Chapter 6

Thesis summary

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THESIS SUMMARY

Lynch syndrome (LS) is a hereditary cancer predisposition to early onset colorectal and endometrium cancers (1, 2). The underlying cause of LS is an inactivating germ-line mutation in one of the DNA mismatch repair (MMR) genes: *MLH1, MSH2, MSH6* or *PMS2* (3–6). Patients are born heterozygous for the germ-line mutation and develop neoplasms upon somatic loss of the one wild-type copy of the gene (7). In the absence of a functional DNA MMR system, cells cannot detect and correct mismatches that may arise in the genome due to erroneous DNA replication or exposure to DNA damaging agents. Consequently, a mutator phenotype will develop that increases the chances of mutations arising in oncogenes and tumor suppressor genes, and hence the development of malignancies (8).

The diagnosis of LS is essential to offer patients the optimal treatment. To date the diagnosis of LS relies on the clinical phenotype, tumor pathology data and genetic screening results. Typical LS cases have a broad family history of LS-associated cancers that co-segregate with a MMR gene mutation and microsatellite instable (MSI) tumors that lack expression of one of the DNA MMR proteins. There are however also many suspected-LS patients for whom co-segregation and tumor pathology data are unreliable. In such cases genetic screening may reveal whether the patient carries a MMR attenuating mutation. In fact, detection of a pathogenic germ-line mutation affecting one the DNA MMR genes is mandatory to establish a definitive LS diagnosis (9). Many LS-associated sequence variants are nonsense and frameshift mutations that clearly truncate the protein and unambiguously attenuate MMR. Missense mutations that alter a single amino acid are also frequently observed in suspected-LS patients (10). Without clear functional data, the diagnosis of these patients is difficult because it is not evident whether such missense mutations abrogate MMR and contribute to cancer risk. To facilitate the diagnosis of LS patients, techniques must be in place that interrogate the functional implications of MMR gene variants of uncertain significance (VUS) (11).

In the present thesis we divulge a tool for the characterization of DNA MMR gene variants.

In *Chapter 1* we provide a comprehensive overview of the DNA MMR system and how loss of DNA MMR activity predisposes to cancer. Furthermore, we describe the clinical management of LS and highlight the importance of a correct diagnosis.

In *Chapter 2* we reveal the oligonucleotide-directed mutation screen we created for the identification of pathogenic *MSH2* DNA MMR gene variants. The screen was developed in mouse embryonic stem cells (mESCs) with a single endogenous *Msh2* allele. Introduction of the desired *Msh2* sequence variant into the single functional *Msh2* allele gave rise to expression of solely the variant protein and enabled immediate investigation of its pathogenic phenotype. To determine the MMR attenuating effect of the introduced variant, cells were exposed to the DNA damaging agent 6-thioguanine (6TG). DNA MMR-deficient cells are 6TG-resistant while MMR-proficient cells die in response to 6TG exposure. Hence, solely mESCs expressing MMR abrogating variants should survive the 6TG selection. In a proof of principle study with 12 known pathogenic

mutations and 10 non-pathogenic variants, we demonstrated the ability of the genetic screen to distinguish (weak and strong) pathogenic *MSH2* variants from polymorphisms. Subsequently, we used it to analyze the functional implications of 59 *MSH2* VUS found in suspected-LS patients. Nineteen of the 59 VUS were found to lead to 6TG resistance; functional assays confirmed all 19 variants indeed abrogate MMR.

In *Chapter 3* the oligonucleotide-directed mutation screen was adapted for the characterization of *MSH6* DNA MMR gene variants. After demonstrating the screen can effectively identify pathogenic from non-pathogenic *MSH6* variants, we used it to investigate the MMR abrogating effect of 26 clinically relevant *MSH6* VUS. Of the 26 variants, 8 were identified as pathogenic. The results from our screen were compared to the clinical and tumor pathology data that were collected for these variants from medical centers in the Netherlands and literature.

In *Chapters 4* we extended the applicability of the oligonucleotide-directed mutation screen to the characterization of *MLH1* DNA MMR gene variants. We validated this approach is capable of distinguishing MMR attenuating *MLH1* mutations from polymorphism, and used it to determine the pathogenic phenotype of 50 *MLH1* VUS. Twenty-six of the 50 VUS were found to lead to MMR-deficiency.

In *Chapter 5* the genetic screen for the identification of pathogenic *MSH2* and *MLH1* variants, described in Chapters 2 and 4, was used to investigate the functional consequences of 18 VUS that were found in the *MLH1* and *MSH2* genes of 21 suspected-LS families from the Erasmus Medical Center Rotterdam and Netherlands Cancer Institute Amsterdam. This study demonstrates the clinical benefit of the genetic screen. Furthermore it proposes the relative simplicity as well as ability to study many variants in parallel makes the genetic screen a good tool for implementation in clinical genetics laboratories that are confronted with suspected-LS patients carrying MMR gene VUS.

GENERAL DISCUSSION

The advances in next generation sequencing have made it possible as well as affordable to identify an individual's entire genetic code and the variations it harbors. Consequently the doors to personalized medicine have been opened (12, 13). To offer a personalized treatment approach, however, the functional implications of the detected sequence alterations must be known. This currently poses a significant hurdle; the phenotypic consequences of many rare genetic variants are unclear. To help clinicians diagnose and treat patients, more methods that interrogate the repercussions of genetic variants must become available (11). The presented thesis reveals one such method: the oligonucleotide-directed mutation screen for the characterization of DNA MMR gene variants and hence the diagnosis of LS patients.

Efficacy of the genetic screen for the identification of pathogenic DNA MMR gene variants. The divulged oligonucleotide-directed mutation screen is capable of distinguishing pathogenic *MLH1, MSH2* and *MSH6* DNA MMR gene mutations from polymorphisms. Proof-of-principle studies with 25 proven pathogenic and 24 non-pathogenic variants demonstrated that combining oligonucleotide-directed gene modification (oligo targeting) in mESCs with 6TG selection and sequence analysis allowed all pathogenic DNA MMR gene mutations to be detected while not one of the polymorphisms was.

Based on the described results, the genetic screen has a sensitivity of >96%. The sensitivity of the screen can be defined as the likelihood that in a deleterious variant is not detected in a cohort of proven deleterious variants; it can be calculated using to the formula:

sensitivity = 1 - false negatives / tested deleterious variants

In our genetic screen, all 25 mutations that were *a priori* selected for their pathogenicity were identified as MMR abrogating in the proof-of-principle studies. Hence:

the sensitivity > 1 - 1 / 25.

The specificity of the genetic screen is a measure of the false positive rate; it is the chance that in a set of proven neutral variants, a variant is detected as MMR-abrogating. The specificity can be calculated using the formula:

specificity = 1 - false positives / tested neutral variants

Considering not one of the 24 non-pathogenic variants in the proof-of-principle studies were found in 6TG-resistant colonies, 3 undetected variants (*Mlh1 A31C, Mlh1 R385C* and *Msh6 G565R*) were demonstrated to be MMR-proficient, and one false positive variant (*Mlh1 P585L*) was identified as MMR abrogating:

the specificity = 1 - 1 / 28, or 96.4%.

Incorporation of screening results in the InSiGHT classification scheme. The International Society for Gastrointestinal Hereditary Tumours (InSiGHT) colon cancer variant database is an international multidisciplinary effort that uses available clinical, *in vitro* and *in silico* data to calculate the likelihood of pathogenicity of DNA MMR gene variants to categorize variants into 5 classes (14, 15):

- Class 5 – Pathogenic, >0.99 probability of pathogenicity
- Class 4 – Likely pathogenic, 0.95-0.99 probability of pathogenicity
- Class 3 – Uncertain, 0.05-0.949 probability of pathogenicity
- Class 2 – Likely not pathogenic, 0.001-0.049 probability of pathogenicity
- Class 1 – Not pathogenic, <0.001 probability of pathogenicity

Based on the specificity of our screen any VUS detected to lead to 6TG-resistance should be considered Likely Pathogenic by InSiGHT. The sensitivity of our screen would allow non-detected VUS to be considered Likely not pathogenic. We however recommend a simplification of the InSiGHT classification scheme to a three-tiered system with solely classes: Pathogenic, Not pathogenic and Uncertain. The Likely pathogenic and Likely not pathogenic classes can be

eliminated as these variants are respectively generally treated as Pathogenic and Not pathogenic in the clinic and should otherwise be considered Uncertain. In the three-tiered VUS classification scheme, any 6TG-resistant variant should be treated as Pathogenic while variants not detected in 6TG-resistant colonies should be considered Not pathogenic.

Comparison of screening results and InSiGHT classifications. Overall we used the oligonucleotide-directed mutation screen to investigate the pathogenic phenotype of 198 DNA MMR gene variants. The variants were brought to our attention through contact with the clinic or by the inspection of the InSiGHT database. In line with the InSiGHT classifications, all 28 Pathogenic mutations analyzed by the screen were identified in 6TG-resistant colonies and thus indicated to abrogate MMR. Of the 19 screened Likely pathogenic variants, only *Mlh1 R385C* was not found to lead to 6TG-resistance. The screen suggested 41 of 115 Uncertain variants attenuated MMR. All of the 17 screened Likely not pathogenic variants, except *Mlh1 P585L*, were indicated to be MMR-proficient. Likewise, all 19 screened Not pathogenic variants, except *Mlh1 K622A*, were not identified in 6TG-resistant colonies.
To investigate the discrepancy between the screening results and the InSiGHT classifications of *Mlh1* variants *R385C, P585L*, and *K622A,* we generated mESCs expressing solely the variant proteins and assessed their MMR capacity. At variance with our screening result but in line with the Likely not-pathogenic InSiGHT classification, independently generated $Mlh1^{P585L/\Delta}$ mESCs were MMR-proficient. Therefore, *Mlh1 P585L* was erroneously detected in our screen. Interestingly, *Mlh1 P585L* was only detected in 1 of 22 sequenced 6TG-resistant colonies; the lowest observed detection rate in our genetic screen. This suggests that we must be careful with the classification of variants that are identified at such a low rate. To avoid misclassification, we propose a variant can only be considered pathogenic when it is detected in at least two independent 6TG-resistant colonies. In case the variant is detected in a sole 6TG-resistant colony, it should be investigated further, *e.g.*, by generating the variant via CRISPR/Cas9 technology as shown in Chapter 4, before classifying it as 0.96 probable of being pathogenic.
The InSiGHT classified Not pathogenic *Mlh1 K622A* variant was identified in 16 of 22 sequenced 6TG-resistant colonies in our screen. Conversely, InSiGHT classified Likely pathogenic *MLH1 R385C* was not detected in any 6TG-resistant colony. Assessment of the MMR capacity of independently generated $Mlh1^{K622A/\Delta}$ and $Mlh1^{R385C/\Delta}$ mESCs confirmed the screening results, suggesting that InSiGHT misclassified these two variants. However, although these variants are located in highly conserved regions, we can at present not fully exclude there is a difference in their phenotype between men and mice. Investigating these variants in a human cell system is mandatory to exclude this possibility.

Studying DNA MMR gene variants in mice. The genetic screen was developed in mESCs. mESCs provide a good study model because the DNA MMR system is highly conserved between species and mouse models can be made from these cells if variants need to be studied *in vivo*.

The human and mouse *MLH1, MSH2*, and *MSH6* DNA MMR genes respectively share 88%, 93% and 86% homology at the amino acid level. Hence most variants detected in suspected-LS patients can be studied by the genetic screen. Variants at non-conserved residues are generally unlikely to have an impact on MMR capacity. Critics have however suggested that the DNA MMR system is not as well conserved as we suggest based on the fact that *Msh2*$^{+/-}$ mice are hardly cancer prone as opposed to *MSH2*$^{+/-}$ humans. It is true that a previous study in our laboratory only found one tumor, of endometrial origin, that showed MSI and loss of the *Msh2* wild-type allele in a cohort of 65 tumors developing in *Msh2*$^{+/-}$ mice (16). But we expect the paucity in tumor development in *Msh2*$^{+/-}$ mice can be explained by the relatively low number of spontaneously arising *Msh2*$^{-/-}$ mice, due to their small size and short lifespan. Lending support to this possibility: in a novel mouse model in which the percentage of *Msh2*$^{-/-}$ intestinal stem cells can artificially be increased to 5%, spontaneous development of intestinal tumor cells was observed in 40% of the cohort (17). Furthermore, *Msh2*$^{-/-}$ mice are highly cancer prone and largely develop the same tumor spectrum as seen in individuals with bi-allelic MMR gene defects, *i.e.*, hematopoietic and intestinal tumors (18, 19). The proof-of-principle studies also confirm pathogenic and not pathogenic variants behave the same in men and mice.

Further improvement of the genetic screen. To fully dissuade the man-mouse conservation argument we will have to develop the genetic screen in human embryonic stem cells (hESCs). Before this can be achieved, however, much time and effort will need to be invested in optimizing the oligo targeting technique and 6TG selection protocol in hESCs. Development of the genetic screen in hESCs will be particularly useful for the analysis of extra-exonic variants. Extra-exonic regions are not as highly conserved between species as exons. We did use the genetic screen in mESCs to study *MLH1 c.546-1G>A* and *MSH6 c.3438+6T>C*; while the variants were located in relatively well conserved intronic areas and the screening results were in line with the InSiGHT classifications as well as splice prediction programs (20–22), it would have been better to study the variants in human cells as it is not completely clear yet how well splice sites are conserved. Development of the genetic screen in hESCs will also demonstrate if any functional differences exist between the human and murine DNA MMR systems.

One issue we have not been able to resolve thus far is the cause of the background colonies that managed to survive 6TG selection despite not carrying the planned mutation. In the case of *Msh6* the majority of these colonies could be ascribed to loss of heterozygosity events causing loss of the wild-type *Msh6* allele. The *Mlh1*$^{+PUR/\Delta}$ and *Msh2*$^{+PUR/\Delta}$ mESCs, however, carry a *puromycin* resistance gene (*PUR*) upstream of the wild-type *Mlh1* and *Msh2* allele, respectively, which should have enabled Puromycin selection to exclude any cell that became 6TG-resistant due to loss of heterozygosity events. Hence the cause of the background 6TG-resistant *Mlh1*$^{+PUR/\Delta}$ and *Msh2*$^{+PUR/\Delta}$ mESCs is mysterious. One hypothesis is that these colonies arose from rare wild-type cells that were temporarily non-cycling and therefore managed to escape killing or permanent arrest by 6TG treatment. This is supported by the fact that background colonies were typically

smaller in size and 6TG survival assays with several of the background *Msh2*$^{+PUR/\Delta}$ colonies revealed the cells were 6TG sensitive. Another theory could be that the background colonies gained 6TG-resistance through partial loss of heterozygosity events that spared the *PUR* gene or because they somehow acquired a different inactivating (epi)mutation. To investigate if the latter theory is the case, Western blot analyses should be performed to determine if DNA MMR and HPRT protein levels were maintained in the background 6TG-resistant colonies. Additionally, RNA from the DNA MMR gene under investigation could be sequenced to identify if an unintended exonic or splice site mutation is present. In the case of *Mlh1*, it may also be interesting to explore if murine *Mlh1*, like human *MLH1* (23–25), can be silenced by promoter methylation. *MSH2* silencing has been shown to occur as a consequence of deletions in the 3' end of the neighboring *EPCAM* gene that give rise to *EPCAM-MSH2* fusion transcripts as well as loss of *MSH2* transcription and promoter hypermethylation (25, 26). In the *Msh2*$^{+PUR/\Delta}$ cell line, the *PUR* is located between the *Epcam* and *Msh2* genes, hence it seems unlikely that silencing of *Msh2* in this manner is causative for the observed background 6TG resistance as cells remained Puromycin-resistant.

Incorporation of the genetic screen in Lynch syndrome diagnostics. The diagnosis of LS is established through several steps. First the patients' cancer family history as well as tumor pathology are analyzed (27). If these are suspicious of LS, *i.e.*, a high family incidence of LS-related tumors, a tumor showing absence of one of the DNA MMR proteins or a microsatellite instable tumor, the DNA MMR genes are sequenced. The identification of a pathogenic germ-line mutation affecting one of the DNA MMR genes manifests LS (28, 29). Consequently patients as well as mutation-carrying relatives can be enrolled in optimal tumor treatment and surveillance programs (9). Surveillance programs have proven to significantly reduce morbidity and mortality of LS-associated mutation carriers (30). They, however, pose unnecessary psychological and physical stress on non-carriers of pathogenic DNA MMR gene variants as well as pressure on preventive healthcare. Hence it is important to establish the phenotypic consequences of DNA MMR variants. If a VUS is found in a suspected-LS patient, approaches that define its pathogenicity must be in place.

To facilitate LS diagnosis, a number of *in silico* algorithms and functional assays that aim to predict the pathogenicity of specific DNA MMR gene variants have been developed. *In silico* algorithms take into account evolutionary conservation as well as physicochemical differences between amino acids to predict a variant's phenotype (31–34). While such computational approaches can accurately predict MMR abrogating mutations, validation is often required to definitively diagnose LS. Functional studies generally investigate the consequences of MMR VUS using assays based on ectopic expression of the mutant MMR gene in MMR-deficient yeast, bacteria or human cells or on *in vitro* reconstituted MMR reactions (35–48). A possible caveat of such functional studies is that they are often performed in distantly related species, and the effect of the mutation may be masked by the unstable genetic background of MMR-deficient cells or over/under-representation of the ectopically expressed or *in vitro* studied proteins (11, 49).

To overcome these limitations, we developed the presented oligonucleotide-directed mutation screen that assesses the pathogenicity of endogenously expressed variant alleles.
The oligonucleotide-directed mutation screen allows many DNA MMR gene variants to be characterized in parallel in a high-throughput manner. Furthermore, the relative simplicity of the screen makes it the ideal tool for clinical genetics laboratories confronted with DNA MMR VUS. Hence we propose that it should be employed in combination with *in silico* prediction tools to define the diagnosis of patients with VUS. To set up the screen in clinical genetics laboratories confronted with suspected-LS DNA MMR VUS, all that is required is a cell culture cabinet and sequencing facility. Our laboratory can provide the required *Mlh1*$^{+PUR/\Delta}$, *Msh2*$^{+PUR/\Delta}$ and *Msh6*$^{+/-}$ mESCs and cell culture protocol. The oligonucleotides, 6TG and cell culture materials can be ordered online. As soon as all materials are present, the high throughput screening can commence; within 3.5 weeks it should be clear if the variant(s) of interest affect MMR capacity.

Broadening the spectrum of the screen to investigate variants in other cancer-susceptibility genes. The presented oligonucleotide-directed mutation screen is the first example of a high-throughput approach for the analysis of cancer-susceptibility mutations that uses oligo targeting (50). Although the site-directed mutagenesis efficacy of oligo targeting is relatively low versus nuclease assisted gene modification techniques (in particular CRISPR/Cas9 assisted), combining it with a strong selection technique allows efficient identification of pathogenic DNA MMR mutations. Furthermore, the low number of false positives and the finding that 6TG-resistant colonies only contained the planned mutation demonstrate that oligo targeting is highly accurate and rarely leads to inadvertent gene-disrupting mutations that may have generated 6TG-resistant colonies. We propose that oligo targeting can also be used to study sequence alterations in other cancer-susceptibility genes provided a selectable readout can be generated. *E.g.*, abrogating *p53* mutations may be identified because they confer resistance to Nutlin3; deleterious mutations in transcriptional repressors such as the tumor suppressor genes *RB1* or *APC* may be detected by activation of an appropriate reporter gene. Techniques that characterize the phenotypic effects of specific allelic variants will become increasingly important with the advances in next generation sequencing. The development of more genetic screens that use oligo targeting in combination with a strong reporter/selection system to characterize the phenotype of sequence variants will facilitate the diagnosis of individuals at risk of specific diseases and hence improve global health.

REFERENCES

1. Lynch HT, de la Chapelle A (1999) Genetic susceptibility to non-polyposis colorectal cancer. *J Med Genet* 36:801–18.
2. Kastrinos F, Stoffel EM (2014) The history, genetics, and strategies for cancer prevention in Lynch syndrome. *Clin Gastroenterol Hepatol* 12:715–27.
3. Bronner CE, et al. (1994) Mutation in the DNA mismatch repair gene homologue *hMLH1* is associated with hereditary non-polyposis colon cancer. *Nature* 368:258–61.

4. Fishel R, et al. (1993) The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. *Cell* 75:1027–38.
5. Miyaki M, et al. (1997) Germline mutation of *MSH6* as the cause of hereditary nonpolyposis colorectal cancer. *Nat Genet* 17:271–2.
6. Plazzer JP, et al. (2013) The InSiGHT database: utilizing 100 years of insights into Lynch syndrome. *Fam Cancer* 12:175–80.
7. Hemminki A, et al. (1994) Loss of the wild type *MLH1* gene is a feature of hereditary nonpolyposis colorectal cancer. *Nat Genet* 8:405–10.
8. Fishel R (2001) The selection for mismatch repair defects in hereditary nonpolyposis colorectal cancer: revising the mutator hypothesis. *Cancer Res* 61:7369–74.
9. Lynch HT, Snyder CL, Shaw TG, Heinen CD, Hitchins MP (2015) Milestones of Lynch syndrome: 1895–2015. 15:181–94.
10. Peltomäki P, Vasen H (2004) Mutations associated with HNPCC predisposition -- Update of ICG-HNPCC/ INSiGHT mutation database. *Dis Markers* 20:269–76.
11. Heinen CD, Rasmussen JL (2012) Determining the functional significance of mismatch repair gene missense variants using biochemical and cellular assays. *Hered Cancer Clin Pract* 10:9.
12. Meldrum C, Doyle MA, Tothill RW (2011) Next-generation sequencing for cancer diagnostics: a practical perspective. *Clin Biochem Rev* 32:177–95.
13. Shyr D, Liu Q (2013) Next generation sequencing in cancer research and clinical application. *Biol Proced Online* 15:4.
14. Plon SE, et al. (2008) Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. *Hum Mutat* 29:1282–91.
15. Thompson BA, et al. (2014) Application of a 5-tiered scheme for standardized classification of 2,360 unique mismatch repair gene variants in the InSiGHT locus-specific database. *Nat Genet* 46:107–15.
16. de Wind N, Dekker M, van Rossum A, van der Valk M, te Riele H (1998) Mouse models for hereditary nonpolyposis colorectal cancer. *Cancer Res* 58:248–55.
17. Wojciechowicz K, et al. (2014) Temozolomide increases the number of mismatch repair-deficient intestinal crypts and accelerates tumorigenesis in a mouse model of Lynch syndrome. *Gastroenterology* 147:1064–72.
18. Whiteside D, et al. (2002) A homozygous germ-line mutation in the human *MSH2* gene predisposes to hematological malignancy and multiple cafe-au-lait spots. *Cancer Res* 62:359–62.
19. Ricciardone MD, et al. (1999) Human *MLH1* deficiency predisposes to hematological malignancy and neurofibromatosis type 1. *Cancer Res* 59:290–3.
20. Reese MG, Eeckman FH, Kulp D, Haussler D (1997) Improved splice site detection in Genie. *J Comput Biol* 4:311–23.
21. Pertea M, Lin X, Salzberg SL (2001) GeneSplicer: a new computational method for splice site prediction. *Nucleic Acids Res* 29:1185–90.
22. Desmet FO, et al. (2009) Human Splicing Finder: an online bioinformatics tool to predict splicing signals. *Nucleic Acids Res* 37:1–14.
23. Herman JG, et al. (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci USA* 95:6870–5.
24. Hitchins MP, et al. (2011) Dominantly inherited constitutional epigenetic silencing of *MLH1* in a cancer-affected family Is linked to a single nucleotide variant within the 5'UTR. *Cancer Cell* 20:200–13.
25. Hitchins MP (2013) The role of epigenetics in Lynch syndrome. *Fam Cancer* 12:189–205.
26. Ligtenberg MJ, et al. (2009) Heritable somatic methylation and inactivation of MSH2 in families with Lynch syndrome due to deletion of the 3' exons of TACSTD1. *Nat Genet* 41:112–7.
27. Umar A, et al. (2004) Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. *J Natl Cancer Inst* 96:261–8.
28. Giardiello FM, et al. (2014) Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the US Multi-Society Task Force on Colorectal Cancer. *Am J Gastroenterol* 109:1159–79.
29. Hampel H, et al. (2008) Feasibility of screening for Lynch syndrome among patients with colorectal cancer. *J Clin Oncol* 26:5783–8.
30. Järvinen HJ, et al. (2000) Controlled 15-year trial on screening for colorectal cancer in families with hereditary nonpolyposis colorectal cancer. *Gastroenterology* 118:829–34.
31. Adzhubei IA et al. (2010) A method and server for predicting damaging missense mutations. *Nat Methods* 7:248–9.

32. Ali H, Olatubosun A, Vihinen M (2012) Classification of mismatch repair gene missense variants with PON-MMR. *Hum Mutat* 33:642–50.
33. Chao EC, et al. (2008) Accurate classification of MLH1/MSH2 missense variants with multivariate analysis of protein polymorphisms-mismatch repair (MAPP-MMR). *Hum Mutat* 29:852–60.
34. Kumar P, Henikoff S, Ng PC (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* 4:1073–82.
35. Christensen LL, et al. (2009) Functional characterization of rare missense mutations in *MLH1* and *MSH2* identified in Danish colorectal cancer patients. *Fam Cancer* 8:489–500.
36. Drost M, et al. (2012) A rapid and cell-free assay to test the activity of Lynch syndrome-associated MSH2 and MSH6 missense variants. *Hum Mutat* 33:488–94.
37. Gammie AE, et al. (2007) Functional characterization of pathogenic human MSH2 missense mutations in *Saccharomyces cerevisiae. Genetics* 177:707–21.
38. Lützen A, de Wind N, Georgijevic D, Nielsen FC, Rasmussen LJ (2008) Functional analysis of HNPCC-related missense mutations in *MSH2. Mutat Res* 645:44–55.
39. Mastrocola AS, Heinen CD (2010) Lynch syndrome-associated mutations in *MSH2* alter DNA repair and checkpoint response functions *in vivo. Hum Mutat* 31:E1699–708.
40. Ou J, et al. (2007) Functional analysis helps to clarify the clinical importance of unclassified variants in DNA mismatch repair genes. *Hum Mutat* 28:1047–54.
41. Ollila S, et al. (2006) Pathogenicity of *MSH2* missense mutations is typically associated with impaired repair capability of the mutated protein. *Gastroenterology* 131:1408–17.
42. Ollila S, Bebek DD, Jiricny J, Nyström M (2008) Mechanisms of pathogenicity in human *MSH2* missense mutants. *Hum Mutat* 29:1355–63.
43. Drost M, et al. (2010) A cell-free assay for the functional analysis of variants of the mismatch repair protein MLH1. *Hum Mutat* 31:247–53.
44. Shimodaira H, et al. (1998) Functional analysis of human *MLH1* mutations in *Saccharomyces cerevisiae. Nat Genet* 19:384–9.
45. Andersen SD, et al. (2012) Functional characterization of *MLH1* missense variants identified in Lynch syndrome patients. *Hum Mutat* 33:1647–55.
46. Raevaara TE, et al. (2005) Functional significance and clinical phenotype of nontruncating mismatch repair variants of *MLH1. Gastroenterology* 129:537–49.
47. Heinen CD, et al. (2002) HNPCC mutations in hMSH2 result in reduced hMSH2-hMSH6 molecular switch functions. *Cancer Cell* 1:469–78.
48. Guerrette S, Acharya S, Fishel R (1999) The interaction of the human MutL homologues in hereditary nonpolyposis colon cancer. *J Biol Chem* 274:6336–41.
49. Rasmussen LJ, et al. (2012) Pathological assessment of mismatch repair gene variants in Lynch syndrome: past, present, and future. *Hum Mutat* 33:1617–25.
50. van Ravesteyn TW, et al. (2016) LNA modification of single-stranded DNA oligonucleotides allows subtle gene modification in mismatch-repair-proficient cells. *Proc Natl Acad Sci USA* 113:4122–7.