Molecular aspects of HNPCC and identification of mutation carriers
Niessen, Renée Cecil

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Chapter 2

Hereditary nonpolyposis colorectal cancer: identification of mutation carriers

and assessing pathogenicity of mutations

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Chapter 2

ABSTRACT

Hereditary nonpolyposis colorectal cancer (HNPCC), also referred to as Lynch syndrome, is an autosomal dominantly inherited disorder that is characterised by susceptibility to colorectal cancer and extracolonic malignancies, in particular endometrial cancer. HNPCC is caused by pathogenic mutations in the mismatch repair (MMR) genes, which play an important role in maintaining genomic stability during DNA replication. Identification of MMR gene mutation carriers is important as this enables them to enrol in surveillance programs, thus reducing their risk of cancer and increasing survival. Clinical criteria as well as non-clinical criteria have been formulated to select patients for mutation analysis. In this paper we review the approaches to select patients for mutation analysis. Mutation analysis in the MMR genes may yield mutations of which the pathogenic nature is unclear. Criteria to determine the pathogenicity of such variants are discussed, as well as differences in design of functional assays to assess pathogenicity.

1. General introduction

Hereditary nonpolyposis colorectal cancer (HNPCC), also referred to as Lynch syndrome, is an autosomal dominantly inherited disorder of cancer susceptibility with high penetrance (80-85%)\(^1\)\(^-\)\(^4\). HNPCC is one of the most common inherited cancer syndromes identified in humans so far. It is caused by a defect in the DNA mismatch repair (MMR) system, which maintains genomic stability during DNA replication. Germline mutations have been identified in the following human MMR genes: \(MLH1\), \(MSH2\), \(MSH6\), \(PMS2\), \(PMS1\), \(MLH3\) and \(EXO1\)\(^5\)\(^-\)\(^16\). Mutations in three of these genes, \(MSH2\), \(MLH1\) and \(MSH6\), account for the majority of mutations found in HNPCC families. The diagnosis HNPCC can be made on a molecular basis, by identification of a pathogenic mutation, or on clinical grounds, by meeting the Amsterdam criteria I or II (Table 1)\(^17\),\(^18\). Before the discovery of the role of the MMR system in HNPCC, the diagnosis of HNPCC could only be based on family history. As a result, the true incidence of this disease and the underlying molecular defects were difficult to establish. The estimations of the proportion of HNPCC of the total of colorectal cancers, based on clinical cases, varied from less than 1% in Finland to 13% in the United Kingdom\(^19\),\(^20\). Recently, five population-based studies concluded that genetically proven HNPCC, i.e. cases with a detected germline MMR mutation, accounts for 0.3% to 2.7% of colorectal cancer cases at the population level in several areas\(^21\)\(^-\)\(^25\). The variation in frequencies may be explained by geographic differences in the occurrence of HNPCC and more so by differences
Hereditary nonpolyposis colorectal cancer

Table 1. Amsterdam criteria I and II 17,18

<table>
<thead>
<tr>
<th>Amsterdam criteria I</th>
</tr>
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<tbody>
<tr>
<td>At least three relatives should have histologically verified colorectal cancer:</td>
</tr>
<tr>
<td>One should be a first-degree relative of the other two</td>
</tr>
<tr>
<td>At least two successive generations should be affected</td>
</tr>
<tr>
<td>At least one of the relatives should be diagnosed before the age of 50 years</td>
</tr>
<tr>
<td>Familial adenomatous polyposis should be excluded</td>
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</table>

<table>
<thead>
<tr>
<th>Amsterdam criteria II</th>
</tr>
</thead>
<tbody>
<tr>
<td>At least three relatives should have an histologically verified HNPCC-associated cancer (colorectal cancer, cancer of the endometrium, small bowel, ureter or renal pelvis)</td>
</tr>
<tr>
<td>One should be a 1st-degree relative of the other two</td>
</tr>
<tr>
<td>At least two successive generations should be affected</td>
</tr>
<tr>
<td>At least one of the relatives should be diagnosed before the age of 50 years</td>
</tr>
<tr>
<td>Familial adenomatous polyposis should be excluded in colorectal cancer case(s)</td>
</tr>
</tbody>
</table>

in study design. The true prevalence of mutations in the MMR genes may be higher because screening of the MSH6 gene was not included in any of these studies. Another factor is that in a proportion of undeniable HNPCC families, MMR gene mutations, e.g. in regulatory sequences, will not be detected with most current mutation detection designs.

As stated earlier, the MMR genes contribute to maintaining genomic stability during DNA replication. Carriers of an MMR gene mutation have one mutated allele and one wild-type (normal) allele of the involved MMR gene in normal tissues. In most MMR mutation carriers, physical or functional loss of the wild-type allele will occur in the course of time at HNPCC-associated sites, usually through loss of heterozygosity (LOH).26,27 When both alleles are mutated (one germline and one somatic mutation), MMR will become deficient, leading to genetic instability. This genetic instability is usually referred to as microsatellite instability (MSI) as the expansion or contraction of simple repetitive sequences (microsatellites) is one of the characteristic features of HNPCC-associated tumours. More than 90% of these tumours show MSI.28-31 Deficiency of the MMR system is responsible for a rapid accumulation of somatic mutations in non-coding repetitive sequences, but also in repetitive sequences in oncogenes, tumour suppressor genes, signal transducing genes and genes responsible for apoptosis, which play crucial roles in tumour initiation and progression. It is this accumulation of mutations in coding sequences that causes tumour development.
Table 2. Cumulative cancer risks (%) up to 70 years of age in MLH1 and MSH2 mutation carriers \(^{32,33}\)

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Aarnio et al. (1999)</th>
<th>Vasen et al. (2001)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MLH1 and MSH2(^{2})</td>
<td>MLH1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MSH2(^{3})</td>
</tr>
<tr>
<td>Colorectal</td>
<td>82</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Endometrial(^{1})</td>
<td>60</td>
<td>24.7</td>
</tr>
<tr>
<td></td>
<td>37.5</td>
<td></td>
</tr>
<tr>
<td>Gastric</td>
<td>13</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>Ovarian(^{1})</td>
<td>12</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>10.4</td>
<td></td>
</tr>
<tr>
<td>Small bowel</td>
<td>0.0</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>3.7</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Biliary tract, gallbladder</td>
<td>2.0</td>
<td>-</td>
</tr>
<tr>
<td>Urinary tract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(including renal pelvis)</td>
<td>7.3</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\)Women only. \(^{2}\)Pooled results for 360 mutation carriers from 47 families with an MLH1 mutation and 3 families with an MSH2 mutation. \(^{3}\)Results from 362 putative MLH1 mutation carriers and 301 putative MSH2 mutation carriers.

HNPCC is characterised by the development of colorectal cancer and extracolonic malignancies, particularly cancer of the endometrium, renal pelvis/ureter, stomach, small bowel, ovary, biliary tract, brain and also sebaceous tumours of the skin (Muir-Torre variant). The lifetime risks for these cancers are listed in Table 2.

In two-thirds of cases the colorectal cancers are located in the proximal part of the colon and 35% of patients have multiple (synchronous or metachronous) colorectal cancers. The average age at cancer diagnosis is 45 years. As compared with sporadic colorectal cancer, tumours in HNPCC are usually poorly differentiated, with an excess of mucoid and signet-cell features, the presence of tumour infiltrating lymphocytes and a Crohn-like reaction (lymphoid nodules, including germinal centers, located at the periphery of infiltrating colorectal carcinomas).\(^ {34-37}\)

Although multiple adenomas may be observed in HNPCC, florid polyposis is not a feature. The adenomas that occur in HNPCC tend to develop at an early age, to have villous components, and to be more dysplastic than adenomas detected in the general population, in particular those located in the proximal colon.\(^ {38,39}\) The progression of an adenoma to malignancy can be relatively rapid, sometimes within as short a time as two years.\(^ {40}\) The features of HNPCC are summarised in Table 3. The higher the number of these features observed in a given family, the higher the suspicion of HNPCC.\(^ {18}\)
Table 3. ICG Definition of HNPCC 18

1. Familial clustering of colorectal and/or endometrial cancer
2. The occurrence of HNPCC-associated cancers: cancer of the stomach, ovary, ureter or renal pelvis, brain, small bowel, hepatobiliary tract and skin (sebaceous tumours)
3. Cancer diagnosis at an early age
4. Development of multiple cancers
5. Features of colorectal cancer: 1) predilection for proximal colon; 2) improved survival; 3) multiple colorectal cancer; 4) increased proportion of mucinous tumours, poorly differentiated tumours, and tumours with marked host-lymphocytic infiltration and lymphoid aggregation at the tumour margin
6. Features of colorectal adenoma: 1) one to few colorectal adenomas; 2) villous growth pattern; a high degree of dysplasia; 4) probably rapid progression from adenoma to carcinoma
7. High frequency of MSI
8. Immunohistochemistry: loss of MLH1, MSH2, or MSH6 protein expression
9. Germline mutations in MMR genes (MSH2, MLH1, MSH6, PMS1, PMS2)

ICG = International Collaborative Group; HNPCC = hereditary nonpolyposis colorectal cancer; MSI = microsatellite instability; MMR = mismatch repair.

2. How to identify HNPCC

The identification of MMR gene mutation carriers is important as they are eligible for surveillance programmes to detect neoplasms at the earliest possible stages. It has been shown that periodic colonoscopy reduces the risk of colorectal cancer and decreases overall mortality.41,42 However, mutation analysis is presently costly and labour-intensive and because of this, mutation analysis in the MMR genes cannot be offered to all patients with colorectal cancer or endometrial cancer. Therefore, there is a need for sensitive and specific criteria to select patients for mutation analysis. Clinical as well as non-clinical criteria can be used for selection, as is reviewed below.

2.1 The Amsterdam criteria I and II

In 1990, the International Collaborative Group on HNPCC (ICG-HNPCC) formulated the Amsterdam criteria to define HNPCC and to make research on this subject more comparable.17 Soon the criteria were also used as a selection method for mutation analysis in the MMR genes and for clinical diagnosis of HNPCC, especially when families had to be advised on endoscopic screening. However, the Amsterdam criteria were soon felt to be too stringent as a significant proportion of families carrying MMR germline mutations does not fulfil these criteria. There are several reasons to explain this. First, it was noted that extracolonic tumours occur frequently in HNPCC families and the original Amsterdam
criteria do not include these tumours. Second, a decrease in family size, as is occurring in many countries world-wide, results in fewer families fulfilling the Amsterdam criteria. Furthermore, the histological confirmation is sometimes difficult to obtain, especially when family members died a long time ago. De novo mutations, although to our knowledge reported only rarely, will certainly be missed and limited penetrance of mutations may be another complicating factor.\textsuperscript{43,44} Other factors may affect the fulfilment of the Amsterdam criteria by influencing the expression of MMR gene mutations. First, germline mutations in different MMR genes may cause different propensities for disease expression. For example, mutations in \textit{MSH6} are associated with atypical, that is non-Amsterdam, pedigrees, with a higher age of onset of colorectal and endometrial cancer and with higher frequencies of endometrial cancer.\textsuperscript{10,11,45,46} Second, it is possible that certain types of mutations such as missense mutations, of which the pathogenicity is uncertain, have lower penetrance and could thus explain non-Amsterdam pedigrees. Third, polymorphisms of genes, e.g. those involved in carcinogen metabolism, i.e. not associated with the MMR system, may influence the expression of MMR gene mutations.\textsuperscript{47,49} And finally, environmental factors can probably affect the expression of MMR gene mutations.\textsuperscript{50} Sometimes the use of the Amsterdam criteria can lead to over-diagnosis of HNPCC when large pedigrees fulfil the Amsterdam criteria as the result of accidental clustering of three 1st-degree family members with cancer, while other family members have neither colorectal nor other HNPCC-associated cancers.\textsuperscript{51}

To identify as many mutation carriers as possible, several variants of less strict selection criteria have been formulated in the past. Berends et al. reviewed the attempts of various groups to define new criteria to select patients for mutation analysis.\textsuperscript{52} The primary goal was to find as many patients as possible with a relevant germline mutation, without unacceptably increasing the percentage of negative results. This is important because of the high costs of mutation analysis and because of the psychological and social burdens that the possibility of genetic analysis and its result may impose on a patient and on his/her family. In 1999 the Amsterdam criteria were revised. These now include extracolonic malignancies with the highest relative risks likely to occur in mutation carriers, i.e. cancers of the endometrium, small bowel, ureter or renal pelvis.\textsuperscript{18} Several studies have shown that the Amsterdam criteria II are still too stringent and that carriers of germline mutations in MMR genes can be missed and excluded from cancer surveillance and proper management when using these criteria for selection for mutation analysis.\textsuperscript{22,53-57}
When applying clinical criteria to diagnose HNPCC and/or to select patients for molecular studies, it should be appreciated that self-reported family history of cancer is not necessarily accurate and should be verified by checking medical records. Katballe et al. evaluated the accuracy of a suspected HNPCC family history reported by patients with colorectal cancer. They found that when patients with colorectal cancer report a family history that clinically meets the Amsterdam criteria I or II, the information is correct in most cases, but that the risk of misclassification is relatively high.

2.2 Microsatellite instability analysis

Testing for microsatellite instability (MSI) has been put forward as a selection criterion for subsequent mutation analysis. This is based on the finding that the large majority of colorectal cancers from patients with a MSH2 or MLH1 mutation exhibit this feature, as mentioned previously. Conversely, only a small minority of colorectal cancers and other tumours displaying MSI are due to a germline mutation in one of the MMR genes. The reason for this is that in most of the microsatellite instable tumours, MMR deficiency is caused by somatic mutations in both alleles of a MMR gene. In 1996 the Bethesda guidelines were formulated for the identification of tumours that should be tested for MSI in order to select patients for subsequent MMR mutation analysis (Table 4). To ensure uniformity, a reference panel of the five most sensitive microsatellite markers was developed and validated to perform MSI analysis in colorectal cancer.

<table>
<thead>
<tr>
<th>Table 4. Bethesda guidelines for testing of colorectal tumours for microsatellite instability</th>
</tr>
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<tbody>
<tr>
<td>1. Individuals with cancer in families that meet the Amsterdam Criteria</td>
</tr>
<tr>
<td>2. Individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers (endometrial, ovarian, gastric, hepatobiliary, small-bowel cancer and transitional cell carcinoma of the renal pelvis or ureter)</td>
</tr>
<tr>
<td>3. Individuals with colorectal and a 1st-degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or colorectal adenoma; one of the cancers diagnosed at age &lt;50 years, and the adenoma diagnosed &lt; 40 years</td>
</tr>
<tr>
<td>4. Individuals with colorectal cancer or endometrial cancer diagnosed at age &lt;45 years</td>
</tr>
<tr>
<td>5. Individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/cribiform) on histopathology diagnosed at age &lt; 45 years</td>
</tr>
<tr>
<td>6. Individuals with signet-ring cell type colorectal cancer diagnosed at age &lt;45 years</td>
</tr>
<tr>
<td>7. Individuals with adenomas diagnosed at age &lt;40 years</td>
</tr>
</tbody>
</table>

HNPCC = hereditary nonpolyposis colorectal cancer
Syngal et al. investigated the sensitivity and specificity of the Bethesda guidelines, as compared with the Amsterdam criteria I and II, as a selection method for the detection of MSH2 and MLH1 mutations in 70 families with suspected hereditary colorectal cancer. The sensitivity and specificity were 94% and 25% for the Bethesda guidelines, 61% and 67% for the Amsterdam criteria I and 78% and 61% for the Amsterdam criteria II. It was also found that the sensitivity of the combination of the first three criteria of the Bethesda guidelines was 94% and the specificity 49%. The prevalence of MSH2 and MLH1 mutations if these three criteria only were used was 39% and the investigators therefore suggested to proceed directly to mutation analysis when one of these criteria is fulfilled. They proposed to use MSI screening only for those patients that are less likely carrying a mutation, such as the early onset sporadic cancer patients. One of the limitations of this study is that mutation analysis was only performed for MSH2 and MLH1. It is now known that mutations in MSH6 are often associated with tumours that do not exhibit a high degree of MSI (MSI-H) and when mutation analysis is performed only in patients with MSI-H tumours, a substantial proportion of the MSH6 mutation carriers will be missed.

2.3 Immunohistochemical staining for MMR proteins
Several investigators have suggested the use of immunohistochemistry (IHC) as a prescreening method for mutation analysis of the MMR genes. In 1996 Leach et al. and Thibodeau et al. first reported the use of monoclonal antibodies directed against MSH2 and MLH1 in the immunohistochemical analysis of colorectal cancers. Studies in colorectal cancers and endometrial cancers have shown the diagnostic value of MSH2, MLH1 and MSH6 protein staining. Significant correlations were found between the absence of staining of these proteins and the presence of a truncating germline mutation in the corresponding genes. However, if a missense mutation in an MMR gene still results in an immunohistochemically detectable aberrant protein, then carriers of such a mutation will be missed.

2.4 The Groningen study on selection criteria
In 1997 we started a collaborative study with the goal to define better selection criteria for mutation analysis. The study design has been described previously. Three large groups of patients were studied, patients with colorectal cancer diagnosed before the age of 50, patients with endometrial cancer diagnosed before the age of 50 and patients with multiple HNPCC-related cancers, irrespective of age. In 344 patients mutation analysis was performed in
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*MSH2, MLH1* and *MSH6*. Immunohistochemical staining for the proteins of these genes and MSI analysis were performed in tumours whenever material was available. A total of 24 patients were found to have pathogenic mutations. In all three examined groups a positive 1st-degree family history increased the risk of having a germline mutation markedly. In about one third (8/25) of all families fulfilling the Amsterdam criteria II, pathogenic mutations were found. The remaining 16 mutations were found in 319 non-Amsterdam families. Thus, in our study, the sensitivity and specificity of the Amsterdam criteria II were 33% (8/24) and 95% (303/320) respectively. Besides the individual and family history, MSI analysis and, to a seemingly greater extent, immunohistochemical analysis appear to be sensitive indicators for the presence of an MMR mutation. MSI analysis failed to detect a proportion of the pathogenic mutation carriers (all *MSH6* mutation carriers), while immunohistochemical analysis missed only one. It was concluded that MSI analysis is an unreliable tool to detect patients with *MSH6* mutations and that immunohistochemical analysis might be the first choice for detection of possible carriers of pathogenic mutations (Berends et al. unpublished).

**3. Assessing pathogenicity of mutations in MMR-genes**

The database established by the International Collaborative Group on HNPCC (http://www.nfdht.nl) currently contains information on more than 300 different mutations occurring in over 500 HNPCC kindreds, mainly affecting the *MLH1* (49%), *MSH2* (38%), and *MSH6* (9%) genes. Most mutations result in premature termination of translation and thus in loss of function. However, a significant proportion gives rise to single amino acid substitutions (~10% of *MSH2*, ~30% of *MLH1*, and ~37% of *MSH6* mutations) or in-frame deletions (~10% of both *MSH2* and *MLH1* mutations). The pathogenicity of these variants, and the same holds true for intronic variants, is often unclear. Criteria defining the pathogenic nature of mutations in general and in particular of mutations associated with HNPCC have been reported in several publications (Table 5). Some of these criteria may be difficult to apply. Segregation analysis, for instance, is often impossible, as large numbers of affected family members are not available. The same applies to the availability of tumour material for LOH, MSI and immunohistochemical analysis. Previous inclusion of the mutation in an HNPCC mutation database might be misleading, as most variants have not been tested properly. The contribution of immunohistochemistry to solve the riddle of pathogenicity may be limited, as the missense (instead of truncating) mutation-coded protein may cause positive nuclear staining. In many cases, the only criteria for pathogenicity that can be used are de novo appearance, occurrence of the amino acid change in an evolutionary conserved domain,
absence of the mutation in a control population and changes in amino acid polarity charge or size in the encoded peptide. Meeting the above-mentioned criteria can be regarded as circumstantial evidence. More direct evidence for the effect that possibly causative mutations have on the functioning of the MMR proteins is sought through functional assays.

Table 5. Criteria to define the pathogenic nature of MMR gene variants

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<table>
<thead>
<tr>
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<tbody>
<tr>
<td>1.</td>
<td>De novo appearance of a mutation</td>
</tr>
<tr>
<td>2.</td>
<td>Segregation of the mutation with pedigrees</td>
</tr>
<tr>
<td>3.</td>
<td>Absence of the mutation in control individuals</td>
</tr>
<tr>
<td>4.</td>
<td>A change of amino acid polarity charge or size in the encoded peptide</td>
</tr>
<tr>
<td>5.</td>
<td>Occurrence of the amino acid change in a domain which is evolutionarily conserved between species and/or shared between proteins belonging to the same protein family</td>
</tr>
<tr>
<td>6.</td>
<td>Loss of the non-mutated allele in tumour material of the patient (loss of heterozygosity, LOH)</td>
</tr>
<tr>
<td>7.</td>
<td>Absence of immunohistochemical staining for the corresponding protein in tumour material</td>
</tr>
<tr>
<td>8.</td>
<td>Presence of MSI in tumour material of the patients</td>
</tr>
<tr>
<td>9.</td>
<td>Effect of the mutation on MMR capacity in functional assays</td>
</tr>
<tr>
<td>10.</td>
<td>Previous inclusion of the mutation in disease-specific mutation databases</td>
</tr>
</tbody>
</table>

MMR = mismatch repair; MSI = microsatellite instability

3.1 Protein-protein binding studies

MMR functioning is based on the proper functioning of heterodimers of human homologues of the *Escherichia coli* MutL and MutS. For instance, MLH1 forms heterodimers with PMS1/2 and MSH2 with MSH3/6. (Missense) mutations could change the binding capacity of the proteins and thus have an effect on the functioning of the heterodimers, resulting in a loss of MMR activity. The binding of the different proteins to each other can be measured and this test is often used in combination with other tests to determine pathogenicity of MMR gene mutations. Mismatch repair capacity but do not alter the ability to form dimers.

3.2 In vitro analyses

In vitro analyses are being used to determine whether MMR activity of proteins isolated from an MMR-deficient cell line (cell extracts) can be restored by adding (mutated) MMR proteins. It has been proven that cell extracts from MMR-proficient cell lines can be used to repair mutations in DNA in vitro. When a mutated protein is added to protein extracts of MMR-deficient cell lines one can see whether the protein extract is able to repair the DNA
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mutations. If not, it is assumed to be pathogenic; in other words, the mutation results in a non-functional or less functional MMR protein.

3.3 Yeast-based functional assays

Another way to test the functional consequences of MMR mutations is the introduction of mutated MMR proteins (by transfection with expression vectors that produce these mutated proteins) in MMR-proficient or -deficient yeast strains. Many different assays have been described and all are based on the assumption that the MMR system is evolutionary conserved between humans and yeast. Different types of yeast-based functional assays can be distinguished. In one method, MMR-deficient yeast cells are complemented with mutated yeast MMR genes. When MMR is not restored, the mutation is classified as pathogenic.64,91-93 Another method is based on the fact that MMR-proficient yeast cells, transfected with wild-type human MMR genes, show loss of MMR through a not yet fully understood dominant negative effect of the normal human proteins. When mutated human MMR genes are expressed in these MMR-proficient cells and this does not result in loss of MMR function, it suggests that these mutations are pathogenic.94,95 Recently, a yeast-based two-hybrid system was described with high specificity to determine loss of function of mutant MMR proteins.84,85 In all these yeast-based studies, reporter genes with unstable repetitive sequences such as \textit{Lac-Z} and \textit{URA3} are used to measure MMR activity.

Assessing pathogenicity with a yeast-based assay seems an attractive method for functional analysis. However, as there is only a moderate homology between human and yeast MMR proteins, a mutant human allele in a yeast environment may not have the same effect as a mutant human allele in a human environment. This may explain discrepancies between the results of different functional assays. For example, Kolodner et al. performed a \textit{S. cerevisae}-based assay to assess the pathogenicity of the Ser144Ile missense variant.64 This missense variant was introduced in the yeast \textit{MSH6} gene. The mutagenised gene was thereafter introduced in a yeast strain deficient for \textit{MSH6} and \textit{MSH3}, which resulted in persistence of MMR deficiency. In contrast, in an in-vitro MMR assay that studied the ability of the mutant proteins to form MSH2-MSH6 heterodimers, the same \textit{MSH6} variant appeared as functional as the wild type.81
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3.4 A human expression system

Trojan et al. wanted to overcome the limitations of the yeast-based functional assays and designed a human expression system to express MLH1 variants and characterise the biochemical properties of their protein products. Their functional assay was based on transient transfection of MLH1 complementary DNA (cDNA) into a human embryonic kidney fibroblast cell line, which lacked intrinsic MLH1, which in turn caused degradation of PMS2. The investigators showed that co-transfection with wild-type MLH1 and PMS2 constructs resulted in the expression of both MLH1 and PMS2, which fully restored the MMR capacity in their in vitro assay. In contrast, the products of several HNPCC-related MLH1 missense mutations were unable to restore MMR function and were therefore classified as pathogenic.

Another way to measure the effect of mutated MMR proteins is by introducing a mutated human MMR gene in a MMR-deficient human cell line and measure the effect of the mutated protein by looking at the expression of that MMR gene both on mRNA and protein level and by looking at proliferation and apoptosis of these transfected cells.

4. Conclusion

Identification of MMR gene mutation carriers is important as this gives them the opportunity to enrol in surveillance programs and thereby reducing their risk of cancer and decreasing mortality. Currently, it is not possible to offer mutation analysis in the MMR genes to all patients with colorectal cancer. For this reason, there is a need for sensitive and specific criteria to select patients for mutation analysis. The use of MSI analysis and, possibly more so, the use of immunohistochemical staining for MMR proteins in tumour tissue are promising tools that can aid in the selection of HNPCC-suspected patients for mutation analysis. Knowledge of pathogenicity of MMR gene variants, such as missense mutations, can contribute towards optimising selection criteria. As stated earlier, different approaches are used to determine pathogenicity of MMR gene variants. Differences in design of functional assays, such as the organisms used and the way MMR function is monitored, can lead to different test outcomes. Human expression systems might be able to overcome some, or even most of the limitations of yeast-based functional assays. It is clear, however, that more research is needed before functional assays for MMR gene mutations can be introduced in clinical practice.
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