Molecular aspects of HNPCC and identification of mutation carriers
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Chapter 8

General discussion and future perspectives
GENERAL DISCUSSION

Identification of HNPCC

In this thesis, several issues regarding hereditary nonpolyposis colorectal cancer (HNPCC), or Lynch syndrome, are addressed. Possibly the most important issue, physicians dealing with HNPCC suspected patients are struggling with, is which (HNPCC suspected) patients should be offered screening for mutations in the mismatch repair (MMR) genes. Different sets of criteria have been formulated, such as the Amsterdam criteria II, to clinically define HNPCC and to select HNPCC suspected patients for mutation analysis in the MMR genes.\textsuperscript{1-4} However, numerous studies have shown that these criteria are too stringent and that, as a consequence, mutation carriers are missed when these criteria are used to select patients for mutation analysis.\textsuperscript{3-16} One of the objectives of this study was to formulate criteria with a higher sensitivity and specificity compared to the ones previously published. Therefore, we investigated a group of HNPCC suspected patients with either colorectal cancer (CRC) before the age of 50 or with CRC and another HNPCC associated tumour, for mutations in the mismatch repair genes $\text{MLH1}$, $\text{MSH2}$ and $\text{MSH6}$ (see chapter 3). The sensitivity of the Amsterdam Criteria II for the prediction of presence of a mutation in this group was 36%. This is similar to most other studies, however, sensitivities in these other studies range from 13\% to 78\%.\textsuperscript{17-23} Differences between the studies may be explained by differences in inclusion criteria. For instance, in some studies a positive family history was one of the inclusion criteria.\textsuperscript{24-26} The study which found a sensitivity of 13\% had included CRC patients, unselected for family history or age of onset, which may be the explanation for the low sensitivity rate.\textsuperscript{27}

On the basis of our study data, if the Amsterdam Criteria II would have been used as a selection criterion for mutation analysis, 64\% of the mutation carriers would have been missed. Therefore, more sensitive criteria are needed. The positive predictive value of the Amsterdam Criteria II, on the other hand, was in our study 43\%, meaning that in 43\% of the Amsterdam Criteria II positive families a mutation in $\text{MLH1}$, $\text{MSH2}$, $\text{MSH6}$ was detected. Immunohistochemical (IHC) staining for the mismatch repair proteins MLH1, MSH2 and MSH6, however, proved to have a much higher sensitivity, closely followed by microsatellite instability (MSI) analysis. Our data suggest that preselection by means of IHC and/or MSI-analysis before mutation analysis is justified in the group of patients with either CRC before the age of 50 or with CRC and another HNPCC associated tumour. In addition, we found that the occurrence of two or more HNPCC-associated cancers all above the age of 60 years is
rarely due to germline MMR gene mutations. However, as another study on patients with multiple HNPCC associated tumours showed that 20% of the mutation carriers had an age of diagnosis of the first tumour above the age of 60 years, it seems not correct to exclude these patients for mutation analysis.28

**Sporadic tumours**

In addition to the prescreen methods used for inclusion for mutation analysis mentioned above, protocols have been proposed to exclude patients from screening. Most of them are protocols trying to identify non-HNPCC tumours. Previously, it had been shown that detection of the \(BRAF\)-Val600Glu mutation in CRC is most likely suggesting a sporadic origin of the disease, and the absence of germline alterations of \(MLH1\) and \(MSH2\).29-33 We investigated tumours from \(MSH6\) mutation carriers and tumours from MMR gene mutation-negative patients from Amsterdam criteria-positive families for the \(BRAF\)-Val600Glu mutation and concluded that this mutation is absent in these tumours as well. These data, in combination with the \(BRAF\) mutation data in \(MLH1\) and \(MSH2\) mutation carriers, suggested a potential use of the somatic \(BRAF\)-Val600Glu mutation (in tumours) as an exclusion criterion for HNPCC or as a molecular marker of sporadic cancer.34

Another marker for the identification of sporadic tumours, in the context of HNPCC, is hypermethylation of the \(MLH1\) promoter, a phenomenon that is characteristic for sporadic MMR deficient CRCs. However, there are several pitfalls for the use of this marker. One of them is the presence of hypermethylation of the \(MLH1\) promoter in lymphocytes, suggesting a germline defect. Such epigenetic events have been found responsible for a small fraction of the HNPCC suspected cases as eight CRC patients were reported with hypermethylation of the \(MLH1\) promoter in lymphocytes.35-38 Tumours of these patients are MSI-high and show loss of MLH1 expression, just like the tumours of \(MLH1\) mutation carriers. A small fraction of tumours which show \(MLH1\) promoter methylation, will therefore be caused by this phenomenon. We also identified one such patient (chapter 6). The use of \(MLH1\) promoter hypermethylation as an exclusion criterion for mutation analysis could therefore be a pitfall in diagnostic testing of HNPCC suspected patients. This may be overcome by adding methylation analysis of the \(MLH1\) promoter in lymphocytes to the standard methylation analysis in tumour material. Another pitfall may be that finding hypermethylation in the tumour (and not in lymphocytes), does not necessarily imply that it is a sporadic tumour: there are reports of patients with a pathogenic germline \(MLH1\) mutation in combination with somatic hypermethylation of the \(MLH1\) promoter, which is probably the second hit in these
patients according to the Knudson’s two hit model. \textsuperscript{39-41} However, the exact prevalence of \textit{MLH1} promoter hypermethylation in \textit{MLH1} mutation carriers is not yet clear: reported prevalences range from 0\% to 44\%.\textsuperscript{42-46} On the basis of these findings, we question the screening strategy recently proposed by Hampel et al. in which mutation analysis in \textit{MLH1} is excluded if the tumour shows somatic \textit{MLH1} promoter hypermethylation.\textsuperscript{47}

**Candidate genes**

Another issue we have tried to address in this thesis is, whether mutations in other genes, or combinations of mutations in MMR genes, can lead to an HNPCC phenotype. This knowledge is essential for the design of the perfect selection strategy to identify HNPCC patients. If mutations in other genes are shown to contribute to HNPCC, these genes should be included in the screening procedures. For instance, recently it was shown that germline mutations in \textit{PMS2} play a far more important role in HNPCC than initially thought.\textsuperscript{48-53} In the study described in chapter 5, three carriers of pathogenic \textit{PMS2} mutations were identified in a group of 73 HNPCC suspected patients. These 73 patients had an MSI-high tumour and/or were from an Amsterdam Criteria II positive family, nevertheless, no germline mutation in \textit{MLH1}, \textit{MSH2}, or \textit{MSH6} had been identified previously. The tumour of the one mutation carrier with an informative PMS2 staining showed loss of PMS2 expression. On the basis of these findings, in combination with previous studies on \textit{PMS2}, it was concluded that IHC analysis for the PMS2 protein and mutation analysis in \textit{PMS2} should be implemented in the current screening protocols.

For other candidate genes, the evidence for an important causative role is less convincing. In HNPCC suspected patients, germline mutations have also been reported in \textit{MLH3}, \textit{EXO1}, \textit{PMS1}, and \textit{TGFβRII}.\textsuperscript{54-57} However, other studies either did not detect any pathogenic mutations in these genes in their study population or questioned the pathogenicity of the encountered mutations.\textsuperscript{58-64}

**Combinations of mutations**

All studies, so far, have considered HNPCC as a clear Mendelian disease, one mutation causing the syndrome in an autosomal dominant way with incomplete penetrance. Non-mendelian inheritance, especially in the group of patients we have been focusing on, can however not be excluded. It is conceivable that certain mutations are harmless on their own, but deleterious in combination with mutations in other genes. In a previous publication we have shown already that combinations of MMR gene mutations exist.\textsuperscript{65} Three of the twelve
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MLH3 missense mutation carriers we reported, also carried an MSH6 mutation of unknown pathogenicity. Other combinations have been reported (e.g. MLH3 with MSH2, PMS1 with MSH2, and MSH6 with MSH2), but real proof that the combinations indeed caused cancer development in the reported patients is limited or nonexisting.\textsuperscript{66-68} Besides combinations of mutations in MMR genes, mutations in other repair genes could be involved as well, such as MUTYH, a base excision repair gene. This idea is based on the observation that the MUTYH protein interacts with the MMR protein MSH6 which is part of the MSH2/MSH6 heterodimer (hMutS\(\alpha\)).\textsuperscript{69} It was shown that MutS\(\alpha\) stimulates the DNA binding and glycosylase activities of MUTYH. Considering this interaction between the MUTYH and MSH6 proteins, we hypothesised that a monoallelic MUTYH mutation might contribute to cancer susceptibility in carriers of an MMR gene (in particular MSH6) mutation (chapter 6). We therefore investigated the prevalence of monoallelic MUTYH mutations in carriers of a germline MMR mutation, and observed that MUTYH mutations occur more frequently in MMR gene missense mutation carriers than in controls. The results suggested that the presence of a monoallelic MUTYH mutation combined with a missense MMR gene mutation, of MSH6 in particular, leads to an increased cancer risk. As the possible interaction of the observed missense mutations is presently unknown at the functional level, we did not reach any definite conclusions yet as to its clinical significance. However, if larger studies confirm these results, and conclude that combined mutations cause a clinically relevant increased tumour risk, then this could have implications for management of patients with monoallelic MUTYH mutations: they should all be examined for mutations in MSH6 and conversely, MSH6 missense mutation carriers should all be screened for MUTYH mutations.

\textbf{Figure 1.} Strategy for identification of CRC patients with a pathogenic mismatch repair gene mutation
A strategy to detect MMR gene mutations in HNPCC suspected CRC patients

When patients are diagnosed with CRC, an assessment should be made whether or not the patient is at risk for HNPCC. It is recommended to get an accurate impression of the family history for HNPCC associated tumours, bearing in mind that self reported family history is not necessarily accurate. In case families fulfil the Amsterdam Criteria II, or are highly suspected of HNPCC (e.g. early-onset cancer, multiple HNPCC associated tumours, family history, particular tumour characteristics), referral to a department of clinical genetics is advised.

CRC patients who are not directly referred to a department of clinical genetics

CRC patients in whom the suspicion for HNPCC is less pronounced, will often not be directly referred to a genetics clinic. Patients who fulfil the Bethesda criteria should in our opinion be screened directly for the $BRAF$-Val600Glu mutation and MSI. From an ethical point of view these methods allow screening of the tumours without the necessity of genetic counselling prior to this screening (in contrast to IHC analysis, where absence of MMR protein staining is strongly predictive for the presence of a germline MMR gene mutation). If an MSI-high tumour in combination with a negative $BRAF$ mutation status is found - which is indicative for a MMR gene defect - genetic counselling should be offered. If the tumour harbours the $BRAF$-Val600Glu, or is MSI-low and negative for the $BRAF$-Val600Glu mutation, no further genetic analysis will be performed and genetic counselling is not indicative. When patients decide, after genetic counselling, that they want to know whether or not they are carriers of a MMR gene mutation, the strategy (with IHC analysis as the next step), as presented here, can be followed by the collaborating genetics and pathology laboratories. However, some $MSH6$ mutation carriers may be missed, as a proportion of tumours of $MSH6$ mutation carriers do not exhibit MSI. $MSH6$ mutation carriers will not be missed when patients are referred to a department of clinical genetics and the strategy is strictly followed as described hereafter and as depicted in Figure 1.

CRC patients who are directly referred to a department of clinical genetics

In the first step, colorectal tumours should be investigated for the $BRAF$-VAL600GLU mutation. In this way, non-HNPCC tumours can be identified, as the manifestation of the $BRAF$-VAL600GLU mutation in the tumour makes the presence of a germline mutation in the patient highly unlikely. When patients with the $BRAF$-VAL600GLU mutation in their tumour are not from an Amsterdam Criteria II positive family, no further action is necessary.
On the other hand, if the family of the patient does fulfil the Amsterdam Criteria II, a second tumour from the same patient or from a family member should be investigated, if possible. It is conceivable that the index patient had a sporadic tumour (a phenocopy) whilst the family members had tumours caused by an MMR gene mutation.

**Microsatellite instability and immunohistochemical analysis**

*BRAF*-VAL600GLU negative tumours should hereafter be investigated for microsatellite instability (using the 5 consensus markers) and immunohistochemical stainings should be performed for the proteins MLH1, MSH2, MSH6 and PMS2. For this step in the diagnostic process, different outcomes are possible, which are discussed hereafter.

1. **Patients with an MSI-low tumour with retained protein expression of the four MMR proteins.** MSI-low tumours with retained protein expression of MLH1, MSH2, MSH6 and PMS2 are considered non-HNPCC and therefore, no further mutation analysis is performed.

2. **Patients with a tumour with loss of MLH1 expression.** Loss of MLH1 expression in a tumour is not only suggestive for a germline mutation in MLH1, but also for tumours with somatic or germline hypermethylation of the MLH1 promoter. For patients with such tumours, we recommend to perform mutation analysis in MLH1 first. We have considered performing methylation analysis before proceeding to mutation analysis in MLH1 in order to exclude the sporadic tumours, which were not filtered out by the *BRAF* analysis, but we chose not to, in view of the possibility of somatic hypermethylation as the second hit in MLH1 mutation carriers.

   If no pathogenic MLH1 germline mutation is detected, and when concomitant loss of PMS2 expression in the tumour is observed, PMS2 analysis is indicated. If still no pathogenic mutation is demonstrated, methylation analysis of the MLH1 promoter is indicated in the tumour and in lymphocytes, in order to identify sporadic tumours or, more importantly, patients with MLH1 promoter methylation in lymphocytes, which have the same cancer risks as HNPCC patients with a pathogenic germline mutation in MLH1.

3. **Patients with loss of PMS2 expression in the tumour.** In case of absent PMS2 staining in the tumour, mutation analysis in PMS2 is performed. If no causative mutation is detected, mutation analysis in MLH1 is indicated, as it was shown that patients with germline MLH1 mutations can have loss of PMS2 expression and retained MLH1 expression.

4. **Patients with loss of MSH2 and/or MSH6 expression in the tumour.** When a tumour shows loss of MSH2 or MSH6 expression, mutation analysis is performed in the indicated gene. As MSH2 and MSH6 mutation carriers often have tumours which show concomitant
loss of MSH2 and MSH6 expression, both genes will have to be screened in some patients.\textsuperscript{92,93}

It may occur that despite the observation of loss of expression of one of the four MMR proteins, no pathogenic germline mutation can be detected in the MMR genes. Of course, it cannot be excluded that a mutation was missed with the mutation analysis or that the stability of the protein was affected by absence of another MMR protein.

5. Patients with an MSI-high tumour and retained expression of the four MMR proteins.

When the MSI in the tumour suggests a MMR gene defect, but IHC analysis shows no loss of protein expression, a variant of unknown significance (unclassified variant or UV) – e.g. a missense mutation - may be present in the germline. It is conceivable that such a variant affects the function of the protein, e.g. by a conformational change or diminished binding capacity, and thus causes mismatch repair deficiency. Missense mutations do not influence translation and usually do not interfere with protein staining if the antibodies do not target the mutated part of the protein. With the possibility of a UV in mind, mutation analysis in all four MMR genes is advised in case a MSI-high tumour with retained protein expression is observed. When subsequently still no pathogenic mutation is detected, the microsatellite instability may have been caused by a germline mutation in another MMR gene.

Functional assays

In several publications, criteria have been formulated to evaluate the pathogenic nature of a UV\textsuperscript{94,95}. Although these criteria can help, the final proof of pathogenicity likely should come from functionally analysing the identified UV. For this purpose, different types of functional assays should be used, such as protein-binding studies, in vitro cell systems, yeast-based functional assays and human expression systems, as is discussed in Chapter 2.\textsuperscript{96} These assays can also be used to investigate the effect of combinations of mutations in different genes which are detected in HNPCC suspected patients.

The strategy proposed here has some limitations. First, going through the whole scheme until a pathogenic mutation is detected or a UV is proven to be pathogenic, is time-consuming when the scheme is followed in a step-by-step way. One might prefer to screen the MMR genes simultaneously to save time. However, screening four genes in every HNPCC suspected patient is also time-consuming (and costly), and therefore hardly feasible. Whether the screening strategy proposed above is cost-effective has not been calculated. Another limitation is that functional assays are not yet routinely available for the evaluation of
pathogenicity of UVs. Therefore, one might choose not to screen all four MMR genes in the patients with MSI-high tumours with retained MLH1, MSH2, MSH6 and PMS2 protein expression.

Overall, we feel that the strategy described here, is useful for diagnostic laboratories to identify MMR gene mutation carriers. In addition, we believe it can be used for all patients who present themselves with CRC, independently of age of onset or family history.

FUTURE PERSPECTIVES

This thesis addressed several issues concerning HNPCC. Existing clinical criteria to select patients for mutation analysis in the MMR genes were investigated for sensitivity and specificity and new clinical and molecular criteria were formulated to identify MMR gene mutation carriers. In addition, it was shown that germline mutations in \textit{PMS2} can, and combinations of monoallelic mutations in \textit{MUTYH} with missense mutations in the MMR genes may, cause HNPCC. Also, germline hypermethylation of the \textit{MLH1} promoter was proven to be causative for HNPCC.

All this lead to a protocol which can be used to identify CRC patients with germline MMR gene mutations. When patients decide, after counselling or genetic prescreening that they want to know whether or not they are carriers of a MMR gene mutation, the strategy, as presented here, can be followed by the collaborating genetics and pathology laboratories, in order to identify all the MMR gene mutation carriers. It would be interesting to investigate whether or not the strategy presented here also holds for patients with extracolonic HNPCC associated tumours. Introduction of new techniques may change the cost aspects of the screening procedures and thereby may influence the proposed screening strategies and the inclusion criteria of patients for genetic analysis of the MMR genes.

Naturally, this thesis did not investigate all issues concerning HNPCC which are of interest. One of the greatest challenges in HNPCC research lies in the development of simple functional assays that test the pathogenicity of UVs that have been encountered in HNPCC suspected patients; tests that can be setup and used by every diagnostic lab. When such tests become available for routine diagnostic purposes, it likely will have enormous impact on the clinical management of HNPCC families, as a proportion of UVs will turn out to be pathogenic. More patients (and their family members) who are at risk for developing HNPCC-associated cancers will then be identified and enrolled in surveillance programmes to prevent cancer and to detect it in the earliest possible stages. The other way around, other
patients can be discharged from surveillance if their UVs turn out to be harmless variants. Another subject of interest to investigate by means of these functional assays, is whether or not combinations of MMR gene mutations which were observed in HNPCC suspected patients, affect mismatch repair capacity more severely than the mutations separately. Diagnostic functional assays will not only change the clinical management of HNPCC suspected patients, but also will likely broaden our knowledge on genotype-phenotype relations and might give us tools to examine identified compound mutations.

From a clinical point of view, it will be interesting to know whether or not missense mutations lead to a different, or milder, HNPCC phenotype when compared to the clear-cut pathogenic MMR gene mutations. Clearly, if it will be shown in the future that UVs are playing a major role in HNPCC, existing protocols for identification of HNPCC patients will need to be adjusted. This does not apply to the strategy proposed in this thesis, as this strategy takes pathogenicity of missense mutations into account.

The identification of “new” genes which play, either alone or in combination with other genes, a role in HNPCC is another subject of interest. The major question here is whether or not other HNPCC genes exist. Most investigators have doubts about their existence and at least doubt whether other genes than MLH1, MSH2, MSH6 and PMS2 play a major role in HNPCC. We do believe, however, that other genes are involved. This is mainly based on the finding of patients (with a positive family history) with tumours that have an MSI-high phenotype and show normal protein staining for MLH1, MSH2, MSH6, and PMS2, and in whom no mutation or UV has been identified. Clearly, in these cases, the MSI-high status suggests the involvement of an MMR defect, which is not likely to be detected in the four known MMR genes. Recently, we started searching for candidate genes by whole genome micro-array CGH to investigate tumours for loss of heterozygosity (LOH). LOH in tumours is believed to be an indicator for regions harbouring cancer causing genes.97-102 Therefore, finding regions of LOH in the selected tumours will help in the identification of regions and candidate genes that are involved in the development of MMR-related tumours. An alternative method to identify HNPCC associated genes, may be to perform linkage analysis in large MMR gene mutation-negative families which show a dominantly inherited pattern of HNPCC-associated cancers. However, such families are rare.

Finally, epigenetic phenomenons are an intriguing subject. For instance, the heritability and the mode and time of acquisition of germline hypermethylation of the MLH1 promoter could be studied more extensively in the nine families which have been reported so far.103-106
In conclusion, future challenges in the genetic analysis of HNPCC lie in the development of diagnostically applicable functional assays, in identification of candidate genes and in finding out whether or not combinations of mutations in different genes may be causative for disease. In addition, the screening strategies to identify MMR gene mutation carriers should be investigated more extensively for patients with extra-colonic HNPCC associated tumours. Over the next few years, our group will try to elucidate these matters into more detail, in order to further optimise current clinical management strategies for HNPCC (suspected) patients.

REFERENCES


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