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

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# Summary

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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.