Molecular Genetic Background of Lynch Syndrome in Hungary

Abstract of the Ph.D. Thesis

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Background

Colorectal cancer is the second most prevalent cancer type worldwide, there are over one million new cases annually. Besides, colorectal cancer is one of the most common neoplastic disorders in Hungary. The storage of excreta and the extremely quick proliferation of colonic crypts, present in the whole length of the large intestine, are central risk factors for this disease.

The stem cells residing in the crypt bottom produce progenitor cells. They divide in every 12-16 hours, generating 200 cells per crypt every day. The resulting cells differentiate, migrate to the surface epithelium, die and shed in a few days. The processes of proliferation, differentiation and apoptosis require a balanced regulation, and any impairment of this harmony may result in neoplastic transformation.

Among all colorectal cancers, sporadic cases account for the 70-80%, while 10-15% is seen as familial accumulation of cancer cases and the another 10-15% meets the criteria of colorectal cancer syndromes.

The most common colorectal cancer-associated syndrome is Lynch syndrome (hereditary non-polyposis colorectal cancer – HNPCC). According to published data, the prevalence of this syndrome accounts for 1,9-15% of all colorectal cancer cases. These wide margins can be explained with the great variety of criteria used in the clinical definition of this syndrome and the difficulty of it's diagnosis since, besides colorectal and endometrial cancer, other tissues (such as the small intestine, stomach, ovaries, and the urogenital system) can also be affected. Moreover, in Lynch syndrome associated mutation carriers the risk of developing colorectal cancer before age 50 is approximately 180-fold higher in men and 100-fold in woman as compared with the general population. Autosomal dominant inheritance and younger age at disease onset are other typical characteristics of the Lynch syndrome.

The tumors associated with this clinical phenotype are caused by germline mutations in the genes of the DNA mismatch repair (MMR) system – mainly in MLH1 and MSH2 – and among their distinctive features in the molecular level are microsatellite instability (MSI) and lack of the protein in the cells' nucleus, as can be shown using immunihostochemical (IHC) analysis. Thus, routine application of MSI and IHC analyses of tumors in cases suspected of Lynch syndrome (patients with positive family history and/or early age at cancer onset) can

facilitate accurate diagnosis and direct molecular geneticists towards targeted mutation testing.

The mutation spectra of the mismatch repiar genes was shown to present a different pattern in distinct populations or ethnic groups, but it was not assessed in Hungarian Lynch syndrome families yet.

Aims

The aims of the Ph. D. study can be summarized as follows:

- To determine the mutation spectra of MLH1 and MSH2 genes in Hungarian Lynch syndrome families.

- To characterize the sequence variants on different levels (including the determination of evolutionary conservation of the affected DNA/protein regions and prediction of the variant's effects on both mRNA processing and protein function) in order to evaluate their relation with the disease (pathogenic mutations, polymorphisms, or variants with unknown pathogenicity).

- To reveal genotype-phenotype correlations in mutation carriers.

- To introduce and adapt new methodologies and approaches in those cases where previously applied, routine diagnostic procedures give no result – thus widening the possibility to uncover novel potential genetic susceptibility factors.

- To improve and broaden the scope of conventional test methods and make them applicable to future genetic screening setups.

Materials and Methods

: 55 families diagnosed with Lynch syndrome from different regions of Hungary were recruited to this study.

: Blood samples were obtained from all consenting subjects and DNA was extracted with the classic phenol-chloroform method.

: The entire MLH1 and MSH2 coding regions and splice junctions were amplified by PCR from genomic DNA.

: In our preliminary studies systematic screening for point mutations in both genes was performed using conformation-sensitive gel electrophoreses (heteroduplex analysis and single-strand conformation polymorphism analysis) on all amplicons. The studied fragments were silver-stained and analyzed laser-densitometrically (Personal Densitometer SI, Molecular Dynamics). For the running of the gels we used a Protean II xi Cell (Bio-Rad) apparatus.

: All samples showing altered migration patterns were subjected to direct bi-directional sequencing. The sequencing reactions were carried out using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Capillary electrophoresis of the samples was performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) equipment, with the use of POP-7 polymer. The evaluation of the data was done with the ABI PRISM Sequencing Analysis 5.2 (Applied Biosystems) software.

: Both MLH1 and MSH2 coding regions were screened for genomic rearrangements using the multiplex ligation-dependent probe amplification (MLPA) technique. The PCR products were separated and analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems). To determine the yields of peak heights commensurable to the original copy number we used GeneMapper 4.0 and Peak Scanner 1.0 softwares (Applied Biosystems).

: When genomic deletions affected more than one exon, the breakpoints were confirmed with the combination of restriction mapping and XL PCR (Expand Long Template PCR System, Roche), according to the manufacturers' instructions.

: The extent of deletions 5' from the MSH2 gene was determined by Q-PCR (with the use of Power SYBR Green PCR Master Mix, Applied Biosystems). The reaction conditions were as recommended in the kit protocol.

: In the QMPSF (*Quantitative Multiplex PCR of Short Fluorescent Fragments*) reaction 100 ng DNA was amplified with Qiagen Multiplex PCR Kit. The reaction was carried out according to the manufacturer's instructions. For the semiquantitative evaluation we terminated the amplification after the 22nd PCR cycle. The analysis of the PCR products was similar to the MLPA reactions.

: RNA isolations were carried out with the use of Ambion RNAqueous or RNAqueous Micro Kit (Applied Biosystems), following the recommendations of the manufacturer. For cDNA production the High Capacity cDNA Reverse Transcription Kit was applied (Applied Biosystems).

: For the quantification of relative RNA expression of TACSTD1 and MSH2 genes we applied fluorescent TaqMan technology, according to the manufacturer's recommendations (TaqMan Universal PCR Master Mix, Applied Biosystems). The Q-PCR reactions were run on an ABI 7900HT device, the results were evaluated using SDS 2.0 software (Applied Biosystems).

: We performed the haplotype analysis on DNA samples of 11 individuals from two families affected with deletions with the same 5' and 3' breakpoints. Fluorescent PCR products were separated on an ABI 3130 Genetic Analyzer (Applied Biosystems), data were evaluated with GeneMapper v4.0 software (Applied Biosystems).

: Microsatellite instability (MSI) analysis was performed on matched normal and tumor DNA pairs using the International HNPCC Reference Marker Panel. The separations were performed on an ABI 3130 Genetic Analyzer (Applied Biosystems). We analyzed the data with GeneMapper v4.0 software (Applied Biosystems).

: The immunohistochemical (IHC) stainings predicting the presence of mutations were done at the Department of Molecular Pathology, National Institute of Oncology, with the supervision of dr. Zoltán Szentirmay.

: For the bioinformatics analyses of the sequence variants we used databases and softwares freely available on the internet.

Results

The results of our investigations can be summarized as follows:

- In 55 families meeting various clinical criteria for Lynch syndrome we analyzed the MLH1 and MSH2 genes and revealed their mutation spectra for the first time in Hungary. Also, we initialized the mutation screening of the MSH6 gene.

- With the combined use of prescreening methods (HDA, SSCP and MLPA) and direct sequencing we uncovered the presence of 49 different variants.

- Among these variants twenty-nine (59%) different disease-causing mutations (12 in MLH1, 12 in MSH2, 1 in MSH6 and 4 in the TACSTD1 gene, 5' from MSH2) and three variants with unknown pathogenicity were found, in addition to 17 frequent polymorphisms.

- Eighteen variants (39%) were novel, in the majority of them (16) bioinformatics analysis, examination of effects in RNA level, cosegregation with the disease and the loss of protein function rendered the connection with the disease probable – thus, the pathogenicity is still uncertain for 2 cases.

- The above mentioned analysis of thirteen previously described missense mutations, and the comparison of our results with bibliographical data suggested that 6 of these variants could be classified as pathogenic mutations, while we recommend the other 7 variants to be handled as polymorphisms.

- Half of the 20 pathogenic point mutations was disclosed in the MLH1, while the other half in the MSH2 gene.

- These 20 point mutations occurred in 21 different families, the mutation c.350C>T (p.Thr117Met) of the MLH1 gene was revealed in two cases – meaning the first recurrent pathogenic mutation in MLH1 gene in Hungary. Similarly, the nonsense

mutation c.1264G>T (p.Glu442X) in exon 12 of MSH2 is the first recurrent mutation in this gene.

- In addition to pathogenic point mutations, the presence of three large genomic deletions and one large genomic insertion in the coding region of MLH1 and MSH2 were brought to light. Since all of them are novel, verification of their pathogenicity was achieved using several different approaches.

- We identified the first large genomic deletion in the MSH6 gene in the Hungarian Lynch syndrome population.

- We classified four different large genomic deletions far upstream of MSH2 (in the TACSTD1 gene) as pathogenic mutations, thus presenting a novel mechanism for Lynch syndrome. This so-called transcriptional interference means inhibition of a given gene by another promoter 5' *in cis* – also, this promoter can be the part of another gene. This phenomenon was not previously known as an inactivation mechanism in connection with any cancer susceptibility gene.

- We demonstrated the presence of one of the 5' (TACSTD1) deletions in two families, proving the existence of another recurrent mutation in our population. These two deletions share a common haplotype, thus representing the first, verified founder effect in the Hungarian Lynch syndrome population.

Conclusions

- We determined the mutation spectra of the MLH1 and MSH2 genes in 55, clinically defined Lynch syndrome families.

- The revealed 49 different variants scattered throuhgout the coding region of the genes involved, thus no mutation hot spot is present in either.

- Besides this lack of mutation hot spots, we detected the first recurrent pathogenic mutations in the MLH1 and also in the MSH2 gene in the Hungarian Lynch syndrome families (c.350C>T (p.Thr117Met) in exon 4 of MLH1 and c.1264G>T (p.Glu442X) in exon 12 of MSH2).

- The proportion of pathogenic mutations is more prevalent in families meeting more stringent selection criteria (the proband has a mutation in 79% of families fulfilling Amsterdam I criteria, in contrast to only 26% of the probands in families matching Bethesda guidelines).

- As TACSTD1 genomic deletions represent a mutation mechanism new in the pathogenesis of Lynch syndrome and as their prevalence was shown to account for 16% of all pathogenic mutations, we proposed that this type of mutations should be screened in familial cancer syndromes.

- The large genomic aberrations were revealed in families fulfilling more stringent criteria – additionally, together with TACSTD1 deletions, these account for 28% of the pathogenic mutations. Thus, the inclusion of screening for genomic deletions or duplications is especially important.

- The use of MSI and IHC analysis as prescreening methods is pivotal: in every case where we had a chance to test the tumor of a patient carrying a variant with suspected pathogenicity, these examinations confirmed our hypothesis – the lack of protein in the cell nuclei and the presence of microsatellite instability underlined the existence of a pathogenic aberration.

- We proposed the application of MSI/IHC analysis as a pre-genetic screening method in Hungarian Lynch syndrome families.

The results of this study support our understanding of the mechanisms underlying inactivation of the mismatch repair system, presenting evidence that damages of regions outside cancer susceptibility genes could cause errors in the regulation of these genes and have a role in the predisposition to syndromes associated with them. Our results can be applied to increase the detection rate of predisposing mutations in other cancer syndrome genes as well.

The clinical use of the results is also of great importance, as for a patient with a cancer family syndrome the determination of mutation status is essential for the accurate diagnosis, genetic counselling and adequate treatment decision.
Moreover, the most important practical benefit of our study could be that molecular screening makes the identification of patients at high risk for developing the disease possible even before malignant transformation occurs, thus opening the way before the application of effective treatment strategies for members of cancer syndrome families.

Publications related to this Ph. D. thesis:

Articles in peer-reviewed journals:

Janos PAPP*, Marietta E. KOVACS*, Edith OLAH:
"Germline MLH1 and MSH2 Mutational Spectrum Including Large Genomic Aberrations in Hungarian Hereditary Non-Polyposis Colorectal Cancer Families: Implications for Genetic Testing"
World Journal of Gastroenterology 2007; 13(19):2727-2732. IF(2008): 2,081
**co-first authors*

Marietta E. KOVACS*, Janos PAPP*, Zoltan SZENTIRMAY, Szabolcs OTTO, Edith OLAH:
"Deletions removing the last exon of TACSTD1 constitute a distinct class of mutations predisposing to Lynch syndrome"
Human Mutation 2009; 30: 197-203. IF(2008): 7,033
**co-first authors*

Conference abstracts:

Lecture:

KOVÁCS Marietta Éva:
„Molecular alterations in mismatch-repair genes MLH1 and MSH2 in Hungarian HNPCC families" - in Hungarian
National Undergraduate Students' Science Conference XXVII, Biology Section, March 24th 2005, Pécs, Hungary (program book page 115, summary: GENET-4)

KOVÁCS Marietta Éva, PAPP János, OLÁH Edit:
„Genetics of Hereditary Non-Polyposis Colorectal Cancer (HNPCC) in Hungary" - in Hungarian
Magyar Onkológia 2005; 49(3S): 43.

Poster:

Marietta Éva KOVÁCS, János PAPP, Edith OLÁH:
"Germline mutations and large genomic rearrangements in mismatch repair genes predisposing to HNPCC in Hungary"
19th Meeting of the European Association for Cancer Research, Programme/Proceedings 2006; 76: 243. (Abstract No. 386)

KOVÁCS Marietta Éva, PAPP János, OTTÓ Szabolcs, SZENTIRMAY Zoltán, OLÁH Edit:
"Novel alleles conferring susceptibility to Lynch-syndrome (HNPCC) in Hungary" - in Hungarian
Magyar Onkológia 2007; 51(4): 349.

Marietta E. KOVACS*, Janos PAPP*, Zoltan SZENTIRMAY, Szabolcs OTTO, Edith OLAH:
„Deletions in TACSTD1, far upstream of MSH2: a new aspect for genetic testing for Lynch syndrome"
In: Proceedings of the 100th Annual Meeting of the American Association for Cancer Research; 2009 Apr 18-22; Denver, CO. Philadelphia (PA): AACR; 2009. Abstract nr 3039