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Discovery of genetic defects in unexplained colorectal cancer syndromes

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Chapter 8

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Summary

Colorectal cancer (CRC) is the third most common cancer in men and second most common cancer in women worldwide. An estimated 20-30% of all CRC patients have a positive family history of colorectal cancer, which in 3-5% of all CRCs can be explained by inherited germline variants in highly penetrant CRC genes. The most common form of hereditary CRC is Lynch Syndrome, caused by pathogenic germline variants in the mismatch repair (MMR) genes, *MLH1*, *MSH2*, *MSH6* and *PMS2*. MMR-deficient tumors characteristically show instability in the microsatellites, small repeated sequences in the DNA, and immunohistochemical loss of the MMR gene that is mutated. Additionally, in approximately 20-25% of patients with immunohistochemical loss of *MSH2* but without a germline *MSH2* variant a germline deletion in the *EPCAM* gene is found. This deletion results in transcriptional inactivation of the *MSH2* gene, that is directly upstream from *EPCAM*. MMR-deficiency can also occur sporadically, through *MLH1* promoter hypermethylation, shown in >85% of MMR-deficient tumors. Still, in up to 60% of patients with MMR-deficient tumors without *MLH1* promoter hypermethylation no germline pathogenic MMR gene variant is detected. These patients are referred to as 'suspected Lynch Syndrome' (sLS) patients and clinical management of these patients and their families remains difficult. This thesis focusses on explaining the MMR deficiencies and finding underlying genetic causes in these patients and their relatives. We hypothesize that these sLS patients can be explained by (1) missed variants in the MMR genes, (2) biallelic somatic inactivation of the MMR genes or (3) variants in other genes that subsequently lead to secondary MMR-deficiency.

Accordingly, in **Chapter 2**, we describe an effort to detect missed germline intronic or promoter variants in the *MLH1*, *MSH2*, *MSH6* and *PMS2* genes, but also germline variants in other CRC susceptibility genes, such as *MUTYH*, *BMRP1A*, *PTEN* and *APC*. Whole Gene Capture on leukocyte DNA of 45 sLS patients showed 1979 germline variants, of which the majority (97%) was intronic. One patient was found to carry a missed variant in *MLH1*, resulting in a 29 amino acid incorporation in the protein-interacting domain. This patient showed solitary *PMS2* protein loss in the tumor, and *MLH1* had not been screened before. Additionally, germline variants of uncertain significance (VUS) were found in *EPCAM*, *MSH3*, *MUTYH* and *AXIN1*, but no further testing was done to assess the functional relevance of these variants.

In **Chapter 3** we describe a more prevalent explanation for these suspected Lynch Syndrome patients. Of 62 sLS patients leukocyte and tumor DNA was analysed with next-generation sequencing (NGS) to detect somatic and germline variants in the MMR genes, but also the *POLE* and *POLD1* genes. Variants in the exonuclease domain (EDM) of these genes encoding for polymerase ϵ and δ respectively, had been described in microsatellite stable tumors before but the prevalence of these type of variants in MMR-deficient/MSI-H tumors was unknown. With NGS 10 tumors were found to carry two somatic aberrations (two pathogenic variants or one pathogenic variant with concomitant loss of heterozygosity) in the MMR gene that showed immunohistochemical loss. Additionally, in nine patients a germline- (n=2) or somatic (n=7) variant was detected in the EDM of *POLE* or *POLD1*. All *POLE/POLD1* mutated tumors seemed to show a hypermutated phenotype, concordant with previous studies on *POLE/POLD1* EDM variants.

In **Chapter 4** we present a method to verify the predicted splicing effect of splice site variants found in *MLH1*, *MSH2*, *MSH6*, *APC* and *BRCA1*. For 11 variants RNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissue blocks, and for two additional variants RNA was isolated from EBV-transformed B-cells. Of the total 13 splice site variants, eight variants were previously described to result in splicing, while five were only predicted to result in splicing, but functional evidence was lacking. For all variants specific primers were designed, and cDNA was synthesized and analysed with PCR. For six out of eight samples carrying known splice site variants RNA could be successfully isolated, and all six showed the previously reported aberrant splicing. Notable, one variant for which only RNA from EBV-transformed cell lines was available only showed an aberrantly spliced product after formalin fixation of the cells, indicating that formalin fixation inhibits RNA degradation. For four of the five variants predicted to result in aberrant splicing of RNA cDNA products could successfully be amplified and aberrant splicing was seen in three. It could be concluded that these variants should be regarded as (likely) pathogenic and not of uncertain significance. A simple addition to the analysis toolkit can thus answer the potential pathogenicity of variants involved in aberrant splicing.

In **Chapter 5** we describe a practical guide to detect and analyse variants in *PMS2* in DNA isolated from FFPE tissue. DNA isolated from FFPE is often very fragmented, and the standard diagnostic routine for sequencing leukocyte DNA is not feasible in these samples. This brings extra complexity for the analysis of *PMS2*, which in itself is already complex due to the presence of multiple pseudogenes. Fourteen *PMS2*-pseudogenes share a high homology with the 5' end of *PMS2* (exon 1 to 5), while a fifteenth pseudogene (*PMS2CL*) shares high homology with *PMS2* exon 9 and exon 11 to 15. *PMS2* is distinguishable through paralogous sequence variants (PSVs), a small number of nucleotides that are specific for *PMS2* and not present in the pseudogenes. By designing a custom NGS FFPE-suitable library with small amplicons containing one or two PSVs each, exon 1 to exon 11 can be reliably sequenced.

In **Chapter 6** we aimed to detect the underlying genetic cause in unexplained adenomatous polyposis patients negative for germline defects using conventional testing for CRC susceptibility genes. DNA of two or more colonic adenomas of each patient was tested for *APC* variants with NGS, with the hypothesis that if multiple adenomas carry an identical *APC* variant, this might indicate underlying mosaicism. In nine of 18 patients, and two positive controls, all with 21 to 100 adenomas an identical *APC* variant was detected in multiple adenomas. Testing of DNA from different germ layers (mesoderm, ectoderm and endoderm) could help identify the approximate time point at which the mosaicism arose. Different patterns of mosaicism were identified. In three patients the *APC* variant present in the adenomas could be detected in leukocyte DNA with a very low variant allele frequency, in one patient the *APC* variant was confined to the colon and in six patients the variant was not present in leukocyte, and no normal colonic mucosa was available or testing was not possible. In one patient the variant was only detected in adenomas but not in leukocyte DNA or normal colonic mucosa. In this patient we propose an underlying mechanism of field cancerization where one tumorigenic clone migrates through the colon. Lastly, there was one patient that had mixed mosaic adenomas with sporadic adenomas with unique *APC* variants.