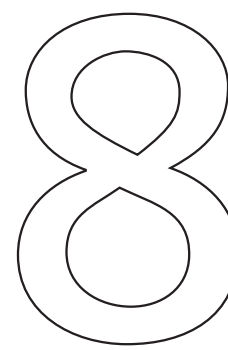
General Discussion and Future Perspectives



Variants of Uncertain Significance (VUS) pose a major problem not only in Lynch syndrome diagnostics, but in clinical genetics in general [Spurdle, 2010; Rasmussen et al., 2012; Sijmons et al., 2013]. A lack of diagnostic assessment of VUS leaves carriers and both affected and unaffected relatives in uncertainty regarding their disease status. This, combined with the surveillance procedures that such patients and all relatives (both carriers and non-carriers) are enrolled in pose a physical and psychological burden on families, but also a burden on the preventive health care apparatus.

The current record of variants in DNA mismatch repair genes is a reflection of sequencing efforts from the last few decades [Fokkema et al., 2011; Plazzer et al., 2013]. From a diagnostic point-of-view, LS families with many disease cases were probably genetically tested first. This likely has resulted in an ascertainment bias where mainly mutations that completely inactivate MMR and are highly penetrant have been identified, such as nonsense of frameshift mutations. The advent of Next Generation Sequencing technologies has made genetic testing easier, cheaper and faster. This will likely lower the criteria for sequencing, which will further increase the incidence of VUS [Heinen, 2010; Rasmussen et al., 2012; Sikkema-Raddatz et al., 2013]. For these reasons, the development and use of robust and accessible approaches to diagnose pathogenic VUS is becoming increasingly important. This will enable the implementation of preventive and curative healthcare that is targeted only to carriers of pathogenic MMR gene VUS. Meanwhile, unaffected relatives of such carriers can be liberated from the burden associated with the uncertain pathogenicity of the VUS while unwarranted inflow into preventive healthcare systems is reduced.

Integration Of Novel Assays For Functional Analysis In Current Lynch Syndrome Diagnostics

Current LS diagnosis protocols are aimed at determining the inactivating mutation in a DNA MMR gene through genetic testing (Chapters 1 and 2). Once such an inactivating mutation has been found, LS diagnosis is completed. In case a VUS is found, LS diagnosis is halted. To assist geneticists in subsequent diagnostics, we and others have proposed additional steps to the current diagnostic tree (discussed in Chapter 2; [Couch et al., 2008]). We have proposed that, once a VUS has been found, the allele is first tested in an *in vitro* MMR assay (Chapters 3-5) and is simultaneously evaluated using *in silico* analyses [Tavtigian et al., 2008; Thompson et al., 2012b]. As the *in vitro* MMR assay covers (nearly) all aspects of canonical MMR and is relatively quick, this assays seems the most feasible approach. Furthermore, owing to the relative simplicity of this assay it may be implemented in multiple labs globally. Based on preliminary data the approach proposed above appears to be a powerful one for the diagnostic assessment of MMR gene VUS [Kansikas et al., 2011].

To integrate the *in vitro* assay in current LS diagnostics, its output may be integrated in a five-class classification system [Plon et al., 2008]. These classes can replace poorly defined terms such as "VUS", "neutral" and "pathogenic" and may aid clinical management by defining specific recommendations for each category. Those five classes are as follows:

- Class 5 variant - Pathogenic, > 99% probability of pathogenicity
- Class 4 variant - Likely Pathogenic, 95-99% probability of pathogenicity
- Class 3 variant - Uncertain, 5-95% probability of pathogenicity
- Class 2 variant - Likely Neutral, 0.1-5% probability of pathogenicity
- Class 1 variant – Neutral < 0.1% probability of pathogenicity

A five-class classification system can be established by combining multiple criteria for assessing pathogenicity to VUS, including well-established ones such as family history, immunohistrochemistry and microsatellite instability (MSI), but also *in silico* assays [Tavtigian et al., 2008] and the *in vitro* MMR assay [Drost et al., 2010, 2012]. These data sets are then integrated into a single statistical model [Plon et al., 2008]. For the assays integration and validation, the assay should be evaluated prosprectively by large-scale validation efforts that include performing the assay on a large number of substitutions, classified in either class 1 or class 5, without the use of functional assays. Such a validation effort should aid in determining positive and negative predictive values and Likelyhood Ratios, which will eventually help to determine a posterior probability of pathogenicity [Thompson et al., 2012a, 2012b].

The *in vitro* MMR assay in its current status has a binary outcome. MMR variants can be either repair deficient (not significantly different from a repair deficient control) or repair proficient (significantly higher than a repair deficient control). When integrating the *in vitro* MMR assays' output into a five-class classification system, its readout (percentage of repair compared to wild type) could be calibrated to be translated into a probability in favor of pathogenicity and may serve as a constant variable.

The *in vitro* MMR assay could be a powerful tool to diagnose VUS in MMR genes, however, the *in vitro* nature of the assays has its pitfalls. The assay is not able to pick up certain *in vivo* defects and therefore, in case a VUS is repair proficient *in vitro* the diagnostic value of the *in vitro* MMR assay is inconclusive and more specialized assays, such as protein stability [Perera and Bapat, 2008], nuclear localization assays [Raevaara et al., 2005; Andersen et al., 2012; Borràs et al., 2013] and splicing assays [Tournier et al., 2008] are required to assess pathogenicity [Rasmussen et al., 2012]. In this regard, the Reverse Diagnosis Catalogs (RDC) may contribute to diagnostic assessment. The *a priori* knowledge of essential residues in MMR proteins may be valuable information in identifying pathogenic missense mutations. In case a human VUS is identified in the RDC, this would imply a high probability of pathogenicity of that VUS. Once Likelyhood Ratios for the RDC are determined and the RDC is incorporated in a five-class classification system, the identification of a certain substitution in the RDC should serve as strong evidence to classify a VUS as class 4 or 5.

To integrate both the *in vitro* assay and the Reverse Diagnosis Catalogs (RDC), these assays could be cross-validated. The RDC may then serve as a "golden standard" of pathogenic alleles, to which the *in vitro* assay may be calibrated against. In this manner, the RDC are a substitute for patient-derived inactivating alleles. Taking this one step further, the cell lines made in the RDC screens could be used to generate mice to determine penetrance of the alleles, which then can be linked to *in vitro* repair efficiencies.

To continue along this line, the RDCs may serve as a standard not only to validate the *in vitro* assay, but also to validate *in silico* algorithms such as MAPP-MMR [Chao et al., 2008], PolyPhen [Adzhubei et al., 2010] or the combination of both [Thompson et al., 2012b]. Once the RDC protocol is adapted to also identify disrupting intronic variants or splice site mutations (see below), the data from the RDC may also aid in validating *in silico* splice site prediction software [Vreeswijk et al., 2009; Vreeswijk and van der Klift, 2012].

Towards Comprehensive Reverse Diagnosis Catalogs

This thesis describes the generation of pilot RDCs for Msh2 (Chapter 6) and Msh6 (Chapter 7). In order to generate saturated, comprehensive catalogs the methodology described in this thesis may be adapted in several ways.

First, mutations are introduced into the MMR genes using the mutagen N-ethyl-N-nitrosourea (ENU). Even though ENU is a relatively wide-spectrum mutagen, using a single chemical for mutagenesis may not suffice to saturate a RDC. Higher saturation of the catalog could be reached by using additional drugs with a complementary mutagenic activity, such as 4-nitroquinoline *N*-oxide [Ryu et al., 1999] or 7,12-dimethylbenz[a]anthracene [Manjanatha et al., 1996]. ENU is not only a suited mutagen due to its diverse mutagenic activity, but also due to its property to induce only low amounts of loss of heterozygosity (LOH). Using other drugs may induce a higher proportion of LOH events, which will decrease the efficiency of the screen. Such an issue may be circumvented by putting selective pressure against LOH, by targeting a selectable marker directly next to the non-disrupted *Msh2* and *Msh6* alleles and using the generated cell lines for RDC development.

Second, the current protocol selects for MMR deficiency using 6-thioguanine (6-TG). Our experimental data suggests that the selection conditions applied may have been quite harsh, as only fully (*Msh2*$^{-/-}$-like) tolerance of MNNG was measured in all generated cell lines (Chapter 6). In future work, selection conditions could be further optimized. By applying a less stringent selection the protocol could be adjusted as such, so that alleles with intermediate levels of methylation tolerance can be selected for. In this way, the RDC could be extended from completely methylation tolerant, probably highly penetrant alleles, towards less penetrant alleles. In this respect, 6-TG could also be replaced by MNNG as a selection agent. Other have shown that cells tolerant to MNNG can still be proficient in repair [Claij and te Riele, 2002]. This could make MNNG a more appropriate drug to select for less penetrant alleles.

Finally, the RDC presented in this thesis are aimed completely at disruptive missense mutations as only those 6-TG-tolerant clones that retain expression of full-length cDNAs were sequenced. From a clinical geneticists' point-of-view, additional types of VUS are of interest, such as intronic variants or splice site variants. To identify these, one would have to apply methods other than cDNA sequencing, such as sequencing of genomic DNA using a classical PCR and Sanger sequencing approach, or to use Next Generation Sequencing methods.

Functional Assays in Fundamental Research

The assays we have developed will not only allow us to assess pathogenicity of VUS in MMR genes, but can also aid in functional studies of MMR. Especially the combination of both the *in vitro* assay and the RDC may help to identify novel functional residues/motifs in MMR proteins that are important for *in vivo* MMR.

A very interesting allele that shows (relevant) discrepancies between *in vivo* and *in vitro* phenotypes was recently described by Li and co-workers [Li et al., 2013]. The PWWP domain in MSH6 helps to recruit MutSα onto chromatin by interacting with the histone mark H3K56me3. Knockdown of SETD2, the transferase that methylates H3K56, gives an MSI phenotype suggesting that SETD2 and H3K56me3 are upstream of MMR *in vivo*. *MSH6* alleles with mutations in the PWWP motif are MMR deficient *in vivo*, but MMR proficient in the *in vitro* MMR assay, which might be explained by the difference in chromatin states of the DNA substrates. The identification and subsequent characterization of this type of alleles may provide new insights into the biochemistry of MMR. Such alleles may be identified in the RDC, by using a readout for *in vivo* MMR and, in parallel, testing those alleles in the *in vitro* MMR assay. The Msh6 RDC may have already identified such alleles. Msh6 alleles D1211E, D1211G and H1246R are repair proficient in the *in vitro* assay but show hallmarks of *in vivo* MMR deficiency (Chapter 7). These are interesting alleles for future research.

In Chapters 6 and 7 several methods are described for measuring MMR *in vivo*, such as MSI assessment and determining spontaneous mutation frequencies at a reporter gene. An alternative approach would be to introduce a fluorescent reporter such as a GFP, rendered out-of-frame due to a microsatellite preceding its ORF. In MMR deficient cells, this microsatellite will be unstable, rendering *GFP* into frame. Therefore, the amount of GFP proficient cells in a cell population will be a measure of the MMR capacity of the MMR allele(s) expressed in those cells [Koole et al., 2013].

Methylation Tolerance: Futile Cycling Or Direct Signaling?

The mechanism of the MMR-mediated toxicity of methylating agents has been under debate for many years now. The MMR field has been divided into two "camps": One supporting the futile cycling model [Stojic et al., 2004; Mojas et al., 2007] and one supporting the direct signaling model [Yang et al., 2004; Yoshioka et al., 2006]. Even though both models are not mutually exclusive, several arguments are in favor of the futile cycling model. First, apoptosis after methylating agents is induced in the second S-phase after treatment, which suggests that a (DNA) intermediate from the first cell cycle is carried over to the second and only then induces apoptosis [Mojas et al., 2007]. Second, proteins such as ATM [Debiak et al., 2004], RAD51 and BRCA1 protect against apoptosis induced by methylating agents, which suggests that a DNA double-strand break is involved, which would be unexpected with the direct signaling model in mind. Third, methylation-induced apoptosis requires a full complement of MLH1 [Cejka et al., 2003] and the catalytic activity of EXO1 [Schaetzlein et al., 2013]. This suggests that a canonical MMR complex is required to induce apoptosis.

The major pieces of evidence in favor of the direct signaling model are 1) Binding of ATR, TopBP1 and CHK1, but not RPA, to chromatin after MNNG treatment in a MutSα and MutLα dependent manner [Liu et al., 2009], 2) Protein interactions between MutSα and ATR [Liu et al., 2009; Pabla et al., 2011], TopBP1 and CHK1, and between MutLα and TopBP1 [Liu et al., 2009], 3) The phosphorylation of CHK1 in the presence of O^6-meG·T mismatches and MMR proteins *in vitro* in the absence of excision [Yoshioka et al., 2006] and 4) Separation-of-function alleles [Lin et al., 2004; Yang et al., 2004]. The relevance of the described protein interactions and chromatin loading briefly after MNNG is questionable, as ATR is only activated at later timepoints [Stojic et al., 2004] and, even if ATR would be activated at earlier timepoints, there is insufficient signaling to trigger apoptosis [Mojas et al., 2007]. The *in vitro* phosphorylation of CHK1 in the presence of O^6-meG·T mismatches and MMR proteins, but in the absence of excision, is prone to artefacts. The absence of excision is measured in an *in vitro* MMR assay which is quantified on an agarose gel [Yoshioka et al., 2006]. In our experience, quantification on gel is not very sensitive and repair percentages of up to 10% can easily stay underdetected, which may suffice to trigger signaling *in vitro*. Therefore, the phosphorylation of CHK1 may still result from single-stranded DNA and a more sensitive method would be required to rule out excision.

Perhaps the strongest evidence for the direct signaling model is the generation and characterization of two separation-of-function alleles in *Msh2* [Lin et al., 2004] and *Msh6* [Yang et al., 2004]. Both of these alleles confer a phenotype that is characterized by repair deficiency, but methylation sensitivity. It should be noted, though, that both described cell lines show high levels of spontaneous apoptosis. This could be a consequence of the ability of these MMR alleles to bind DNA, but their inability to subsequently release the heteroduplex or the inadvertently bound homoduplex. This may lead to a dominant-negative phenotype, in which the persistently DNA-bound MMR alleles impede other cellular processes such as transcription and replication. Therefore, the proficiency to induce apoptosis may not reflect a true separation-of-function phenotype, but a consequence of decreased cellular fitness. An interesting piece of evidence in favor of the direct signaling model would be an allele that has the opposite phenotype: repair proficiency, but methylation tolerance. The RDC protocol would be a perfect approach to find such an allele, as the screen selects for methylation tolerance and, once a GFP reporter is introduced, allows for a high-throughput screening for repair proficiency. The pilot Msh2 and Msh6 RDCs that we generated (Chapters 6 and 7) argues against the existence of such alleles, as we have only found alleles that are repair deficient *in vivo* as well as methylation tolerant. Conversely, this could be a consequence of the limited numbers of alleles identified so far, and increasing the scale of the RDC may suffice to identify such alleles.

Molecular And Clinical Testing And The Future Of Clinical Genomics

Next-generation sequencing technologies are becoming the primary tool in human genetics [Goldstein et. al, 2013; Katsanis et. al, 2013]. These technologies are reaching the point of being able to detect genetic variations at a high accuracy and reduced cost, which may alter

(personalized) medicine. The magnitude of data could lead to weakly justified claims of causality between genetic variants and disease [Goldstein et. al, 2013]. In this thesis, multiple methods are described that allow to determine causality of DNA variants for the cancer predisposition Lynch syndrome. Such methods may be instrumental in facilitating diagnosis in the upcoming age of personalized medicine, aiding in the future of clinical molecular diagnostics.

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