreased responsiveness to IFNγ was observed for MSI-H cell lines compared to the MSS cell lines (Table 1). To study the effect of IFNγ on Fas-induced apoptosis, we measured the fraction of apoptotic cells as quantified by flow cytometry. Again, no impaired IFNγ response was noted in the MSI-H cell lines (Table 1).

Although, the concentrations used are similar to other studies on in-vitro IFNγ responses in colon cancer cell lines [47], we cannot completely rule out a differential response to far lower concentration of IFNγ not yielding maximum response.

In conclusion, we identified a conserved A14 microsatellite repeat within the 3′ UTR of the potential tumour suppressor *IFNGR1* that is mutated in 59% of MSI-H colorectal tumours. Compared to MSI of other conserved, non-coding but transcribed short mononucleotide tracts, the high frequency of instability suggests it to be a valid target during colorectal tumourigenesis. However, *in vitro* analysis did not show an impaired response to IFNγ in any cell line harbouring the mutated gene questioning

		HLA I			*Fas*			*Fas-ind. apoptosis*		
	mRNA	*-*	*+*	*f.i.*	*-*	*+*	*f.i.*	*-*	*+*	*f.i.*
MSS										
SW480	0,1	442,3	1801,8	4,1	28,6	30,3	1,1	17,1	34,1	2,0
SW837	1,1	657,0	1517,4	2,3	16,6	25,6	1,5	11,8	40,5	3,4
MSI-H										
HCT-15	1,0	ne	ne	ne	22,6	32,5	1,4	8,9	85,8	9,6
LoVo	0,9	ne	ne	ne	36,0	22,3	0,9	67,7	81,7	1,2
LS411N	1,3	60,7	375,5	6,2	5,1	22,3	4,4	11,7	61,6	5,3
LS180	1,0	210,6	369,5	1,8	25,0	51,7	2,1	29,2	80,9	2,8

Table 1 IFNγ receptor expression and IFNγ responsiveness of colon cancer cell lines HLA I Fas Fas-induced apoptosis. Abbreviations: f.i., fold increase; NE, not expressed. Displayed are normalized Ct values as derived from IFNGR1 qRT-PCR, fluorescence intensity values of HLA class I and Fas surface expression acquired by flow cytometry and percentages of apopotopic cells after incubation with Fas as determined by propidium iodide staining. + and-: with or without IFNγ stimulation.

the functional consequences of MSI in this repeat. High mutation rates as a consequence of unidentified sequence-dependent factors, chromatin structure or mechanisms associated with transcription may contribute to the high frequency of mutations found in tumours in the absence of selective advantage [38,40,48]. Studying functional consequences seems indispensable to describe the role of these mutations in tumourigenesis rather than the frequency of genomic instability per se [49,50]. Despite a high frequency of MSI of the 3' UTR of *IFNGR1*, it probably does not contribute to colorectal tumour progression.

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High-resolution analysis of HLA class I alterations in colorectal cancer

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1. ABSTRACT

1.1. Background

Previous studies indicate that alterations in Human Leukocyte Antigen (HLA) class I expression are frequent in colorectal tumors. This would suggest serious limitations for immunotherapy-based strategies involving T-cell recognition. Distinct patterns of HLA surface expression might conceal different immune escape mechanisms employed by the tumors and are worth further study.

1.2. Method

We applied four-color multiparameter flow cytometry (FCM), using a large panel of alloantigen-specific anti-HLA-A and -B monoclonal antibodies, to study membranous expression of individual HLA alleles in freshly isolated colorectal cancer cell suspensions from 21 patients.

1.3. Results

Alterations in HLA class I phenotype were observed in 8 (38%) of the 21 tumors and comprised loss of a single A or B alleles in 4 cases, and loss of all four A and B alleles in the other 4 cases. Seven of these 8 tumors were located on the right side of the colon, and those showing loss of both HLA-A and -B membranous expression were all of the MSI-H phenotype.

1.4. Conclusion

FCM allows the discrimination of complex phenotypes related to the expression of HLA class I. The different patterns of HLA class I expression might underlie different tumor behavior and influence the success rate of immunotherapy.

2. BACKGROUND

The high morbidity and mortality of colorectal cancer underscores the need for new, more effective adjuvant treatment strategies. One promising approach centers on boosting cell-mediated anti-tumor immune responses. Numerous tumor-associated peptides have been identified that can be specifically targeted with vaccine-based immune therapy strategies [1]. Specifically, such strategies may be employed for colorectal tumors with a so-called microsatellite instability-high (MSI-H) phenotype [2,3]. These tumors are characterized by a deficient DNA mismatch repair mechanism which results in an abundance of frame shift mutations of (coding) microsatellite repeat sequences. The peptides produced by such frame shift mutations can evoke specific anti-tumor immune responses [4-6]. Nevertheless, however promising the approach, its success is dependent on the extent of operational immune surveillance mechanisms in the tumor.

Human Leukocyte Antigen (HLA) class I molecules are of major importance for cell-mediated anti-tumor immune responses. Expression of HLA class I/β2-microglobulin (β2-m) complexes carrying tumor-specific peptides is a prerequisite for adaptively matured cytotoxic T cells (CTLs) to be able to recognize tumor cells [7]. HLA class I antigens are encoded by a family of highly polymorphic genes, with each allele responsible for a different repertoire of antigen presentation. Thus, even the loss of a single

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allele could potentially allow the escape from an antigen-specific anti-tumor response. Loss of expression of HLA class I molecules has been frequently reported for colorectal tumors [8-10]. This would therefore represent a serious limitation for vaccine-based anti-tumor therapies.

However, these studies have primarily been based on immunohistochemical analyses (IHC) and therefore have a number of intrinsic limitations [11]. HLA staining by IHC is often strongly cytoplasmic, which could potentially obscure functionally relevant membranous co-expression and result in a false negative interpretation. Additionally, adequate study by IHC is hampered by the limited choice of antibodies (Abs) available for the analysis of formalin-fixed, paraffin-embedded tissue and importantly, despite the fact that the use of fresh frozen tissue allows the employment of a higher number of Abs, complete panels of the latter are not yet readily available. Another important consideration is that certain HLA class I complexes also mediate inhibitory signals through receptors expressed by CTLs and natural killer (NK) cells, averting the so-called 'missing self' recognition [12,13]. Hence, an effective tumor immune escape mechanism could occur through a subtle alteration of the tumor cell HLA phenotype, circumventing both CTL and NK cell attack. Thus far, it has not been technically feasible to study these intricacies comprehensively, and the molecular mechanisms proposed to generate HLA alterations have been contradictory, as they could not always account for the observed phenotypic alterations.

In an attempt to overcome these limitations, we have employed four-color multiparameter flow cytometry (FCM) on freshly isolated tumor cells using a large panel of human alloantigen specific monoclonal antibodies (mAbs). This approach has numerous advantages: i) it allows for the study of complex phenotypes, ii) FCM is much more sensitive than IHC, and iii) it is possible to study membranous HLA expression alone. Using the FCM technique we were able to distinguish two distinct patterns of altered HLA expression restricted to specific colorectal tumor subsets.

3. METHODS

3.1. Patient material

Fresh tumor samples, macroscopically identified by a pathologist (HM), were collected from 21 patients with surgical resections between 2001 and 2003 at the department of Surgery of the Leiden University Medical Center. Peripheral blood samples were collected from 15 patients pre-operatively. There was no pre-selection of the patients included in this series. All patients agreed to participate and provided written informed consent, and the study was approved by the local ethical review committee.

3.2. HLA genotyping

DNA was extracted from peripheral blood leucocytes using a standard salting-out procedure. Patients were *HLA-A* and *-B* genotyped using PCR-sequence-specific oligonucleotide probes (Dynal Biotech Ltd., Wirral, U.K.), as described previously [14]. Patient HLA genotype is shown on Table 2.

3.3. Tissue dissociation

Minced fresh tumor samples were weighed, cut in fragments of approximately 1 mm^3, and incubated over night at 4°C with 5 ml/g serum-free RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with 50 IU/ml penicillin-streptomycin (ICN Biomedicals Inc., Aurora), 128 U/ml collagenase type I (Sigma-Aldrich, St. Louis, MO), 100 U/ml hyaluronidase type V Sigma-Aldrich), and 250 U/ml DNAse I (Sigma-Aldrich). The next day, the same suspension was incubated for

mAb	species	isotype	HLA specificity	*reference*
116.5.28	M	IgG2a	Bw4	*
126/39	M	IgG3	Bw6	*
A11.1M	M	IgG3	A11, A24	*18*
BB7.2	M	IgG2b	A2, Aw68	*18*
Bbm1	M	IgG2b	β 2-m	*18*
GAP.A3	M	IgG2a	A3	*18*
MA2.1	M	IgG1	A2, B17	*18*
SFR8.B6	R	IgG2b	Bw6	*18*
W6/32	M	IgG2a	HLA -A,-B,-C	*18*
BV 8E9	H	IgM,κ	A28, A33, A34	*16*
BVK 5B10	H	IgM,κ	B8	†
BVK 5C4	H	IgM,κ	A80, A9	*21*
DMS 4G2	H	IgG1,λ	B62, B35	*17*
FVS 4G4	H	IgM,κ	B15, B17, B5, B37, B16, B18, B35	*16*
GK 31F12	H	IgM,κ	B13	*16*
GV 5D1	H	IgG1,λ	A1, A9	*16*
GVK 10H7	H	IgM,λ	B5, B35, B18, B37, B38, B14, B77, B72, B53	†
GVK 4H11	H	IgM,κ	B35, B62, B5, B16, B18, B37, B53, B70, B14	†
HDG 2G7	H	IgG1,κ	A19, B17, B63, B47	*16*
HDG 8D9	H	IgG1,λ	B51, B35	*16*
IN 2D12	H	IgM,λ	B15, B35, B21, B70	*16*
JOK 3H5	H	IgM,λ	B40, B21, B13, B12, B41, B70	†
KAL 3D5	H	IgG1,λ	B51, B52, B77	*17*
KG 30A7	H	IgM,λ	B27, B12, B14, B49	*18*
KLL 5E10	H	IgG1,κ	B51, B52	†
MUS 4H4	H	IgG1,λ	Bw4	†
Nie 44B8	H	IgM,κ	A10	*16*
OK 1C9	H	IgM,λ	A3, A11, A33, A31, A26	*18*
OK 2F3	H	IgM,κ	A3	*16*
OK 2H12	H	IgM,κ	A11, A3, A36, A32, A1	*16*
OK 3C8	H	IgM,κ	A3, A11, A32, A36, A31	*16*
OK 4F10	H	IgM,κ	A1, A3, A11, A31, A33, A26, A29, A30	†
OK 4F9	H	IgM,κ	A1, A36, A3, A11, A34, A66, A26, A29, A30, A31, A33	*16*
OK 5A3	H	IgM,λ	A1, A3, A11, A24, A36	*18*
OK 6H10	H	IgM,κ	B15, B21, B56, B35, B72	*16*
OK 6H12	H	IgM,κ	B21, B56, B70, B35, B62	*18*
ROU 9A6	H	IgG3,λ	B12, B13, B40, B21, B41	†
SN 607D8	H	IgG1,κ	A2, A28	*16*
SN 66E3	H	IgM,κ	A2, A28	*16*
vD1F11	H	IgM,λ	B62, B35, B57, B21, B56, B70, B55	*16*
VTM 1F11	H	IgG1,κ	B27, B7, B60	†
VTM 4D9	H	IgG1,κ	B7, B27	†
VTM 9A10	H	IgG1,κ	B7, B27	†
WAR 5D5	H	IgG1,κ	B7, B27, B42, B55	†
WIM 8E5	H	IgG1,κ	A1, A10, A11, A9, A29, A30, A31, A33	†
WK 3D10	H	IgM,κ	A2, A3, A23, A31, B7, B13, B17, B21, B40, B62	†
WK 4E3	H	IgM,λ	A locus (not A1, A24)	†

Table 1. Monoclonal anti-human antibodies used for flow cytometry analysis of HLA Class I expression. M, Mouse; H, Human; R, Rat; *, kindly donated by Dr. K. Gelsthorpe, Sheffield, U.K.; †, this paper.

90 min at 37°C followed by two washing steps with RPMI including 10% FCS (Invitrogen) to block proteolysis, and Hank's Balanced Salt Solution (Sigma-Aldrich). Cells were incubated for another 15 min at 37°C with 0.25% Trypsin (Invitrogen), 1 mM EDTA in Hank's Balanced Salt Solution, chilled to 4°C, and washed with RPMI with 10% FCS. Finally, the suspension was filtered through a 100 µm sieve (Verseidag-Industrietextilien GmbH, Kempen, Germany) and used immediately for flow cytometry.

3.4. Anti-HLA antibodies

A large panel of human and mouse anti-HLA mAbs was used for FCM (Table 1), some of which were previously described [15-17]. To establish the activity of the human mAbs (hu-mAb), peripheral blood lymphocytes were isolated from multiparous women who had developed HLA alloantigen specific antibodies during pregnancy, and the lymphocytes transformed with Epstein Barr virus (EBV). HLA antibody producing EBV cell lines were stabilized by electrofusion and rigorous cloning [18]. The specificity of hu-mAbs was determined by testing these antibodies with a large (n > 240) panel of HLA-typed peripheral blood mononuclear cell suspensions in a conventional complement dependent microcytotoxicity (CDC) assay [19]. On the basis of the mAb reactivity patterns, suitable HLA alloantigen-specific sub-panels were chosen for each colorectal cancer patient's HLA phenotype, as well as proper negative isotype controls. Several mAbs were used to address the same HLA allelic products to rule out artifacts caused by differences in mAb in specificity/affinity.

case	A.1	A.2	B.1	B.2
40	A2	A3	B15	B38
43	A2	A3	B18	B49
44	A2	A24	B7	B55
45	A1	A2	B8	B62
48	A3	A3	B7	B62
55	A1	A3	B8	B62
56	A1	A30	B8	B51
58	A2	A32	B44	B60
59	A1	A2	B7	B62
61*	A2		Bw4	Bw6
63*	A2		Bw4	Bw6
69	A2	A24	B7	B39
106*	A3		Bw4	Bw6
108	A2	A68	B51	B53
109*	A3		Bw4	Bw6
110	A1	A23	B8	B50
120	A2	A3	B7	B44
122	A1	A24	B44	B56
124*	A2		Bw4	
179	A3	A3	B7	B35
191	A2	A24	B15	B40

Table 2. HLA genotype and phenotype of the 21 patients whose tumor tissue was used for flow cytometry. A.1, A.2, B.1 and B.2 indicate the different HLA A and B alleles. * HLA genotype was not known prior to tumor resection, phenotype based on flow cytometry of vim+ cells with mouse monoclonal antibodies (Table 1).

3.5. Flow cytometry

A seven-step, four-color staining procedure was performed as described previously [20]. Simultaneous labeling of HLA, β2m, DNA, and the intermediate filaments keratin and vimentin enabled us to discriminate HLA expression in keratin positive (tumor) epithelial cells from those in vimentin positive 'normal' cells (leucocytes and fibroblasts). Importantly, we specifically analyzed membranous expression since cells were only permeabilized after HLA immunostaining making the cytoplasm inaccessible to the anti-HLA Abs. In 15 cases, we used a complete panel of human and mouse anti-HLA mAbs, and in another 5 cases we used mouse mAbs (mu-mAb) because the HLA genotype was not known prior to tumor resection. Reagents used were biotinylated goat anti-mouse and anti-human mAbs (Southern Biotechnology Associates, Birmingham, AL), Streptavidin-allophycocyanin (APC) (Molecular Probes, Eugene, OR), goat F(ab')2 anti-mouse IgG1 and IgG2a-RPE, and goat F(ab')2 anti-mouse IgG2b or IgG1-FITC polyclonal antibodies (SBA), paraformaldehyde (Merck, Whitehouse Station, NJ), L-α-lysophosphatidylcholine (lysolecithin, Sigma-Aldrich), RNase (Sigma-Aldrich), and propidium iodide (Calbiochem) were used. We used three different combinations of anti-keratin and anti-vimentin mAbs to prevent cross-reactivity of the secondary reagents with the anti-HLA antibody. The first combined anti-keratin IgG1 mAbs M9, M20, and AE1/AE3 (Chemicon International Inc., Temecula, CA) and anti-vimentin Ig2b mAb V9; the second combined anti-keratin IgG2a mAbs CAM5.2 (BD Biosciences, San Jose, CA) and anti-vimentin IgG1 mAb V9; and the third combined anti-keratin IgG2a mAb CAM5.2 and anti-vimentin Ig2b mAb V9. Clones M9, M20, and V9 were established at the Department of Pathology, Leiden University Medical Center, The Netherlands, and are commercially available from ARA, Alphen a/d Rijn, the Netherlands. For each sample, measurements from 10,000–20,000 single cells were collected using a standard FACSCalibur™ flow cytometer (BD Biosciences). Data were analyzed using WinList 5.0 and ModFit 3.0 software (Verity Software House Inc., Topsham, ME). After electronic gating on keratin and vimentin content, single parameter histograms were obtained for DNA content and HLA expression. Geomean APC fluorescence intensity values (MFI) were subtracted by MFI values from proper negative controls. These ratios were used to calculate the relative expression value (REV) of keratin positive (ker+) cells compared to vimentin positive (vim+) cells.

REV = MFI[ker+;HLA]-MFI[ker+;negative control]/MFI[vim+;HLA]-MFI[vim+;negative control]

3.6. Immunohistochemistry

A standard three-step, indirect immunohistochemistry was performed on consecutive 4 µm formalin-fixed, paraffin-embedded tissue sections mounted on aminopropylethoxysilane coated glass slides. Antigen retrieval was performed in boiling citrate 10 mM (pH 6.0) for 20 min followed by a peroxidase block with 0.03% hydrogen-peroxide methanol and an endogenous avidin-binding activity-block by incubation with avidin solution for 15 min (one chicken egg white resuspended in 100 ml PBS) and 15 min in biotin solution (pasteurized cow milk), and di-aminobenzidine development. Antibodies used included biotinylated rabbit anti-mouse (DAKOCytomation, Glostrup, Denmark), goat anti-rabbit (DAKOCytomation), biotinylated-peroxidase streptavidin complex (SABC; DAKO-Cytomation), anti-HLA-A heavy chain mAb HCA2, anti-HLA-B/C heavy chain mAb HC10 (supernatant kindly provided by Dr. J. Neefjes, NKI, Amsterdam, The Netherlands and Dr. H. L. Ploegh, MIT, MA, at 1:400 and 1:100 dilutions, respectively) [21,22], rabbit anti-β2-m polyclonal

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Ab (A 072; DAKOCytomation; 167 μg/l), anti-MLH1 (clone G168-728; BD Biosciences Pharmingen; 20 mg/l), and anti-PMS2 (clone A16-4; BD Biosciences PharMingen, San Diego, CA, USA; 10 mg/l). Loss of expression was defined as complete loss of staining in both the membrane and cytoplasm (HCA2, HC10, and anti-β2-m) or in the nucleus (anti-MLH1 and anti-PMS2) with concurrent expression in normal epithelium, stroma, or infiltrating leukocytes.

3.7. Microsatellite instability analysis

Microsatellite instability (MSI) analysis was performed and scored as described previously [23] using the markers BAT25, BAT26, BAT40, D2S123, D5S346, D17S250, *MSH3* and *MSH6*. β*2m* frameshift analysis was performed with primers (available upon request) build around coding repeats of the β*2m* gene; the (CT)4 repeat in exon 1 and two (A)5 repeats in exon 2[24]. Genomic DNA of both normal and tumor tissue was isolated from 3 tissue cores of 0.6 mm diameter of formalin-fixed, paraffin-embedded material using the Chelex extraction method [25].

3.8. Statistics

Values of significance were calculated using the software package SPSS 10.0.7 (SPSS Inc., Chicago, IL, USA). We performed Pearson Chi-square tests or, when expected count was below 5, Fisher's exact 2-sided tests. The independent sample t-test was performed to test equality of means of the age at operation.

4. RESULTS

4.1. Two patterns of alteration in HLA class I phenotype as observed by flow cytometry

Loss of expression of HLA class I alleles was observed in 8 of 21 (38%) colorectal cancer cases studied (see additional file 1). Two distinct patterns of HLA loss were identified; the loss of a single HLA-A or -B allele which was observed in 4 of 8 cases (cases 61, 63, 109, and 191), and the loss of both HLA-A and -B alleles which was observed in the remaining 4 cases (cases 55, 56, 120, and 179). Importantly, in the latter group, membranous expression of β2-microglobulin (β2-M) and remaining HLA class I antigens was retained in the 4 tumors, but was diminished as determined with the mAbs BBM.1 and W6/32. In these samples, the average Relative Expression Values (REV, see Methods section) was 0.43 and 0.38, respectively, compared to the average REVS of 2.9 and 2.2, respectively, in the other 17 cases (Figure 1; see also additional file 1). This would suggest the retention of at least one of the other HLA class I alleles, i.e. HLA-C, -E, -F or -G.

4.2. Alterations of HLA phenotype are found in specific subsets of colorectal cancer

The 7 of 8 cases with HLA alterations were all right sided ($p = 0.024$). Interestingly, all 5 MSI-H cases in the series (5/21) demonstrated HLA alterations [see 1]. Four of the 5 cases demonstrated loss of HLA-A and -B ($p = 0.028$); in 1 of the 5 cases, we observed the loss of a single HLA allele (case 191). Loss of expression of the heterodimer of MLH1 and PMS2 was observed in the 4 MSI-H cases through IHC, and was not observed in any other cases [see 1]. These patients do not fulfill any criteria indicative of HNPCC are thus most likely sporadic. Other clinicopathological features typical of sporadic MSI-H tumors [26] were also present; they occurred in elderly patients (mean age 75 years, $p = 0.043$), 3 of 4 were located in the caecum ($p = 0.019$), and 3 of 4 were peri-diploid ($p = 0.053$). The other MSI-H tumor (case 120) was located in the sigmoid, occurring in the setting of the Hereditary Non-Polyposis Colorectal Cancer (HNPCC) syndrome.

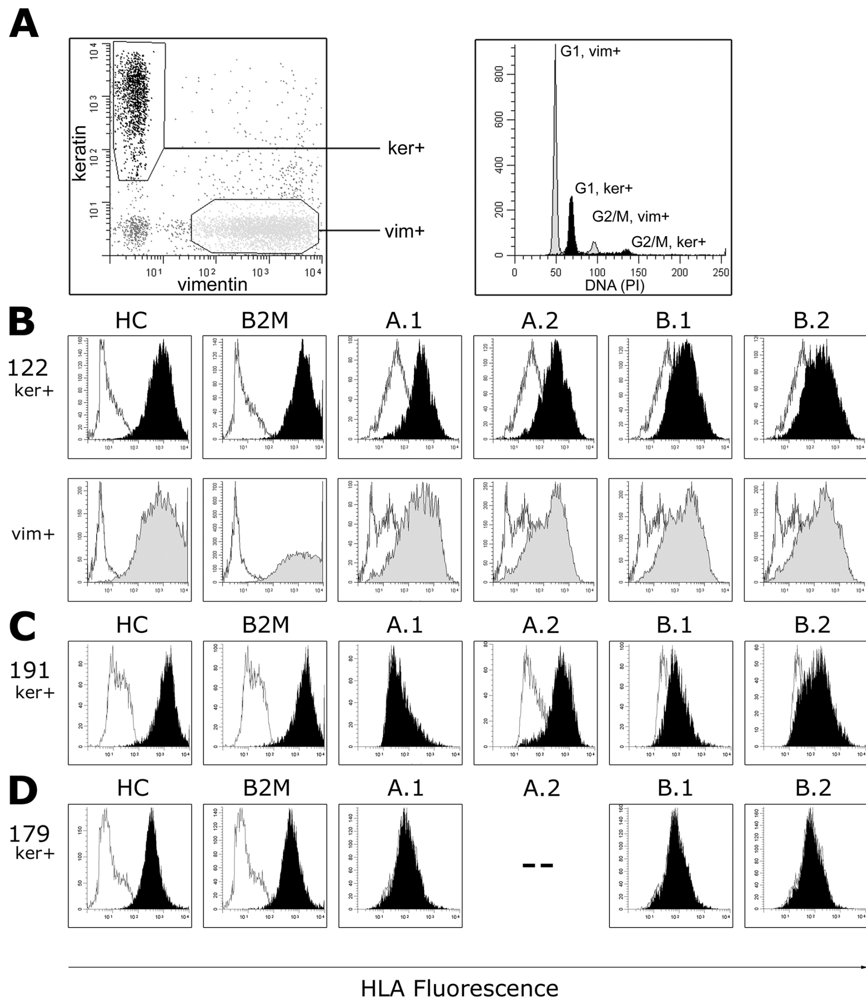

Figure 1. Membranous HLA class I expression analysis by four-color multiparameter flow cytometry of colorectal tumor cell suspensions. Fluorescence intensities of HLA class I with antibodies W6/32 (HC), BBM.1 (B2M), and alloantigen-specific antibodies against single A and B alleles (A.1 – B.2; see Table 2) are displayed in filled curves; corresponding negative controls are in non-filled curves. A, keratin positive (ker+) epithelial cells are distinguished from vimentin positive (vim+) stroma cells and infiltrating leukocytes. DNA ploidy analysis of case 122 reveals an aneuploid ker+ tumor cell fraction (black) compared to the normal diploid vim+ cells (light gray). B, Expression of all HLA molecules tested in case 122 of both ker+, tumor (black), and vim+ 'normal' (gray) cells. C, loss of the single A.1 (HLA-A2) allele was observed for tumor cells in case 191. D, loss of expression of all 4 HLA-A and -B alleles in tumor cells, but retention of HC and B2M, in case 179. The patient is homozygous for HLA-A3 (--;see Table 2).

A mutation in the *Mut-S-Homologue 2 (MSH2)* gene (G674R, exon 13 2020G>C) segregated in this particular family, a variant that is probably pathogenic. The abrogation of MSH2 expression in this tumor was confirmed by IHC. Since all the 4 MSI-H tumors that lost HLA A and B expression showed diminished membranous expression of β2m we have screened the microsatellite

sequences of the corresponding gene but failed to find any frameshift mutation.

One case (case 55, MSI-H, multi-ploid) was particularly intriguing. Ploidy studies indicated a peri-diploid tumor cell population as well as an aneuploid population (Figure 2). Interestingly, while the aneuploid population retained HLA expression for all HLA alleles examined, the diploid population was characterized by two populations of cells; one population that retained HLA expression, and one population (the largest tumor cell population) that demonstrated a loss of expression of both HLA-A and -B alleles.

The 4 cases showing loss of both HLA-A and -B expression have not presented with lymph node or distant metastases to date, while 3 of the 4 cases with loss of a single allele (including 1 MSI-H case) and 10 of the remaining 13 cases without alterations of the HLA phenotype, did metastasize ($p = 0.035$) to either lymph nodes or to distant extranodal sites.

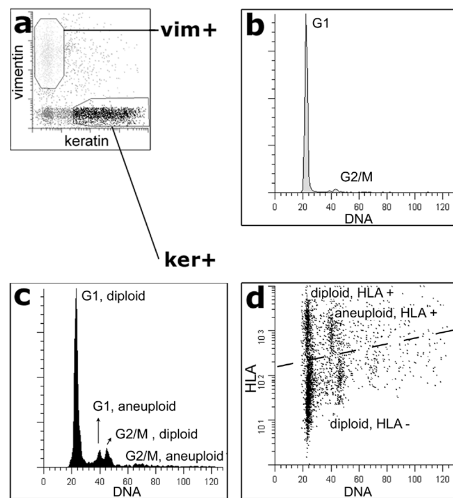

Figure 2. HLA phenotype alteration is associated with DNA ploidy of the cells within one tumor. Flow cytometry analysis of case 55 reveals a synchronous divergence of tumor cell populations with DNA ploidy and HLA expression. Keratin positive (ker+, black) epithelial cells are distinguished from vimentin positive (vim+, light gray) stroma cells and infiltrating leukocytes (a). Compared to the diploid vim+ cells (b), peri-diploid and aneuploid cell populations of ker+ cells are revealed (c). By plotting HLA expression to DNA content, 3 ker+ cell populations can be discriminated, including a HLA negative DNA diploid tumor cell population and a HLA positive aneuploid population (d).

4.3. Comparison of FCM with conventional immunohistochemistry

Using FCM, we detected a significantly lower frequency of HLA class I aberrations than previously reported [8-10,27]. Consequently, we decided to compare our FCM results with those obtained using conventional IHC. The human anti-HLA mAbs used for flow cytometry could clearly not be used for the IHC analyses due to high background staining caused by non-informative cross-reactivity of the secondary anti-human antibody. Consequently, we chose to use a panel of mouse anti-HLA antibodies (HCA2, HC10, anti-β2-m) for the IHC analyses, although these are not alloantigen specific. Loss of expression of a single HLA allele could not be detected using these antibodies.

IHC of cases 55, 56, 120, and 179 (the 4 HLA-A and -B negative cases) showed complete absence of HCA2 and HC10 staining in tumor cells, and decreased anti-β2-m staining which was primarily cytoplasmic (see Figure 3) and, consequently, probably irrelevant since cytoplasmic localization of HLA molecules is not functional. However, membranous staining of HCA2, HC10, and anti-β2-m was clearly observed in the adjacent stroma cells (positive internal control). The FCM data would thus appear to corroborate the IHC findings. Loss of staining was not observed in the remaining cases studied by IHC (see additional file 1). A heterogeneous staining pattern, i.e. both positively and negatively staining tumor fields within the same section, was frequently observed; such heterogeneity in membranous expression was

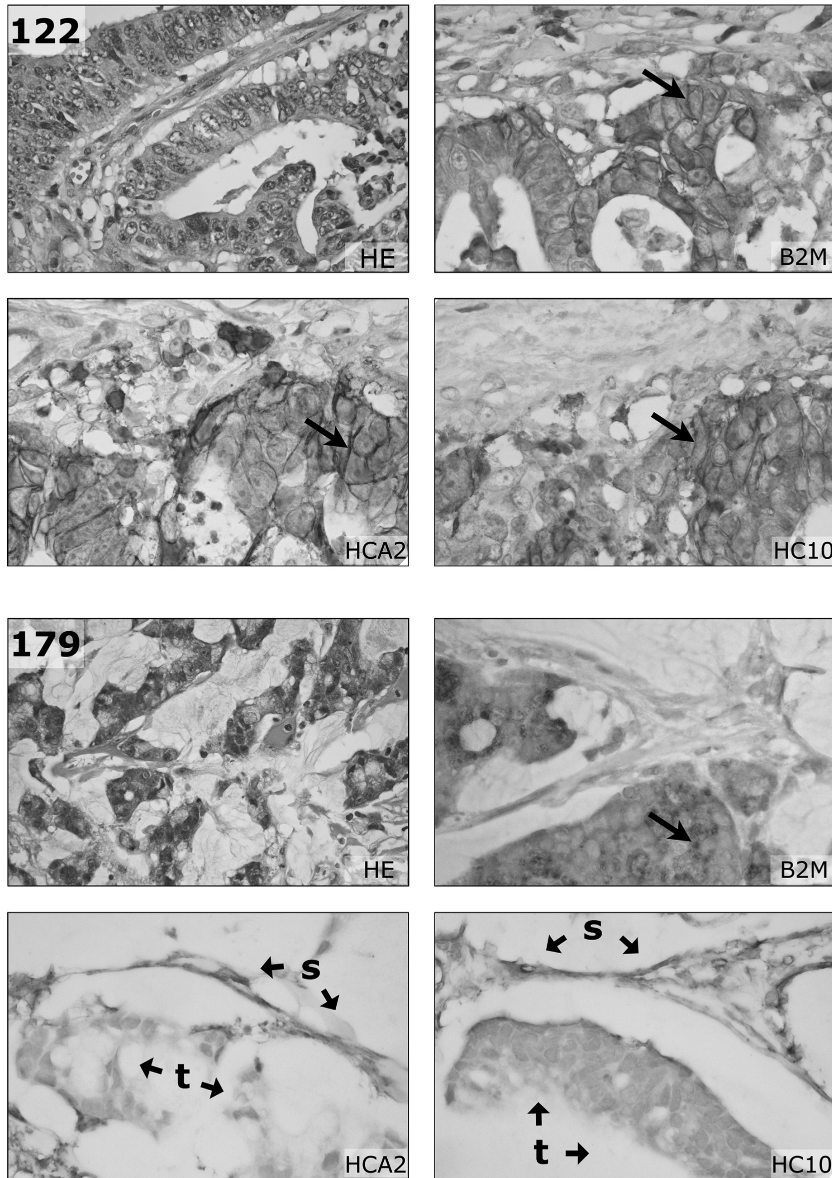

Figure 3. Conventional HLA immunohistochemistry of formalin-fixed paraffin-embedded tissue. Using HCA2 (HLA-A heavy chain), HC10 (HLA-B/C heavy chain) and anti-B2M antibodies, loss of HLA A and B expression as detected by flow cytometry (see Figures 1 and 2) could be confirmed. In the top panel, membranous staining (arrows) of B2M, HCA2, and HC10 is observed in the moderately differentiated sigmoid adenocarcinoma of case 122. In the bottom panel, loss of HLA-A and -B is illustrated for the mucinous caecum adenocarcinoma of case 179. Tumor B2M staining was mainly restricted to the cytoplasm (arrow), but some membranous expression of B2M was observed by FCM (see Figure 1). The HCA2 and HC10 staining was completely lost in tumor epithelium (t). Typically, the retained HLA expression of stroma cells (s) resulted in an inverted staining pattern in comparison to case 122. HE, haematoxylin and eosin staining. Pictures were made at 400 × magnification, HEs at 100×.

not observed as discrete populations (peaks) in our FCM data.

5. DISCUSSION

We used four-color multiparameter FCM to study complete HLA-A and -B phenotypes in colorectal tumors using a large panel of alloantigen-specific mAbs. FCM allows the detection of variation in expression over a dynamic range of 4 logarithmic decades and therefore FCM appears to be very sensitive. Loss of membranous expression was detected in 38% (8 of 21 cases) of tumors in our study. Previous studies based on IHC demonstrated variable alterations in up to 73% of cases [8-10,27]. This discrepancy may be explained by the different technical approaches used and/or by the different composition of the groups under study (e.g. microsatellite stable/MSI-H distribution, tumor staging).

Except for one case (case 55) our FCM data did not provide evidence for discrete intratumoral epithelial subpopulations of differing HLA expression as was observed using IHC, although the lognormal distribution of the measured fluorescence may obscure these variations in expression. Nevertheless, the heterogeneous IHC staining patterns are difficult to interpret, and may be falsely regarded as loss of expression.

Using FCM, we characterized two distinctive alterations in colorectal tumors. We found 4 cases demonstrating loss of a single HLA-A or B allele (alterations not identified through immunohistochemistry), and 4 cases demonstrating loss of expression of both the A and B alleles, but not loss of all HLA class I molecules. The expression of HLA I antigen complexes is a prerequisite for the optimal targeting of tumor cells by CD8+ CTLs. However, NK cells and CTLs also carry inhibitory receptors for specific HLA class I alleles. Immunoselection thus relies on a delicate balance between HLA allele loss and retention. Examples of tumor immune escape due to such intricate alterations of HLA phenotype have been described recently [16,28]. In tumors showing loss of a single allele in our study, this loss was restricted to HLA-A2 in 3 of the 4 cases. HLA-A2 is not a ligand for inhibitory receptors. Those tumors may have effectively shed (HLA-A2) restricted epitopes and remained inhibitory to NK cell attack. Additionally, the 4 cases demonstrating loss of both HLA-A and -B alleles (also applicable to the cases showing loss of a single allele) retained expression of some total HLA class I molecules, which was detected through the W6/32 antibody and might indicate retention of HLA-C or other non classical HLA molecules like HLA-G or -E. Of these, HLA-E is normally expressed in colon epithelial cells [29] and predominantly function as inhibitors of NK-cell function [30]. These tumor cells may escape CTL attack and attack by a subset of NK cells. HIV-1 infected cells, for example, avoid both CTL and NK cell-mediated lysis through specific HLA-A and -B downregulation caused by the HIV-1 gene product nef [31]. These different phenotypic alterations may reflect differences in local immune selective forces between the tumor subsets. Such differences remain to be identified.

All but one of the tumors with an altered HLA phenotype were located on the right side of the colon. The exception was a left sided tumor arising in the setting of HNPCC. Furthermore, the loss of both HLA-A and -B alleles was found exclusively in tumors with the MSI-H phenotype. Mismatch repair deficiency has previously been associated with frequent mutations in the β2-m gene (B2M) [24,32-35]. This gene harbors multiple short tandem repeats that are preferentially mutated, leading to total loss of membranous HLA class I expression. However, we did not observe frame shift mutations in these repeats in the MSI-H tumors with HLA-A

and -B loss, which is in line with the retention of membranous staining (although diminished) with the W6/32 and anti-β2-m mAbs. Yet, the common loss of expression of HLA-A and -B in 3 of 4 sporadic MSI-H tumors, which is not due to *B2M* frame shift mutations, would suggest that another general mechanism may cause this altered phenotype.

Sporadic MSI-H tumors are usually relatively large, but rarely disseminate [3,36,37]. This favorable tumor behavior has been associated with an increased intra-epithelial CD8+ CTLs and CD57+ NK cells infiltrate when compared with the microsatellite stable tumors. CD4+ cells are not frequently found in the intraepithelial infiltrate of MSI-H tumors. [24,38-42]. The MSI of numerous coding microsatellites results in a large repertoire of immunogenic frame shift peptides which can give rise to specific anti-tumor immune responses [4-6]. However, these infiltrating lymphocytes may lead to the selection of HLA alterations in tumor cells. High levels of infiltrate were present in the HLA-A and -B negative cases in this study as observed by microscopy. Alternatively, the observed T cell infiltrate could represent an innocent bystander effect. Previously, we identified loss of HLA-A and -B expression in 6 of 88 cases of sporadic colorectal cancer using IHC that, surprisingly, did not present with recurrences or metastases during follow-up [27]. Although we could not determine the exact HLA phenotypic alteration at that time, the previous cases resemble the 3 sporadic MSI-H cases found in this study that lacked HLA-A and -B expression and that have not presented with lymph node or distant metastases to date. The NK cells that reside in the lymph and blood and that specifically kill metastasizing tumor cells (that lack total HLA class I expression) may explain the more favorable prognosis. However, the postulated expression of the remaining non HLA-A and -B alleles by tumor cells may specifically inhibit these NK cells.

6. CONCLUSION

FCM allows the discrimination of complex phenotypes related to the expression of HLA class I. The different patterns of HLA class I expression might underlie different tumor behavior and influence the success rate of immunotherapy.

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	clinicopathology					MMR			FCM	HLA FCM - expression values						HLA IHC		
case	age	sex	loc.	Dukes	F-U	MSI	MLH1	PMS2	ploidy	A.1	A.2	B.1	B.2	HC	B2M	HCA2	HC10	B2M
61	64	F	Ce	C2	DOD	S	+	+	A	0,06		0,70	0,14	1,86	2,41	+	- +	- +
63	57	F	Ce	B2	nr	S	+	+	D	0,16		2,17	4,30	4,51	5,59	- +	- +	- +
109	68	M	A	C2	M	S	+	+	A	1,77		0,16	1,13	1,47	1,90	- +	- +	- +
191	70	M	A	C3	nr	H	-	-	D	0,02	4,21	0,30	0,47	1,52	1,56	+	- +	+
55	74	F	Ce	B2	nr	H	-	-	M	†	†	†	†	0,15	0,35	-	-	c
56	75	M	Ce	B3	DND	H	-	-	D	0,01	0,05	0,20	0,01	0,40	0,28	-	-	c
120	62	F	S	B2	nr	H*	+	+	D	0,01	0,15	0,10	0,08	0,45	0,59	-	-	c
179	90	M	Ce	B2	nr	H	-	-	D	0,07	0,07‡	0,06	0,05	0,53	0,49	-	-	c
40	81	F	R	C3	DOD	na	+	+	A	2,37	3,05	7,27	>15	0,46	2,64	+	+	- +
43	50	M	R	C2	DOD	na	+	+	M	10,71	8,24	8,34	11,50	4,80	7,95	+	+	+
44	57	M	S	B2	nr	na	+	+	A	9,72	10,32	7,17	2,72	5,85	3,87	- +	- +	+
45	64	F	RS	A	nr	na	+	+	D	2,91	3,86	1,36	3,18	1,56	1,00	- +	- +	- +
48	66	F	S	B2	nr	na	+	+	D	5,65	5,65‡	2,75	2,74	1,29	1,76	- +	- +	- +
58	53	F	D	B2	nr	na	+	+	M	1,10	0,72	1,36	1,00	1,12	1,53	- +	- +	- +
59	56	M	R	B1	nr	na	+	+	D	2,15	2,73	1,56	1,35	1,81	2,40	- +	- +	- +
69	74	F	A	B2	M	na	+	+	A	1,87	2,34	1,28	0,90	1,90	2,20	- +	- +	- +
106	61	F	A	C2	M	na	+	+	M	1,41		0,64	0,87	1,00	1,36	+	+	- +
108	52	M	A	C2	M	na	+	+	M	1,37	1,34	0,59	0,71	1,31	1,51	- +	- +	- +
110	74	F	S	C2	M	na	+	+	D	3,00	4,53	1,53	1,86	2,81	4,46	- +	+	- +
122	55	F	S	C2	M	na	+	+	A	1,10	2,54	1,37	1,22	1,16	1,73	- +	+	- +
124	75	F	Ce	C1	nr	na	+	+	A	5,13		1,58		2,98	3,76	- +	- +	- +

Additional File 1. HLA phenotype alterations and clinicopathological features of colorectal tumors and their mismatch repair status. These data provides the HLA phenotypes assessed by flow cytometry of the different tumors and their clinicopathological features and mismatch repair status. Clinicopathology: loc., tumor localization: A, colon ascendens; Ce, caecum; D, colon descendens; R, rectum; RS, rectosigmoid; S, sigmoid; modified Dukes stages [43]; F-U, follow-up (max. 2 years): DOD, dead of disease; DND, dead but not due to disease; M, distant metastasis; nr, no recurrences. MMR, mismatch repair status: MSI, microsatellite instability: H, MSI-High; S, microsatellite stable; *, HNPCC, hereditary non-polyposis colorectal cancer; +, tumor epithelium staining positive as described in text; -, no staining of tumor cells, but staining of 'normal' cells, - +, heterogeneous staining of tumor cells; FCM, flow cytometry: A, aneuploid; D, diploid; M, multiploid; HLA FCM: Relative HLA Expression Values were calculated from flow cytometry analyses as described in the methods section. Depicted in black are cases in which fluorescence intensity of ker+ cells was equal to the negative control (see Figure 1). A.1 – B.2, HLA-A and -B alleles as depicted in Table 2; HC, HLA heavy chain expression detected with the W6/32 antibody. †, HLA-A and -B negative and positive populations are present, therefore REV is not informative (see Figure 2); ‡, homozygous HLA-A genotype. HLA IHC, immunohistochemistry of HLA molecules: +, tumor epithelium staining positive as described in text; -, no staining of tumor cells, but staining of 'normal' cells; c, tumor epithelium staining restricted to the cytoplasm; -+, heterogeneous staining of tumor epithelium.

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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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Chapter 6

1. ABSTRACT

1.1. Background

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.

1.2. Methods

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.

1.3. Results

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 β*2m* and APM components genes.

1.4. 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.

2. BACKGROUND

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

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genes *HLA-A*, *-B* and *-C*, located on chromosome 6p21.3) [5]; mutations in β*2-microglobulin (*β*2m*) [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 *hMLH1*. 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 *TGF*β*-RII* gene. *TGF*β*-RII*'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 *hMLH1*, *hMSH2*, *hMSH6* 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) sub-groups) colon tumors and in HNPCC tumors and studied the mechanisms underlying any abnormalities in these subgroups.

3. METHODS

3.1. Patient material and tissue microarrays

Two tissue microarrays were constructed from formalin-fixed, paraffin-embedded tissues as described previously [24]. One array, previously described [25], 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 *hMLH1* (n = 24), *hMSH2* (n = 18), *hMSH6* (n = 12) or *PMS2* (n = 1), the remaining were MSI-H, without methylation of the *hMLH1* 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 hMLH1 promoter using a methylation-specific MLPA assay as previously described [26]. All MSI-H sporadic cases presented with hypermethylation at the *hMLH1* 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 [27].

3.2. 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 [28,29]; 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) [28,30]; 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 [31-33]; 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).

3.3. Flow cytometric sorting

The flow cytometric sorting procedure, including tissue preparation, staining and flow cytometry analysis was performed as described previously [34]. 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 [35]). 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. [36]. DNA from non-sorted material was isolated using Chelex extraction as described previously [37].

3.4. LOH and fragment analysis

Markers for loss of heterozygosity (LOH) analysis were chosen from the dbMHC database [38] 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 [39]. LOH was defined as allelic imbalance >2 in the HNPCC cases (non-sorted) and allelic imbalance >5 in the sorted RST [40].

To detect frame-shift mutations in the *HLA-A*, *HLA-B*, β*2m*, *LMP2*, *LMP7*, *LMP10*, *TAP1*, *TAP2*, *Calnexin*, *Calreticulin*, *ERp57* and *Tapasin* genes, 28 pairs of primers (Table 1) were constructed surrounding non-polymorphic microsatellite regions within the coding regions.

3.5. Statistics

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

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

Table 1. Primers used in fragment analysis

4. RESULTS

4.1. 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 2).

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

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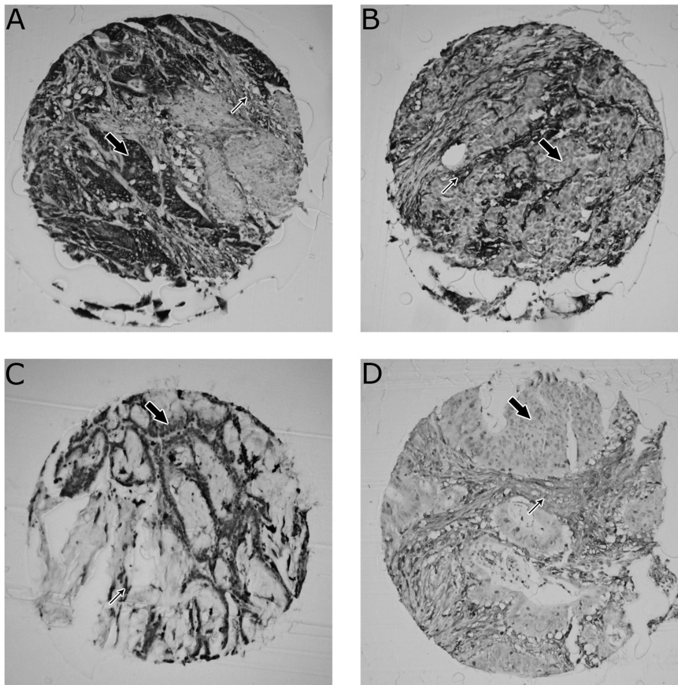


Figure 1. Example of immunohistochemical analysis performed on the RST and HNPCC tumors (Amplification 10×). A, Positive expression of HLA class I antigens detected with the HCA2 antibody. The epithelial (large arrow) membranous expression of HLA class I antigens is identical to the lymphocytic infiltrate (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.

			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%)*

Table 2. HLA, APM and β2m expression in RST and HNPCC colon cancer

* – 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.

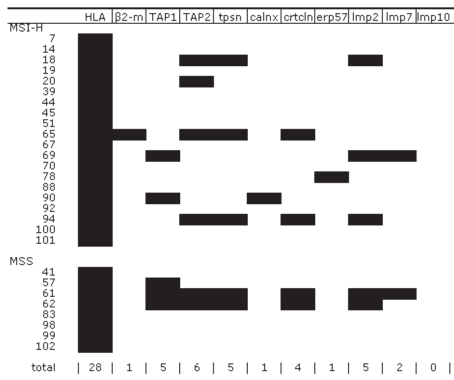


Figure 2. Loss of expression of β2m and different APM members was detected by immunohistochemistry in the RST that presented with HLA loss. The shadowing (in black) is indicative for loss of expression of the respective molecules (tpsn – Tapasin, calnx – Calnexin, crtcln – Calreticulin).

components, occurring in about 37% of HLA-negative tumors regardless of their mismatch repair status (Table 2). 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 2), 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.

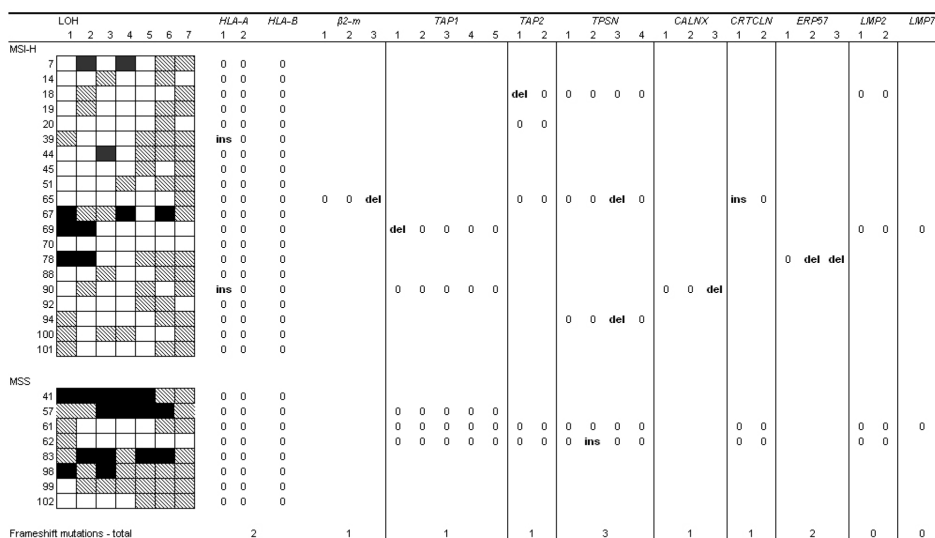
In sporadic RST, the simultaneous loss of more than one APM molecule per case was frequent (Figure 2). Only 3 out of 10 cases lost a single APM component. The TAP2 molecule was most frequently lost (6 cases), followed by TAP1, Tapasin and LMP2 (5 cases), Calreticulin (4 cases), LMP7 (2 cases), and Calnexin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

4.2. 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 5A). 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 ($P < 0.05$) (Figure 3, 4). 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 β*2m* genes was performed on all cases with aberrant HLA class I expression (Figure 5B,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 single sporadic MSI-H case that lost β2m expression also presented with a single frameshift mutation in the β*2m* 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



	HLA-A		HLA-B	β2-m			TAP1					TAP2		TPSN				CALNX			CRTCLN		ERP57			LMP2		LMP7
	1	2		1	2	3	1	2	3	4	5	1	2	1	2	3	4	1	2	3	1	2	1	2	3	1	2	
MSI-H																												
7	0	0	0																									
14	0	0	0																									
18	0	0	0									del	0	0	0	0	0									0	0	
19	0	0	0																									
20	0	0	0									0	0															
39	ins	0	0																									
44	0	0	0																									
45	0	0	0																									
51	0	0	0																									
65	0	0	0	0	0	del						0	0	0	0	del	0				ins	0						
67	0	0	0																									
69	0	0	0				del	0	0	0	0															0	0	0
70	0	0	0																									
78	0	0	0																				0	del	del			
88	0	0	0																									
90	ins	0	0				0	0	0	0	0							0	0	del								
92	0	0	0																									
94	0	0	0											0	0	del	0											
100	0	0	0																									
101	0	0	0																									
MSS																												
41	0	0	0																									
57	0	0	0				0	0	0	0	0																	
61	0	0	0				0	0	0	0	0	0	0	0	0	0	0				0	0				0	0	0
62	0	0	0				0	0	0	0	0	0	0	0	ins	0	0				0	0				0	0	
83	0	0	0																									
98	0	0	0																									
99	0	0	0																									
102	0	0	0																									
Frameshift mutations - total	2			1			1					1		3				1			1		2			0		0

Figure 3. 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);β2m: 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).

	LOH 1	LOH 2	LOH 3	HLA-A 1	HLA-A 2	HLA-B	β2-m 1	β2-m 2	β2-m 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	*hMSH2* 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 4. LOH and frameshift analysis was performed on HNPCC tumors that simultaneously lost HLA class I and β2m expression. LOH markers: (see legend from Figure 3 for key). Frameshift markers:HLA A: 1 – 4th exon 7(C) 2 – 5th exon 3 (GGA); HLA – B: 3(GA) & 3(CA); β2m: 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).

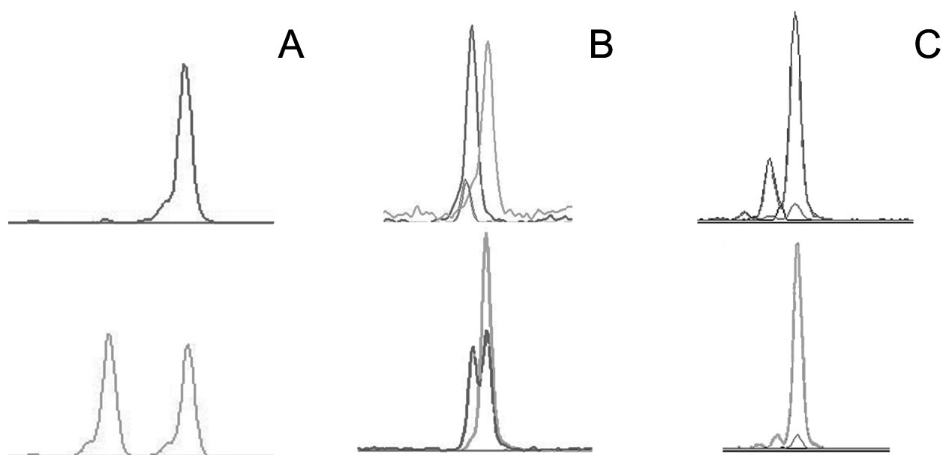


Figure 5. Genetic analysis performed on tumors that have lost HLA class I expression. The different peaks correspond to different sizes from the PCR-amplified products. Peaks corresponding to the normal samples are represented in green whereas tumor samples are represented in blue. A, LOH analysis performed 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 β2m 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.

immunohistochemical staining, one presented with a frameshift in an *HLA* gene (case 39, Figure 3, Table 3) 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 4). 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 4). In neither of the 2 HNPCC cases that immunohistochemically lost the expression of one of the APM components an APM frameshift mutation was found.

5. DISCUSSION

Abnormalities in HLA class I cell surface expression are commonly observed in tumors and are interpreted as a mechanism 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 [41,42]. 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.

RST	Mutation description
18	*TAP2* (341–346)del1 – het
39	*HLA-A* (621–627)ins1 – het
65	*β2m* (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

Table 3. Frameshift mutations description in RST
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.

Applying immunohistochemistry on tissue arrays, we compared HLA class I expression in both sporadic RST (MSI-H and MSS sub-groups) 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 mAb 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 β2m.

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 [43]. Such repeats are also present within the exons of the APM components, β*2m*, *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 [43]; 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 [44]. 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 β*2m* mutations and unfavorable prognosis in colorectal cancer [45].

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 [46]. V600E was distributed equally between tumors that lost vs. retained expression of HLA class I in the sporadic MSI-H cases.

6. 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.

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Concluding remarks and implications for further research

Chapter 7

1. TOWARDS MOLECULAR DIAGNOSIS, TREATMENT AND PREVENTION OF COLON CANCER

Tumours arising from various parts of the colon have long been considered and treated as identical pathological entities. However, accumulating studies on (molecular) tumour development have shown that distinct colon tumour subsets differ in important clinical parameters such as frequency of dissemination and response to adjuvant treatment. Although surgical excision will remain inevitably the major step of treatment irrespective of the tumour type, to design (customized) therapeutic strategies it is essential to discriminate distinct tumour subsets by molecular analyses in advance [1]. Insight into the steps of tumorigenesis may eventually lead to cancer prevention by enabling early detection of precursor lesions, eliminating risk factors, or even vaccination [2, 3].

Concerning the latter, the identification of altered HLA class I expression in colon tumours, especially in those with mismatch repair (MMR) deficiency on a sporadic basis or in the context of the Lynch syndrome, has given immune escape processes a definite position on the roadmap to colon cancer. This observation might support the potential of immune-mediated eradication as a preventive or therapeutic measure. The variation in type of HLA alteration among different tumour subtypes may suggest as yet unresolved differences in immune editing. Such differences probably have repercussions on the applicability of immune therapy approaches for colon cancer.

2. TIMING OF COLON CANCER IMMUNE RESPONSES

The studies presented in this thesis have solely focused on the nature of immune escape mechanisms in primary colon tumours; we did neither study the onset of events during tumour progression, nor did we study tumour metastasis. In other words, the position of immune escape on the roadmap is, to date, unknown. Insight into the position on the timeline may reveal essential information on the type and the effect of both the immune edit and immune escape mechanisms. For instance, the amount and type of displayed tumour-antigens will vary during stages of tumour development, which might determine whether this leads to tumour immune tolerance or attack [4, 5].

Another question unanswered is the onset of the 'danger signal'. This signal is normally evoked at the earliest start of inflammation and is able to define the cascade of following immune response. It may even lead to loss of systemic tolerance [4]. Inflammation is a natural response to any tissue damage and is microscopically already visable in adenomas. Consequently, the danger signal is evoked during the adenoma stage, thus before invasion of the surrounding tissue starts. Interestingly, in Lynch syndrome-adenomas increased tumour-infiltration of lymphocytes was found only in MMR deficient tumours which suggests that this immune response is secondary to the MMR knock-out [6].

Finally, the immune response to metastases needs to be studied. As discussed previously, dissemination is not necessarily a roadblock on the roadmap to cancer. Therefore, metastasizing cancer cells may be challenged by the same type of adaptive immune responses, albeit that they will additionally be confronted more easily by the innate immune system (including natural killer cells) as they migrate through the lymph or bloodstream. Alternatively, they might evoke a second danger signal or give positive-feedback to the primal danger signal as a result of tissue damage at the site of metastasis [7].

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3. MISMATCH REPAIR DEFICIENT TUMOUR IMMUNITY

The relation of immune response evocation and tumour antigen display is of particular interest in DNA repair deficient tumours. We identified frequent HLA alterations in both sporadic MMR deficient tumours, and in Lynch syndrome-related tumours; additionally, it has been frequently identified in *MUTYH*-associated polyposis-related tumours [8]. Due to deficient DNA repair both of these types of tumours accumulate a high amount of DNA errors that potentially could lead to an acceleration of tumour progression compared to DNA repair proficient tumours. Such acceleration has been observed in the adenomatous phase of Lynch syndrome-tumours [9]. In the case of MMR deficiency, proofreading errors result in frame-shift encoded proteins that may be, once processed to peptides, loaded on HLA class I molecules and transported to the cell surface, presented as 'foreign' antigens; some have indeed been shown to be potentially immunogenic [10-12]. Additionally, microsatellite mutations are frequently found within the untranslated regions (UTR) of genes [13]. Such mutations would not lead to an altered protein product. Although we did not observe it for the *IFNGR1*, mutations of the UTR can affect RNA stability leading to functional inactivation as has been shown for the *BMPR2* [14].

The frameshift-mutated antigens need to accumulate into a large enough amount of protein in order to lead the necessary cross-presentation of dendritic cells to effector cytotoxic T cells. Newly designed protein accumulation assays and RNA stability assays have been developed to predict such [15-17]. The results of these pre-screening assays will most likely limit the potential pool of MMR tumour-antigens as well as the repertoire of applicable immunogenic frameshift-mutated antigens for vaccination strategies.

The identification of distinct molecular mechanisms of HLA alteration between the sporadic and hereditary mismatch repair deficient colorectal tumour subsets suggests that they are related to distinct anti-tumour immune responses. What would cause that difference? Patients suffering from the Lynch syndrome may display a mild degree of microsatellite instability throughout the body due to their inherited heterozygous mismatch repair gene mutation [18, 19]. The latter would suggest a role of haplo-insufficiency of MMR defects. In contrast to sporadic MMR deficient tumours, in Lynch syndrome cases a low level of frameshift-mutated antigens may have been displayed to the immune system during or even before tumour formation [20]. The latter might result in a different danger signal. Whether it would lead to immune tolerance or, oppositely, to a stronger immune response is not known.

Active adaptive cell-mediated immune-response (Th1) as detected by intra-tumour infiltrate phenotype and gene expression profiles have been related to a favourable disease-free survival of sporadic colorectal tumours, irrespective of tumour type, clinical classification, or immune escape mechanisms [21]. The protective property of the elevated immune response may be situated locally in reducing the number of disseminating tumour cells, as well as in the periphery in controlling occult tumour growth from dormant metastasised tumour cells [22, 23]. The acquisition of HLA alterations may lead to opposite effects on clinical behaviour and patient survival [24-26]. These studies however are limited and show somewhat contradictory results. In sporadic colorectal tumours it may be associated with a favourable prognosis, whereas in Lynch syndrome-tumours it has been associated with a poor prognosis. Whether this can

explained by the type of immune escape mechanism itself (e.g. the capacity to escape natural killer cell annihilation or not) or by intrinsic co-existing tumour (or immune-response) features could so far not be concluded [27].

Further research is needed to answer these intriguing questions. It would not only help us to understand why the distinct immune escape mechanisms are employed, but would also clarify the immune potential already present in colon cancer patients. This will lead to important clues in designing appropriate adjuvant immunotherapy or even preventive vaccination of colon cancer.

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Summary

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Summary

This thesis is about colorectal cancer, cancer of the large intestine. Colorectal cancer has a high prevalence in the Western World including the Netherlands. The colon is an abdominal organ that consists of multiple regions starting with the cecum (including the appendix), turning into ascending colon, transversing colon, descending colon, then sigmoid en finally the rectum which ends in the anus. Until a decade ago, cancers developing in any of those regions were considered identical. However, after the introduction of new molecular research techniques we know this is not the case. It is now firmly established that colorectal cancer comprises different types of cancer. It all starts with just one cell escaping the 'social control' mechanisms needed to maintain the balance between cell proliferation and cell loss and preservation of normal tissue architecture and function. This cell has gained a growth advantage leading to clonal outgrowth of its offspring. To develop into a malignant tumour (cancer) these cells need to acquire many distinct capacities, but how and when these capacities are acquired depends on the type of cancer. The driving force in cancer development is not unlike Darwinian evolution viz. 'survival of the fittest' on a microscopic scale. Newly acquired properties are passed on to daughter cells by mutations in DNA as well as by stable alterations in the expression of genes (epigenetic changes). Eventually the increasingly dysregulated cells are able to invade surrounding tissue increasing the risk of metastasis.

To respond to this the human body may call for the immune system. But this is far from easy since cancer cells pretty much look like healthy cells. They are hard to recognize. Nevertheless, accumulating evidence has shown that it is possible. Unfortunately, cancers develop ways to circumvent which leads to immune escape.

In this thesis we study immune escape in colon tumours, in particular the alterations in molecules enabling the immune system to recognize the tumour cells. These are so-called Human Leukocyte Antigen class I molecules who also play a major role in organ transplant-rejection. Furthermore we untangled distinct colon tumour subsets, in order to study the tumour-immune interaction more closely and to be able to predict it.

In the general introduction in **chapter 1** some of the current views on the molecular roads to (colon) cancer development relevant for this thesis are summarized. Based on the type of genomic or DNA alterations two main categories of colon cancer can be distinguished. The first category is characterized by chromosomal instability (CIN) which is associated with inherited or acquired mutations of the *APC* tumour suppressor gene. Patients with inherited APC mutations suffer from the Familial Adenomatous Polyposis (FAP) syndrome that is characterized by hundreds to thousands of colorectal polyps and a high risk of cancer. The second category comprises colon tumours caused by defects of DNA mismatch repair leading to instability of microsatellite DNA sequences (microsatellite instability or MIN). Germline mutations in the mismatch repair genes cause hereditary Lynch syndrome, which is characterized by a high risk of cancer including colorectal cancer and endometrial cancer. Next the interplay between tumour cells and the immune system

is outlined. A model of the different processes involved in the genesis of a specialized adaptive anti-tumour immune response is shown. Finally, the distinct ways of immune escape, in particular alterations of the HLA class I molecules are discussed.

In the study described in **chapter 2** we investigated the value of immunohistochemistry for the identification of mismatch repair defects. This involves the use of specific antibodies that recognize mismatch repair gene products and have been labelled with an enzyme producing a coloured reaction product. The presence or absence of staining in tissue sections can be inspected by microscopy and indicates whether the mismatch repair genes function normally or are defective. Mismatch repair defects cause shortening or expansion of repeated DNA sequences called microsatellites and microsatellite instability (MSI) which can be demonstrated by DNA analysis. We compared the performance of immunohistochemistry with that of MSI analysis for the detection of mismatch repair defects on colorectal and endometrial tumours from Lynch syndrome patients. Using antibodies for three different mismatch repair gene products we show that immunohistochemistry reliably detects defects in mismatch repair genes.

In **chapter 3** we extended the immunohistochemistry approach in a large series of hereditary colon tumours. We constructed a tissue micro array using multiple 0.6 mm cores of tumour tissue. The information obtained from these small tissue samples appeared to be sufficient for identification of mismatch repair defects. We now acquired a powerful tool to study large series of colon tumours with preservation of essential information on the molecular subtype.

Next we studied colon tumour immune escape mechanisms. In **chapter 4** we describe the identification of a mutation of the *IFNGR1* gene in MIN tumours. In up to 59 percent of hereditary cases a mutation was found. It concerns a mutation of a microsatellite region within the regulatory region of a gene that codes for a receptor of interferon-gamma (IFN-γ). IFN-γ is a small molecule that immune cells need to prepare for attack. It upregulates the expression of HLA class I molecules and makes target cells more vulnerable to attack by cytotoxic T cells. We were curious whether the mutation would protect tumour cells against such an assault. We tested this on living, cultured cells from both CIN- and MIN tumours (the latter all bearing IFNGR1 mutations). Now we could compare their reactions to IFNγ. We appeared to be wrong. MIN colon tumours remained equally sensitive to IFNγ, with respect to upregulation of HLA class I molecules and vulnerability to one of the attack mechanisms used by T cells. The mutations seemed to have no functional consequence, therefore probably do not contribute to colon cancer development, nor immune escape.

In **chapter 5** we describe the study of HLA class I molecules in colon cancer. HLA class I comprises a large family of proteins, which makes it rather complicated to study. Each HLA class I molecule consists of two chains, a light chain and a heavy chain. The former is identical in all cases. However, the heavy chain is highly variable and is represented by 6 isotypes of which the genes have distinct locations (loci) on the short arm of chromosome 6 and which are indicated *HLA-A to G*. Each locus has many variants, polymorphisms, especially *HLA-A* (over 120) and *HLA-B* (over 250). Every human being has two copies of each locus, alleles, as we inherit one from our father and from our mother. Usually, this concerns two distinct polymorphisms. So the number of allele

combinations is huge and this abundant variability has an evolutionary benefit viz. minimizing the chance of infective pathogens to remain unnoticed.

We set out to study the expression of individual HLA molecules. The use of 4 colour flow cytometry enabled us to study the quantitative expression of distinct alleles in freshly isolated single tumour cells obtained by dissociation of primary tumour tissues. Since tumours consist of multiple cell lineages, including various types of normal cells, we used three colours of fluorescence to discriminate those. The fourth colour was used to label the HLA molecules on the cell surface. The fluorescent dyes were coupled to specific antibodies, of which we needed a large panel.

We identified HLA alterations in 38 percent of the tumours. Furthermore, we discovered that the alterations conferred to either of two patterns. Either just one allele was deleted, or four alleles jointly showed diminished expression (although not all HLA class I molecules were lost). Finally, the distinct patterns were associated with distinct tumour subtypes viz. MIN tumours and tumours from the proximal colon.

In comparison to the results from immunohistochemistry, those from flow cytometry proved to be more consistent. However, the four-allele-diminution pattern as observed in MIN tumours was also distinguishable with use of immunohistochemistry. This opened up the opportunity to study larger series using the tissue micro array technique.

The high frequency of HLA alterations in MIN tumours is remarkable. It suggests a strong selective pressure present forcing tumours to escape the immune system. Previous studies support this hypothesis. MIN tumours are more heavily infiltrated by T cells. Possibly, this is related to the relative abundance of frameshift- and otherwise mutated proteins (even in the absence of any benefit as shown in chapter 4). Microsatellite instability may have its disadvantages. To see if this hypothesis was consistent, we compared sporadic MIN cases to hereditary cases. This is described in **chapter 6**. We analysed large series of sporadic proximal colon tumours, MIN tumours, and Lynch syndrome-tumours. We studied the distinct HLA patterns as well as the underlying molecular mechanisms. We searched for mutations in HLA alleles, in the gene encoding the β2-microglobulin light chain (*B2M*), and in genes encoding proteins of the antigen processing machinery (APM). The latter is needed for the assembly of HLA molecules, and in particular for charging them with small pieces of protein, the antigens. These antigens make the difference as they determine whether a T cell comes into action or not. Without antigens HLA molecules are useless and not even transported to the cell surface. We found mutations in several genes viz. *B2M*, *HLA-A*, as well as in the APM members *TAP1*, *TAP2*, *tapasin*, *calnexin*, *calreticulin*, and *ERP1*. In other cases the entire chromosomal region encoding the HLA-alleles (chromosome 6p21.3) was lost. This is called loss of heterozygosity (LOH). Interestingly, distinct mutations were limited to distinct tumour subsets. *B2M* mutations were found in Lynch tumours. In sporadic MIN cases, mutations of *HLA-A* or APM members were observed. LOH of 6p21.3 was observed in CIN tumours.

What does this tell us? Although the exact reason for the distinct HLA alterations is unknown, it is evident that colon tumours, in particular in the proximal colon are under quite some pressure to modulate the immune-tumour dialogue. In **chapter 7**, this is discussed in more detail. Recent studies have shown that Lynch tumour cells do bear mutation-derived antigens which can give rise to specific cytotoxic T cells that are able to attack

them. Whether feasible therapeutic or preventive immune based modalities can be designed is something to be addressed in the future.

In summary, this thesis describes molecular methods to distinguish separate colon tumour entities. Furthermore, it shows that distinct immune escape mechanisms, in particular distinct mechanisms of corrupting the HLA system, are operational in subsets of colon tumours. The apparent necessity of some colon tumours to circumvent the immune system might underscore the potential of immune based therapy approaches. Alternatively, it may suggest that such therapies will only lead to selection of tumour cells with HLA alterations, limiting the value of these approaches. In general, the identification of distinct tumour types to be targeted by tailor-made therapy is essential study the success of any applied strategy.

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Samenvatting

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Samenvatting

MOLECULAIRE PATHOLOGIE VAN TUMOREN MET DEFECTEN IN HET DNA-MISMATCH-REPAIR SYSTEEM. EEN NADRUK OP IMMUUNMODULATIE MECHANISMEN

Dit proefschrift gaat over dikkedarmkanker. Het komt in de westerse wereld (en dus ook in Nederland) veel voor. Maar wat is het nu eigenlijk?

De dikke darm is een groot orgaan in de buik en bestaat uit meerdere delen die elk hun eigen anatomische naam hebben. De dikke darm begint daar waar de dunne darm ophoudt en heet dan achtereenvolgens coecum (met de blinde darm), colon ascendens (van rechtsonder naar rechtsboven), colon transversum (dwars van rechts naar links), colon descendens (van linksboven naar onder), sigmoïd en tot slot endeldarm (rectum). Dan is de opening van de anus bereikt. Het is eigenlijk verbazingwekkend dat kwaadaardige gezwellen in al deze verschillende gebieden lange tijd op een hoop werden gegooid. Maar door onderzoek met nieuwe, moleculaire technieken weten we dat dit niet terecht is en dat er wel degelijk verschillende subtypen onderscheiden kunnen worden.

Kanker is een gezwel (tumor) van woekerende cellen. Cellen die alsmaar doorgroeien. Het ontstaat in eigen cellen en begint met de ontsporing van één enkele cel. Er is heel wat ontsporing nodig voordat de nakomelingen van deze cel zich tot een kwaadaardig gezwel kunnen ontwikkelen. Tegenwoordig zijn al een groot aantal moleculaire – en celbiologische veranderingen die deze ontwikkeling mogelijk maken in kaart gebracht. Hoe en wanneer deze veranderingen optreden verschilt voor de vele soorten van kanker. Als drijvende factor speelt hetzelfde mechanisme een rol als in Darwiniaanse evolutie, 'survival of the fittest', maar dan op micro niveau. De meest competitieve kankercellen overleven en geven hun eigenschappen door aan dochtercellen via veranderingen in het genetisch materiaal, het DNA. Ook wordt daarbij de gevreesde eigenschap verkregen van groei in het omliggende weefsel dat voor uitzaaiingen kan zorgen.

Hoe komt het dat het lichaam daar niets tegen doet? Het menselijk lichaam kan reageren door het afweersysteem (het immuunsysteem) erop af te sturen. Dat is nog niet zo gemakkelijk. Kankercellen verschillen namelijk maar nauwelijks van gezonde cellen. En dus kan het immuunsysteem ze moeilijk herkennen. Toch zijn er genoeg aanwijzingen dat het in principe wel mogelijk is. Alleen hebben sommige kwaadaardige tumoren weer manieren ontwikkeld om dat te omzeilen. Dat laatste noemen we immuunmodulatie.

In dit proefschrift bespreken wij een manier van immuunmodulatie in dikkedarmtumoren. We bestudeerden veranderingen in bepaalde moleculen die nodig zijn om immuuncellen (leukocyten, witte bloedlichaampjes) in staat te stellen de kankercellen überhaupt te herkennen. Dat zijn de zogenaamde klasse-1-humane-leukocytenantigeen-moleculen (HLA). Deze eiwitten zijn ook betrokken bij afstotingsreacties na transplantatie. Ook hebben we gepoogd om de binnen de groep van darmtumoren verschillende subtypen te onderscheiden op moleclair niveau om zo iets meer te kunnen zeggen over de processen die betrokken zijn bij

immuunmodulatie. En om ze beter te kunnen voorspellen.

In het inleidende **hoofdstuk 1** wordt een globaal overzicht gegeven van de huidige inzichten in ontwikkeling van (dikkedarm)kanker die relevant zijn voor ons onderzoek. In het bijzonder wordt aandacht besteed aan de fasen in het proces waardoor normale cellen zich ontwikkelen tot kankercellen en de veranderingen in het DNA die hierbij een rol spelen. De twee bekendste routes voor het ontstaan van dikkedarmkanker worden besproken, nl. die voor CIN- en MIN-tumoren. Hoewel deze oorspronkelijk geïdentificeerd werden in twee verschillende erfelijke vormen van dikkedarmkanker blijken ook veel niet-erfelijke tumoren via één van deze routes te ontstaan. CIN, voor chromosomale instabiliteit, hangt samen met een verstoorde functie van het APC-eiwit. Geërfde veranderingen (mutaties) in dit gen veroorzaken familiaire adenomateuze polyposis (FAP) dat wordt gekenmerkt door de ontwikkeling van honderden tot duizenden darmpoliepen met een hoog risico op kanker. MIN, dat staat voor microsatelliet instabiliteit, wordt veroorzaakt door defecten in de moleculen die betrokken zijn bij de reparatie van bepaalde DNA-beschadigingen. Mutaties in deze zogeheten 'mismatch-repair'-genen blijken erfelijk te worden overgedragen bij patiënten met het syndroom van Lynch. Deze mensen hebben een verhoogd risico op de ontwikkeling van dikkedarmkanker, maar ook op andere soorten kanker waaronder die van het baarmoederslijmvlies.

Vervolgens wordt de wisselwerking tussen tumorcellen en het immuunsysteem besproken. Een vereenvoudigd model laat de benodigde processen zien die uiteindelijk leiden tot een gerichte aanval van gespecialiseerde witte bloedcellen, de cytotoxische T-cellen, tegen tumorcellen. Het hoofdstuk wordt afgesloten met een beschrijving van de mogelijkheden waarmee tumorcellen deze aanval kunnen pareren (immuunmodulatie). In het bijzonder is er aandacht voor de manier waarop de samenstelling van HLA-moleculen kan worden veranderd.

Het eerste deel van ons onderzoek (**hoofdstuk 2**) had tot doel een methode te ontwikkelen waarmee op een efficiënte en betrouwbare manier MIN-tumoren kunnen worden onderscheiden. Daartoe werden twee verschillende technieken, nl. microsatelliet-instabiliteitsanalyse (MSI) en immunohistochemie getest op een serie baarmoederslijmvliestumoren en dikkedarmtumoren van patiënten met het syndroom van Lynch. Bij MSI-analyse wordt de instabiliteit van bepaalde DNA-sequenties (microsatellieten) onderzocht. Deze microsatellieten veranderen van lengte, ze raken 'instabiel', wanneer in tumorcellen het DNA-mismatch-repairmechanisme niet goed meer werkt. Immunohistochemie maakt het mogelijk om de aanwezigheid van specifieke eiwitten in cellen en weefsels aan te tonen. Daarvoor zijn antistoffen gericht tegen deze eiwitten nodig. Door de antistoffen te koppelen aan een kleurstof kunnen de eiwitten gelokaliseerd worden. Wij gebruikten antistoffen die tegen drie van de mismatch-repair-eiwitten gericht waren. Door dunne plakjes tumorweefsel (weefselcoupes) hiermee te behandelen konden we door microscopisch onderzoek nagaan of die eiwitten verdwenen waren in de tumorcellen. Het bleek inderdaad mogelijk om betrouwbaar mismatch-repair-defecten op te sporen en bovendien het gendefect dat hiervoor verantwoordelijk is te voorspellen.

Vervolgens onderzochten we of we deze methode ook konden toepassen op dikkedarmtumoren van patiënten met het syndroom van Lynch (**hoofdstuk 3**). Dat bleek inderdaad het

geval. Bovendien testten we de toepassing van immunohistochemie op een zogenaamde 'tissue micro array', een verzameling van pijpjes weefsel van veel verschillende patiënten samengebracht in één blokje. Daarmee is het mogelijk om in een keer heel veel (in dit geval 129) darmtumoren tegelijk te onderzoeken. De informatie uit de pijpjes bleek voldoende om de tumoren met mismatch-repair-defecten te onderscheiden.

Nu konden we overgaan tot het onderzoek van mechanismen van immuunmodulatie (**hoofdstuk 4**). Allereerst ontdekten we een veelvoorkomende mutatie van het *IFNGR1*-gen in erfelijke MIN-tumoren. In 59 procent van deze tumoren werd deze mutatie gevonden. Het gen codeert voor de receptor van interferon-gamma (IFN-γ). IFN-γ is een signaalstof die door T-cellen wordt gebruikt ter voorbereiding op de aanval op tumorcellen. Het zorgt ervoor dat tumorcellen meer HLA aanmaken (waardoor ze beter herkenbaar zijn voor het immuunsysteem) en het maakt tumorcellen gevoeliger voor de aanval zelf. We hebben onderzocht of de mutaties hier tegen beschermden. Dit deden we door gekweekte cellen van CIN-tumoren en van MIN-tumoren met *IFNGR1*-gen-mutaties bloot te stellen aan IFN-γ. De CIN- en MIN-tumorcellen bleken in gelijke mate een toename van HLA moleculen te vertonen. Ook waren de MIN-tumorcellen niet meer of minder gevoelig voor één van de door cytotoxische T-cellen gebruikte aanvalsmethoden. Kortom, de veelvoorkomende mutatie in het IFNGR1-gen speelt waarschijnlijk geen rol bij immuunmodulatie.

We besloten om de expressie van HLA-klasse-I-moleculen meer gedetailleerd te onderzoeken voor de verschillende typen dikkedarmtumoren (**hoofdstuk 5**). HLA-klasse-I bestaat namelijk uit een hele familie van eiwitten en dat maakt het behoorlijk ingewikkeld. Ten eerste is elk HLA-molecuul opgebouwd uit twee ketens, een lichte en een zware. De lichte keten is bij alle HLA-klasse-I moleculen hetzelfde maar de zware HLA-klasse-I-ketens worden verdeeld in 6 typen (loci, HLA-A tot en met G). Daarbij zijn er echter per locus zeer veel variaties (polymorfismen), met name van HLA-A (meer dan 120) en HLA-B (meer dan 250). Ieder mens heeft twee versies van elk locus, zogeheten allelen, want we krijgen er een van onze moeder en een van onze vader. Meestal zijn dit twee verschillende polymorfismen. Die variatie is van belang want dit bepaalt het 'repertoire' van eiwitfragmenten dat aan de T-cellen gepresenteerd kan worden. Deze variatie is dus ook van groot evolutionair belang om allerlei (virale) infectie-ziekten het hoofd te kunnen bieden door geïnfecteerde cellen zo snel mogelijk te herkennen en op te ruimen. De zeer vele combinaties van HLA-moleculen vormen dus een essentieel onderdeel van onze afweer tegen virale infectieziekten. Om de expressie van de verschillende typen HLA-moleculen in tumorcellen te onderzoeken hebben we gebruik gemaakt van vier-kleuren-multiparameter-flowcytometrie. Met behulp van deze gevoelige techniek kan de hoeveelheid van de verschillende HLA-moleculen in individuele cellen gemeten worden. In korte tijd worden duizenden cellen geanalyseerd, waardoor een representatief beeld van de tumorcelpopulaties wordt verkregen. De cellen worden losgemaakt uit stukjes tumorweefsel en vervolgens worden in verschillende stappen de HLA-moleculen aangekleurd. Omdat in tumoren naast tumorcellen ook verschillende soorten normale cellen voorkomen, gebruikten we drie kleuren om tumorcellen te onderscheiden en een vierde kleur om de hoeveelheid HLA-moleculen op het celoppervlak te meten. De kleurstoffen werden gekoppeld aan specifieke antilichamen die gericht waren tegen de verschillende soorten HLA-moleculen.

Omdat we afzonderlijk de expressie van HLA-A-moleculen en HLA-B-moleculen onderzochten hadden we vanwege de grote variabiliteit een aanzienlijke verscheidenheid aan antilichamen nodig.

Allereerst zagen we dat veranderingen in HLA bij 38 procent van de dikkedarmtumoren voorkomen. Ten tweede ontdekten we dat deze veranderingen zich beperkten tot slechts twee patronen (in tegenstelling tot de op voorhand berekende grote variatie aan mogelijkheden): of tumoren schakelden één allel uit, of alle vier tegelijk. In de laatste groep was trouwens niet al het HLA verdwenen. Tot slot bleek dat de HLA-veranderingen voorkwamen in twee subtypes tumoren: in MIN tumoren en in tumoren uit het proximale deel van de dikke darm (caecum, colon ascendens en het eerste stuk van de colon transversum).

We vergeleken de met flow-cytometrie verkregen resultaten met die verkregen met immunohistochemie, want die laatste techniek is beter toepasbaar op grote series van tumoren en ook op gearchiveerd tumorweefsel. Het leerde ons dat immunohistochemie in dit geval veel minder betrouwbaar is. Wel konden we er één van de patronen van HLA-afwijkingen mee onderscheiden, nl. het patroon dat wij in MIN-tumoren zagen.

Het grote aantal afwijkingen in MIN-tumoren suggereert dat deze tumoren meer onder druk staan van aanvallen van het immuunsysteem. Dat laatste is ook al eerder beschreven. Zo worden er meer T-cellen in die tumoren gezien. Bovendien zou het heel goed kunnen samenhangen met het feit dat deze tumoren zoveel afwijkende eiwitten produceren (zonder dat ze, zoals we in hoofdstuk 4 lieten zien, bijdragen aan de ontwikkeling van de tumor). Wellicht werkt dat dan toch in hun nadeel. Om te kijken of deze hypothese juist was besloten we na te gaan of er een verschil is tussen spontane-MIN-darmtumoren en erfelijke-MIN-darmtumoren. Dit wordt beschreven in **hoofdstuk 6**. We onderzochten daarvoor een grote groep tumoren. Deels tumoren uit het proximale deel van de dikke darm, waaronder spontane MIN tumoren en deels tumoren van patiënten met het syndroom van Lynch. Ditmaal onderzochten we niet alleen de patronen van HLA-veranderingen, maar zochten we ook naar de mechanismen die deze veranderingen veroorzaakten. We zochten naar mutaties in het DNA. Daarbij richtten we ons niet alleen op de HLA-allelen zelf, maar ook op de lichte keten, het β2-microglobuline (B2M) en op de kandidaten betrokken bij het bewerken van antigenen. Die laatste worden tezamen aangeduid als 'antigen processing machinery' (APM). Het is een hele batterij van eiwitten die betrokken is bij de opbouw van HLA-moleculen en in het bijzonder het verwerken van stukjes eiwit, antigenen, die op HLA-moleculen worden geladen en door deze op het celoppervlak worden 'gepresenteerd' aan patrouillerende T-cellen. Deze antigenen zijn essentieel, want zij bepalen of T-cellen de presenterende cel aanvallen of niet. HLA-moleculen die niet beladen zijn met antigenen hebben geen functie en worden zelfs niet meer naar het celoppervlak toegebracht.

We vonden mutaties in *B2M*, in *HLA-A* en in de APM-leden *TAP1*, *TAP2*, *tapasine*, *calnexine*, *calreticuline* en *ERP1*. Ook zagen we soms dat het hele gebied op chromosoom 6 (6p21.3) dat codeert voor de verschillende HLA-allelen weg was. Dit wordt 'loss of heterozygosity' (LOH) genoemd. Nog interessanter was dat het mutatiespectrum verschillen toonde tussen de verschillende typen tumoren. In Lynchtumoren vonden we met name *B2M*-mutaties, terwijl in spontane MIN-tumoren mutaties in HLA-allelen of in één van de APM-leden de overhand hadden. Verder zagen we in CIN-tumoren LOH van 6p21.3.

Wat de reden is van die verschillen in afwijkingen in HLA is nog onduidelijk. In ieder geval vertelt het ons dat er bij de ontwikkeling van dikkedarmtumoren, vooral die uit het eerste, proximale deel, een grote selectiedruk is om het immuunsysteem te ontduiken. Daar wordt in **hoofdstuk 7** nog een korte beschouwing aan gewijd. Recente literatuur komt ter sprake die laat zien dat Lynchtumoren inderdaad een hoop verschillende antigenen kunnen presenteren die voor T-cellen het signaal kunnen geven om de tumorcel te doden. De resultaten uit dit proefschrift tonen dat een preventieve of therapeutische rol van tumorvaccins bij dikkedarmkanker verder moeten worden onderzocht.

Kort samengevat beschrijft dit proefschrift methoden om verschillende typen dikkedarmkanker van elkaar te onderscheiden. Daarnaast laat het zien dat deze te onderscheiden typen tumoren ook verschillen vertonen in mechanismen van immuunmodulatie, in het bijzonder in de manier waarop het HLA-systeem wordt uitgeschakeld. Deze bevindingen benadrukken de kennelijke noodzaak van tumoren om het immuunsysteem te ontduiken en dat geeft weer hoop op therapievormen of vormen van preventie die gebruik maken van het immuunsysteem om (beginnende) kankercellen te doden. Tegelijkertijd wijst dit proefschrift op het belang om bij het ontwikkelen van therapievormen rekening te houden met de moleculaire variatie tussen de verschillende vormen van dikkedarmkanker.

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Curriculum Vitae

Jan Willem Frederik Dierssen werd geboren op 19 september 1977 in 's-Gravenhage. In 1995 behaalde hij zijn VWO diploma aan het Stedelijk Gymnasium in Leiden. Datzelfde jaar startte hij met de opleiding Biomedische wetenschappen aan de toen nog geheten Rijksuniversiteit Leiden, waarbij hij ook onderzoek verrichte bij zijn huidige promotor. In 1999 begon hij met de opleiding Geneeskunde aan de Universiteit Leiden, waarop hij in 2001 het doctoraal examen haalde. Erna, tot 2005, was hij volledig werkzaam als assistent in onderzoek, waar het huidige proefschrift het resultaat van is. In 2006 hervatte hij de studie Geneeskunde en behaalde in 2007 zijn arts examen. Sinds 2008 is hij in opleiding tot psychiater bij de Parnassia Bavo Groep in Den Haag onder prof. dr. Hans Wijbrand Hoek. Jan Willem is getrouwd met Benita van Lawick. Samen hebben zij twee dochters, Sophia en Valerie.

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