LSHC-CT-2005-18754

MMR-related cancer

Prevention, detection and molecular characterization of mismatch repair-related hereditary cancers of the digestive system

Specific Targeted Project

Priority 1: Life Sciences, Genomics and Biotechnology for Health

Publishable final activity report

Period covered: from 1/2/2006 to 1/8/2009

Date of preparation: 28/9/2009

Start date of project: 1/2/2006

Duration: 42 months

Project coordinator: R.M.W. Hofstra
Project coordinator organisation: University Medical Center Groningen

Revision: Final version

Index

1. Project execution

Summary description of project objectives

The aim of this project was to increase our knowledge on the genetic and molecular basis of two disease entities linked to mismatch repair (MMR) defects, namely Hereditary Non-polyposis Colorectal Cancer (HNPCC, or Lynch Syndrome) and Familial Gastric Cancer (FGC). This increased knowledge has helped us in improving genetic screening and set up comprehensive functional assays, to determine pathogenicity of unclassified variants (UVs) found in MMR genes in patients and their family members. Furthermore, we aimed to improve early detection of polyps/tumours in individuals at risk and when tumours are identified, tumour-mutation-profiles were used as an indicator for disease prognosis and treatment.

Contractors involved

Nr	Contractor	Principal scientist
1	University Medical Center Groningen (UMCG), Department of Genetics, Groningen, The Netherlands (Coordinator)	Prof. Dr. Robert M.W. Hofstra
2	University of Helsinki, Faculty of Medicine, Molecular and Cancer Biology Research Program, Helsinki, Finland	Prof. Dr. Lauri A. Aaltonen
3	Roskilde University, Department of Science, Systems and Models, Roskilde, Denmark	Prof. Dr. Lene J. Rasmussen
4	Leiden University Medical Centre (LUMC), Department of Toxicogenetics, Leiden, The Netherlands	Dr. Niels de Wind
5	University of Porto, Institute of Pathology and Molecular Immunology, Porto, Portugal	Prof. Dr. Raquel Seruca

Coordinator contact details

Prof. Dr. Robert M.W. Hofstra
University Medical Center Groningen
Department of Genetics
Hanzeplein 1
P.O. Box 30001
9700 RB Groningen
The Netherlands
Phone: +31 50 3617100
Fax: +31 50 3617230
E-mail: r.m.w.hofstra@medgen.umcg.nl

Website

www.mmr-relatedcancer-hnpcc.eu

Work performed and results achieved

Background and objectives

This project focuses on hereditary cancers of the digestive system associated with microsatellite instability, i.e. hereditary non-polyposis colorectal cancer (HNPCC) and familial gastric cancer (FGC). In HNPCC, identification of MMR gene mutations has helped in identifying individuals at risk when a clear pathogenic mutation was found. A large proportion of families, however, have mutations of which the pathogenic nature is uncertain (unclassified variants or UVs) or have no mutation at all. These findings made us speculate that more genes might exist and/or that the UVs identified play a more important role in disease development then currently thought.

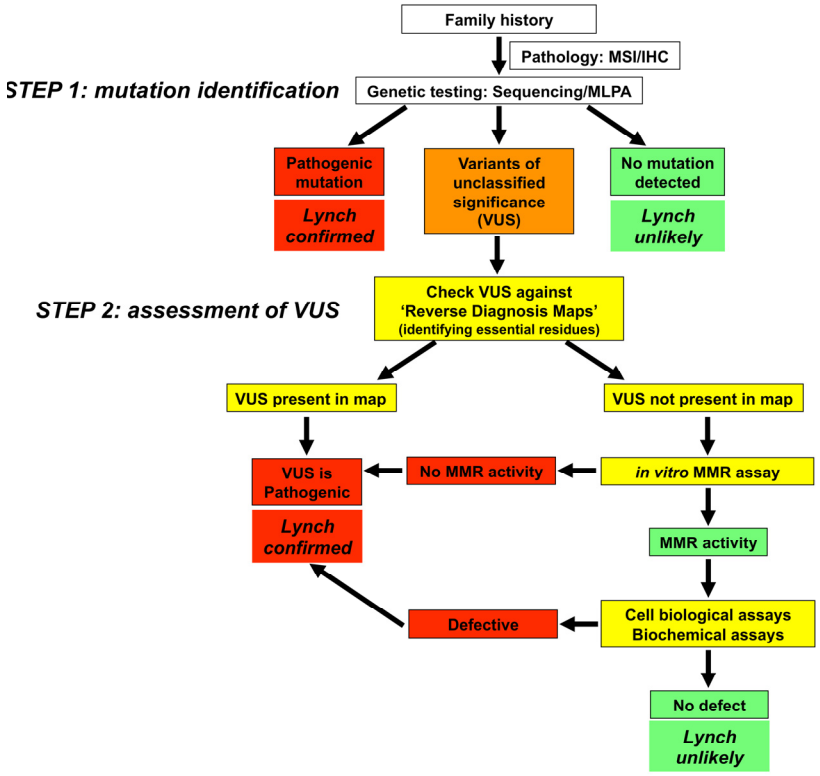

Figure 1. Proposed decision tree for the evaluation of MMR gene VUS

Identification of new MMR genes

As a large proportion of patients do not carry a mutation in one of the known MMR genes we hypothesized that other, yet undiscovered MMR genes should exist. We therefore tried to identify these new HNPCC or FGC-related MMR genes. For this purpose we had available almost 100 selected patients (both HNPCC as well as FGC), patients that had MMR-deficient tumours but had no mutation in the coding sequence of any of the known HNPCC-associated genes. The way to find these new genes was to search for loss of heterozygosity (LOH) in the selected MSI-H tumours. LOH is believed to be an indicator for regions harbouring cancer causing genes. So far for all MMR–related cancers a crucial step in tumour development proved to be loss of the wildtype allele, mostly caused by LOH. Therefore, we hypothesized that finding regions of LOH in the selected tumours would help us in identifying regions and likely genes that are involved in the development MMR-related tumours. To find LOH we made use high resolution genome wide screening with micro-array CGH using bacterial-artificial chromosomes (BACs) and Agilent oligo arrays. Unfortunately during the array experiments we came to the conclusion that these small genomic aberrations did not exist or are extremely rare. We could not identify any good candidate gene.

Identification of new mutations in the known MMR genes

As we had no indication that other, not yet identified, MMR genes do exist we had to conclude that our initial hypothesis was incorrect. It made us wonder whether the known MMR genes

might be involved in a way we have not yet studied. For instance, intronic variants disturbing splicing or the expression of the genes may play an important role in HNPCC. Therefore we decided to extend our mutation screen of the known MMR genes to the non-coding sequences including the promoter region. We therefore sequenced 500 patients and 500 controls over a region of approximately 140.000 basepairs (in total we got over 6000 mB of sequence). At this moment we have generated the data and currently we are analysing them. Subsequently, sequence data of patients and controls will be compared. We hope to find many more variants present in the patient cohort, compared to the controls, indicating that indeed the old genes contain many 'new mutations'
Besides these non-coding mutations it can also not be excluded that methylation in the germline also plays a role. We studied MLH1 and MSH2 promoter hypermethylation. We indeed identified patients with germline MLH1 methylation, however we did not detect any MSH2 germline methylation. As it has been demonstrated very recently that germline deletions of the 3' region of EPCAM cause transcriptional read-through which results in silencing of MSH2 by hypermethylation we also looked for these deletions. We identified 3 patients with somatic MSH2 promoter hypermethylation in their tumors, which was caused by a germline EPCAM deletion. Therefore we had to conclude that methylation of the known genes plays a significant role. Finally, a large set of patients was screened for *PMS2* mutations and involvement of this gene in HNPCC was proven as we identified 3 new mutations.

Our data suggests that mutations in the known MMR genes (MSH2, MLH1, MSH6 and PMS2) are more important than previously thought and that aberrations outside of the coding region, which were not looked at before, can play a role in disease development.

Functional assays
As mentioned, a large proportion of patients carry an unclassified variant (UV) of which the pathogenic nature is unclear. These UVs might also explain a proportion of the patients. To find out whether the UVs found indeed constitute to MMR-related cancer we have set up comprehensive functional assays to determine the role of these UVs in MMR-related disease.

In vitro MMR assay
In the In vitro MMR assay we use a simple complementation assay to investigate whether a patient-derived missense-mutant MMR gene is capable of supporting MMR *in vitro*. If this is not the case, the mutation is considered causative and the patient and his relatives can be correctly diagnosed. We have developed the assays to test the activity of UVs in the MMR genes *MSH2, MSH6* and *MLH1.* In addition to a number of minor improvements, we have modified the assays such that the results can now be analyzed and quantified on an automated fragment analyzer. In addition we have developed a fully *in vitro* procedure for the construction of UVs in *MSH2, MSH6* and *MLH1* genes. Owing to these improvements, the entire diagnostic procedure may now be performed in well-equipped clinical diagnostics laboratories. In addition we have collected a patient panel as well as constructed all mutated *MSH2, MSH6* and *MLH1* genes for testing. Currently, we are finishing testing the mutant genes for their activity (**Figure 2** for a test of 26 UVs in the MLH1 gene). A patent has been applied on the assay and a manuscript will be submitted to a peer-reviewed journal soon.

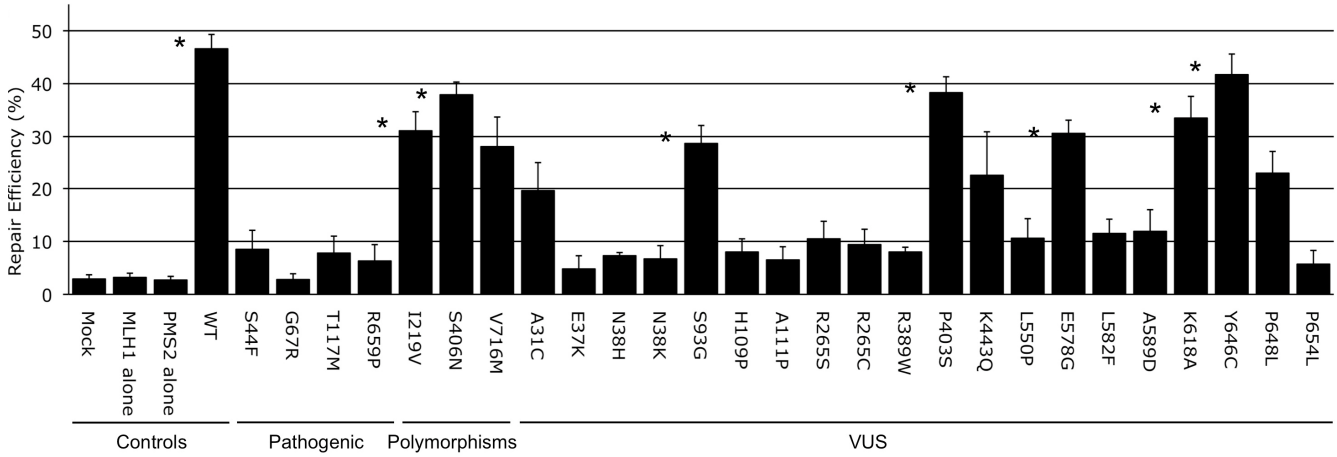

Figure 2. Functional assessment of 27 patient-derived UVs in the MLH1 MMR gene.

ATP binding and hydrolysis are essential for efficient MMR. Both MutSα and MutLα possess an ATPase activity, which is critical for MMR Furthermore, several MSH2 missense mutations conferring defective MMR have been found to lead to altered ATP binding and hydrolysis as well as reduced ADP to ATP exchange and reduced ATP-dependent dissociation from a mismatch.

DNA binding assay

To test whether MSH2 UVs influence mismatch binding, we have set up a DNA binding assay. In this assay we study the ability of the mutant MutSα complexes to bind to homoduplex (H) or G/T mismatched (M) DNA by electrophoretic mobility shift analysis (EMSA). The DNA binding assay has been optimised for testing MSH2 variants.

Pull-down assay

The pull-down assay measures interaction between two proteins. One protein is expressed and purified with a GST-tag (MLH1 or MSH2), the other protein is *in vitro* transcribed and translated (IVTT) using a commercial reticulocyte lysate system (PMS2, EXO1, or MSH6). The GST-tagged protein is bound to glutathione sepharose beads and the ^{35}S-labelled *in vitro* transcribed and translated (IVTT) protein is added. The beads are washed and bound protein is separated on a gel. If the two proteins interact, a signal from the bound ^{35}S-labelled IVTT protein can be detected with a phosphorimager. When investigating many UV-containing MLH1 and MSH2 proteins this procedure has been very time consuming because all UVs needed to be purified as GST-tagged proteins. However, we have set up the assay in the opposite orientation by cloning and purifying GST-tagged PMS2 and MSH6 protein allowing the UVs to be synthesized by IVTT. The assay using GST-PMS2 has been tested by investigating eight MLH1 UVs in both orientations of the assay. The results of the assay were the same regardless of the orientation; five UVs did not interact with PMS2 whereas three did interact. The GST-MSH6 is hard to purify and needs to be concentrated before it can be used in the assay. The assay using GST-MSH6 has been set up and the results from this assay are currently being tested by comparing the results to results from the assay in the opposite orientation.

Nineteen MLH1 UVs have been analysed in the pull-down assay and five of these show no binding to neither PMS2 nor EXO1. Seventeen MSH2 UVs have been tested and all except for one bind to MSH6. Four of the UVs that do bind to MSH6 exhibit no binding to EXO1.

Subcellular localization assay
One aspect of MMR that are not measured in other in vitro assays is the subcellular localization of UVs. By transiently expressing UV proteins with a fluorescent tag (either CFP or YFP) together with the WT protein with a different fluorescent tag (YFP or CFP) it is possible to see whether the wt and UV localize together in the nucleus by confocal laser scanning microscopy. We have investigated 21 UVs in MSH2 and 18 of these localize to the nucleus whereas three stay in the cytoplasm and thus exhibit defective nuclear localization. Of the 14 MLH1 UVs investigated so far in this assay, all but one localize together with WT in the nucleus. All ten MSH6 UVs tested so far localize to the nucleus.

Early detection of tumours
Another problem in the HNPCC field is early detection of tumours in patients or individuals at risk. To identify patients at very early stages we have set up a test that enables us to detect MMR defects (MSI-H) in faeces. We therefore developed a sensitive and specific PCR-based method to detect low frequencies of microsatellite-instable cells in material. We have shown proof of principle of our newly designed method and we intend to further increase the selectivity of the method to enable the highly specific detection of tumour DNA in a large excess of normal DNA.

Mutation profiles in tumours
Last we have made mutation profiles in tumours. The mutations are a consequence of MMR deficiency. This study is performed to obtain a better insight in tumour development, which can be instrumental in clinical management/tumour treatment. After determining the mutational background frequencies we screened large series of tumours and identified a number of highly mutated genes. These genes will help in understanding tumour development and might prove useful in designing new therapeutics.

Two sets of data (list I and list II) were generated and integrated; U133A chip expression data on MSI (hereditary and sporadic) CRCs and normal colorectal mucosa, as well as human genome sequence data. List I was based on genes down-regulated in MSI-H cancers *versus* normal mucosa, having a short repetitive sequence in their coding region (as based on human genome sequence information). List II was based on genes differentially expressed between MSI-H hereditary (HNPCC) and MSI-H sporadic cancers, having a short repetitive sequence in their coding region (human genome sequence information)
To validate these lists we analyzed the list I genes (approximately 700). Mutation analysis of List I genes was initially performed in 30 primary MSI CRCs. The genes that displayed a somatic mutation rate >20% were evaluated in an extended set of 70 MSI CRCs, totalling 100 samples. Normal tissue DNA was evaluated to confirm somatic origin of mutations. MSI status was evaluated in our previous studies using a set of microsatellite markers. The genes which showed a somatic mutation rate of >20% also in the extended series of MSI CRCs were sequenced for somatic mutations in the entire coding region in 30 microsatellite stable (MSS) CRCs.
The sequence analysis of List I genes in MSI CRCs has been completed. We identified 16 putative novel MSI target genes, *MYH11, ABCC5, CLOCK, SEC31L1, NM_032569,*

NM_015023, ROCK1, WASF3, OR51E2, HPS1, TCEB3, PRDM2, HDCMA18P, PIGB, ZNF288 and *AKAP9*. These genes were somatically mutated in 23-55% of primary MSI CRCs and in 8-71% of MSI CRC cell lines. Sequence analysis of the identified 16 target genes in 30 MSS CRCs resulted in identification of somatic mutations of *MYH11* in MSS CRC.
The genes with the highest mutation rates in the primary MSI CRCs, *MYH11 and CLOCK* were selected for further functional studies. Furthermore, all 16 candidate genes were functionally assessed using high-throughput RNAi and transfection experiments and measures of cell cycle and viability.
In addition we have analyzed 114 intergenic region control repeats to examine background mutation rates. High mutation rates up to 81% were detected in presumably neutral intergenic repeats. A strong positive correlation between repeat length and mutation rate was detected, and long C/G repeats were significantly more unstable than A/T repeats.

Mutation database
Furthermore, a publicly available mutation database was set up at *www.mmrmissense.info* The database contains data on functional assays performed for over 560 UVs (more than 3000 records). It is complemented for all UV with in silico data. The database is well visited by over 3000 visits over 1.5 years (1500 first-time visitors)

End results

Our project has made considerable progress in our goal to improve genetic testing, improve early detection of polyps/tumours in individuals at risk for MMR-related cancer syndromes, and improve clinical management of HNPCC and FGC patients.
Specifically:

- We have not found new MMR-related genes
- We came to the conclusion that ‘new types of mutations’ in the old genes likely play an important role in these MMR related cancer syndromes
- Comprehensive functional assays to determine the role of unclassified variants in MMR-related genes are set up and used to test a large set of UVs
- We have shown proof of principle for a very sensitive PCR for MSI testing.
- We have available a mutation profile of MMR tumours, which gives a better insight in tumour development, and can be instrumental in clinical management/tumour treatment
- We have identified 16 putative new MSI target genes.
- We have a database filled with functionally analysed UVs publically available at *www.mmrmissense.info*

2. Dissemination and use

In general

Dissemination of results and (internal/external) knowledge management is done through the following activities:

- 45 publications in national and international scientific journals
- 17 presentations at national and international seminars
- Establishment of a patent
- 2 press releases
- The consortium has organised a HNPCC cancer workshop for interested students/clinicians about the genetic basis of HNPCC at the INSIGHT meeting in Düsseldorf in 2009.

Improved assays for the analysis of UVs

Currently a few thousand UVs in MMR genes are listed in publicly accessible databases (e.g. *www.mmrmissense.info*. It is anticipated that, as the trend towards personalized genomic analysis proceeds, a large number of additional UVs in MMR proteins will be uncovered. The pathogenicity of UVs cannot currently be established and consequently all relatives, whether they carry the UVs or not, enrol in annual cancer screening programs. This results in an enormous burden on suspected Lynch syndrome patients, on their relatives, on individuals with 'coincidentally-detected' UVs, but also on preventive health care systems.
We have developed, and partly validated a widely applicable and completely cell free assay that can be used to measure the activity of MMR gene VUs. The turnaround time of the assay is approximately three days and many UVs can be tested and processed simultaneously. Depending on continued funding, we anticipate the assay to be marketable in 4-5 years.