English summary

Functional analysis of MSH2/MSH6 variants: in tune or off key?

Lynch syndrome (LS) is an autosomal-dominant disorder that predisposes patients to colorectal cancer (CRC), endometrial cancer (EC) and several other cancer types. The risk of developing CRC and EC is up to 10 times increased compared to the general population. LS is caused by inherited defects in the DNA mismatch repair (MMR) system, which main players are two heterodimers, MSH2/MSH6 and MLH1/PMS2. The core function of the MMR system is the recognition and repair of replication errors. For successful repair, it needs to recognize the mismatching base(s), which is done by MSH2/MSH6. Subsequently, it is important to distinguish between the parental and newly synthesized strand to ensure genomic integrity. The MLH1/PMS2 dimer plays an essential role in this process and further orchestrates the removal of the mismatch. Subsequently, the removed sequence is resynthesized. MMR also plays a role in the prevention of homeologous recombination (*i.e.*, recombination between homologous but not identical DNA sequences), most likely by recognition of the mismatches that arise upon the formation of a D-loop. Another main function of MMR is mediating the toxicity of certain DNA damaging agents, among which several chemotherapeutics, by its capacity to recognize methylated bases.

When a patient is suspected of Lynch syndrome, the tumor material is examined for indications of MMR deficiency. This is done by immunohistochemical (IHC) staining for the four main MMR proteins and by microsatellite instability (MSI) analysis, which is a marker for a defect in MMR. When the results point towards a defect, the suspected MMR genes are sequenced for aberrancies. The majority of mutations found are deletions, frameshifts, premature stop codons and other mutations that clearly abrogate protein function. However, there is also a significant proportion that consists of variants of which the effects on protein function are much harder to predict. These are called Variants of Uncertain Significance (VUS) and this group mainly consists of missense mutations.

When a clearly pathogenic mutation is found, counseling the patient and his/her family is relatively straightforward since family members can be tested for the presence of the mutation. Subsequently, carriers are offered regular screening through (bi)annual colonoscopy and gynaecological screening for women. This screening is burdensome but has proven to be effective. In those cases where a VUS is found, counseling is problematic since non-carriers cannot safely be excluded from screening and hence all

family members are advised to undergo screening. This places a burden on all involved, both physically and mentally. Therefore, methods to reliably classify VUS are extremely important and a lot of effort is put into their development.

This thesis presents a novel method to test *MSH2* and *MSH6* VUS found in families with suspected Lynch syndrome. To obtain clear and reliable results we chose to use a system that allowed us to introduce the selected variants into the endogenous gene of a convenient cell type: mouse embryonic stem cells (ESCs). Variants were generated using the oligotargeting technique, which makes use of small single-stranded DNA oligonucleotides. This system closely mimics the human situation since only the codon affected by the missense mutation under study was altered and mouse and human MMR proteins are highly homologous. Modifying the endogenous MMR gene ensures physiological expression levels and avoids overexpression that could mask a partial defect. Subsequent inactivation of the wild-type allele yielded cells that exclusively expressed the mutant allele. To assess the MMR capacity of mutant cells, we performed a set of functional assays spanning the main MMR functions. This way we could directly study the effect of the mutation on the MMR system in an *in vivo* setting.

In **chapter 2** we set up the system by creating four *MSH2* missense mutations found in suspected Lynch syndrome patients. We selected these mutations based on the available literature; one mutation was strongly suspected to be pathogenic, another to be polymorphic. Our results classified these two mutations as pathogenic and polymorphic, respectively, and the other two as polymorphic. The data from the functional assays corresponded with the clinical data that was reported in the literature.

In **chapter 3**, we used a similar experimental setup to see if our method would also obtain reliable results for *MSH6* missense mutations. While a proven deleterious mutation was indeed identified as pathogenic in our assay, three others behaved as wild-type. Again, these results could be reconciled with clinical data, which led to the conclusion that our setup also works for *Msh6*. With the reliability of our approach now confirmed, we moved away from the literature into the clinical setting. Through our cooperation with three Dutch centers for clinical genetics, we obtained requests for the analysis of three *MSH2* missense mutations found in Dutch families with suspected Lynch syndrome. In all three cases, conventional classification using clinical data and *in silico* analysis had been insufficient to classify these VUS, which hampered counseling. In **chapter 4** we presented the clinical data along with the results from our analysis. The two sets of data showed large similarities, once more confirming how closely our system mimics the clinical situation. Surprisingly, two VUS showed a clear but partial MMR defect while the third

behaved as wild-type and was classified as a polymorphism. Even though the defects of the other two VUS were partial, they were significant which led us to believe that these VUS are the underlying cause of the disease although they might be low penetrance alleles.

In **chapter 5** we exploited one of the advantages of using mouse ES cells combined with oligotargeting by making a mutant mouse. We selected a VUS that caused a deletion of the last 60 amino acids of MSH2. For this VUS, we did not limit our study to a single cell line. Because of the underlying fundamental question regarding the role of the C-terminus of MSH2, we made additional cell lines in which we inactivated either *Msh3* or *Msh6.* Our analysis showed that the interaction between the truncated MSH2 protein and its binding partners was affected but not completely lost as there was some remaining MMR activity seen in the functional assays. The mutant mice showed a clear tumor predisposition and a shortened lifespan. In agreement with the functional data, however, the effect was less severe than a complete loss of *Msh2.* This led to the conclusion that the MSH2 C-terminal 60 amino acids are essential for interaction with MSH6 and MSH3 and effective DNA mismatch repair.

We have shown in this thesis that our approach is a valuable alternative to existing functional assays and can aid VUS classification and counselling. Its major advantages include the highly homologous system, the wide range of assays that can be done to study MMR functionality and the physiological expression levels that are obtained through the use of oligotargeting. Furthermore, the ability to make a mutant mouse is an additional asset for those cases in which the effect of the VUS on tumor development cannot be predicted based on the results of the assays. It is however a labour intensive approach that requires specialized knowledge and specific skills. Currently, several improvements have already been made to reduce the time involved, which greatly enhances the applicability and accessibility of our method. We feel the highest value of our approach can be obtained through collaboration between the clinic and the research lab, as shown in chapter 4 where both parties combined their specialized knowledge to achieve the best possible classification.