OUR EXPERIENCE WITH THE OCCURRENCE OF HEREDITARY NON-POLYPOSIS COLORECTAL CANCER. PEDIGREE AND GENETIC ANALYSIS OF THE PROVEN MUTATION CARRIER FAMILIES

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Introduction

The rapid development of molecular biology during the past two decades led to the better understanding of the genetic background and characteristics of more and more malignant diseases. Some sort of familial accumulation has been reported in 10-15% of all colorectal cancers. FAP and its variants (attenuated-FAP, Turcot- and Gardner-syndrome) - which are autosomal dominant obligatory pre-malignant conditions – are accounting for approximately 1% of the colorectal cases. Various publications estimate the proportion of HNPCC in colorectal cancer burden to be 3-6%.

Malignancies are primarily considered as genetic diseases. Development of the malignant transformation originates from the uncontrolled proliferation of a genetically altered cell line.

The importance of HNPCC is further emphasized by the fact that it represents the majority of all inherited colorectal cancers. HNPCC is characterized by right colonic dominance, the frequent occurrence of synchronous and metachronous tumors and predisposition for the development of HNPCC-associated malignancies (stomach, endometrium, ovaries, urether, biliary tract) in the affected families. Those patients with a confirmed mutation have an 80-85% lifetime risk for colorectal cancer which may develop at a relatively young age.

Familial accumulation is common in first-degree relatives and in succeeding generations. Inheritance is autosomal dominant with a penetration of over 80%.

The human Mismatch Repair system (MMR) has 7 known members; their designation comes from the bacterial homologues: MutL homologues (MLH) hMLH1, hMLH3; MutS homologues (MSH) hMSH2, hMSH3, hMSH6 and the postmeiotic segregation genes (PMS) hPMS1 and hPMS2. The MMR proteins form a functional complex with each other to repair any errors on the newly duplicated DNA strand. The most affected genes are the hMLH1 and

hMSH2 which contribute to 95% of all the confirmed pathogenic mutations. Mutation of the hMSH6 was detected in 3-5%, the hPMS1 in around 1% of the cases.

Some authors reported on the hypermethylation of the promoter region of the hMLH1 gene as the basis for the development of the HNPCC phenotype. “Silencing” of the gene leads to the failure of gene transcription and the respective protein will not be expressed. In this case no sequence error can be detected on the gene. This epigenetic development of the HNPCC phenotype was only published in the case of hMLH1.

Aims

The primary aim of our study was to recognize, evaluate and follow our patients with HNPCC who have been screened according to the international criteria. Insufficiency of the Hungarian data inspired us to obtain reliable information on this disease with a population-wide screening program. We paid special attention to ensure that our results could be easily implemented and used in the everyday gastroenterological practice. To achieve all this, we laid down the following goals:

1. We tried to evaluate the percentage of the Amsterdam positive families among the newly diagnosed patients with colorectal cancer. No such data is available from Hungary to date.
2. We strived to assess the feasibility and differences of the international criteria systems when used on the Hungarian population. We examined whether using the Amsterdam criteria alone or their combination with the Bethesda criteria has any impact on the effectiveness of screening the mutation carrier patients.
3. Our main goal was to evaluate the genetic mutations in the Eastern-Hungarian colorectal cancer population and to compare our findings with international

databases. We tried to recognize any possible repetitive MMR gene mutations characteristic to this population, and also to detect any "hot spots" – regions with an unusually high number of mutations – within the genes.

4. Considering that researching the genetic background of this disease requires very expensive and time-consuming examinations, we sought a way to develop a cost-effective and easy-to-use method for the detection of MMR gene mutations responsible for the expression of the HNPCC phenotype, and also to implement this method under clinical circumstances at the University of Debrecen.
5. The clinical feasibility of our work is supported by the pedigree analysis of the mutation carrier families and regular follow-ups of the screened individuals with a proven mutation, thus preventing the manifestation of the malignant disease. Our experience and recent findings help us to improve the effectiveness of our screening and patient care methods.

Patients and Methods

Our present study includes 1127 patients with colorectal cancer primarily diagnosed and surgically treated at the 1st Department of Surgery (later Institute of Surgery), University of Debrecen between 1997 and 2006. From the abovementioned cohort only 809 patients could be actually involved in the data analysis. Unfortunately many patients did not or not adequately respond to our inquiry. Between 1997 and 2003 (1st investigation period) our examinations were performed in a retrospective, between 2004 and 2006 (2nd investigation period) in a prospective manner. We used our questionnaire based on the Revised Amsterdam and Bethesda criteria on all patients. Those patients in whom Familial Adenomatous Polyposis or any of its variants could not be safely ruled out – as determined from the family

history and the removed surgical specimen – were excluded from the study. Those cases where cancer developed on the basis of some kind of inflammatory bowel disease (Crohn's disease, ulcerative colitis) were also excluded. After completing the questionnaires the patients were categorized basically into three groups. The first group comprised of patients in whom colorectal cancer was thought to be of no hereditary origin (**Sporadic cancer group**). Patients in the other two groups were suspected to have HNPCC. The first of these groups was for those patients who clearly fulfilled the Amsterdam criteria based on their family history (**Amsterdam positive group**). The other group collected patients who did not meet the Amsterdam criteria - either because of their family history or the low number of relatives - but certain pieces of information (occurrence of synchronous or metachronous tumor, early onset, etc) otherwise suggested the presence of HNPCC in the patient and his/her family, and necessitated further investigation (**Amsterdam negative group**). After the classification we performed immunohistochemical examinations and the assessment of microsatellite instability on the removed surgical specimen from all patients with suspected HNPCC. In the case of the latter examination we compared the microsatellite instability results of the DNA obtained both from the caner tissue and the peripheral blood of the patient. These two procedures enabled us to narrow the group of patients in whom we tried to confirm the assumed MMR gene mutations responsible for the pathogenetic basis of disease using the most expensive and time-consuming investigation, DNA sequencing. When a pathogenic DNA alteration was found, the relatives of the patient were tested only for that particular mutation.

Immunohistochemistry

Routine 5 micron thick, formalin fixed, paraffin-embedded tissue sections were dewaxed, rehydrated, and treated in the microwave oven in 10 mM citrate buffer (pH 6.4) for 20 min. in order to restore antigenecity. Unspecific protein binding was blocked with 1 %

bovine serum albumin containing PBS for 30 min. at 37 ˚C, then slides were incubated overnight with the primary antibodies (mouse monoclonal anti-MSH2, clone 25D12, Labvision Corp., Fermont, CA, USA at 1:100 dilutions and mouse monoclonal anti-MLH1, clone G168-15, Becton-Dickinson Biosciences, USA, at 1:100 dilutions), respectively. Primary antibodies were detected by a biotin-streptavidine detection kit (LSAB, Dako, Carpinteria, CA, USA) using VIP chromogene. The slides were counterstained with methylgreen. Negative controls were stained with the omission of the primary antibodies.

Microsatellite analysis

The DNA of the cancerous tissues and the corresponding blood DNA were used for testing microsatellite instability (MSI). Two mononucleotide markers (BAT25 and BAT26) and three dinucleotide markers (D2S123, D5S346 and D17S250) were studied according to the international reference panel recommendations [6] by the HNPCC Microsatellite Instability Test (Roche Diagnostics GmbH, Mannheim, Germany). The MSI status was assessed according to the consensus of the National Cancer Institute workshop on Microsatellite Instability for Colorectal Cancer Detection. High level instability (MSI-H) was diagnosed when at least 30 % of the examined markers presented new alleles in the tumor tissue, low level instability (MSI-L) when less than 30 % of the markers carried instability.

Heteroduplex and Single Strand Conformation Polymorphism (SSCP) analysis

All exons of hMLH1 and hMSH2 genes were analyzed in the blood sample of the index patient. Primers and PCR conditions were used as it was published before [7,8]. After denaturation or heteroduplex formation the PCR products were loaded to electrophoresis on MDE gel (Cambrex Bio Science Rockland Inc., Rockland, MA, USA) by the manufacturers' instruction and visualized by silver staining.

Sequencing of hMSH2 and hMLH1 genes

PCR products showing altered migration patterns on MDE gel were purified and sequenced in both directions. Sequencing reactions were performed using BigDye thermocycler sequencing kit v.3.1 (Applied Biosystems, Foster City, CA, USA). The semi-automated fluorescence analysis was performed by the use of ABI-PRISM 310 Genetic Analyzer (Perkin Elmer, Boston, MA, USA).

Large deletion detection

Multiple Ligation dependent Probe Amplification (MLPA)

Genomic deletions were tested by the use of SALSA MLPA Kit P003 MLH/MSH2 (MRC-Holland b.v.) according to the manufacturer's instructions. The amplification products were analysed by capillary gel electrophoresis (ABI-3130). A deletion of one copy of a probe target sequence was stated if the relative peak area for that probe amplification product had been reduced by 35-55% compared to a negative control sample.

Results and Discussion

Results of population screening

From the 809 patients assessed during the whole investigation period 725 cases (89.6%) were considered to be undoubtedly sporadic colorectal cancer. We registered 84 families with suspected HNPCC (10.3%) in the same time period. From these subjects 49 families (6%) were classified as Amsterdam negative and 35 (4.3%) as Amsterdam positive. Between 1997 and 2003 our investigations were performed in a retrospective manner. The posted questionnaires were completed by the patients themselves without supervision from the investigating doctor. From this period we had to exclude a significant portion of the

questionnaires from the study (297 pieces), leaving only 464 cases to be analyzed: 437 patients (94%) had sporadic colorectal cancer and 27 families (5.8%) were considered suspicious to HNPCC, from which 13 (2.8%) were Amsterdam negative and 14 (3%) Amsterdam positive. Between 2004 and 2006 screening of the patients was carried out at the time of the ward admission by the investigating physician using the same questionnaire. In this investigation period only 21 patients had to be excluded from the study and 345 questionnaires were processed. 288 cases (83.4%) were clearly sporadic colorectal cancer with a late onset; 57 patients (16.5%) were suspicious of HNPCC with 36 families (10.4%) being Amsterdam negative and 21 families (6%) Amsterdam positive.

During the whole investigation period microsatellite instability and immunohistochemical assessments were performed in 64 patients. Based on the results of these examinations we planned to carry out DNA sequencing of 25 patients; however, we were actually able to perform DNA sequencing from peripheral blood in 19 cases. We found 10 supposedly pathogenic mutations and several known polymorphisms. From the 10 pathogenic mutations 8 was first identified and reported by our workgroup. Four confirmed mutation carrier families were discovered in the first (1997-2003), 6 in the second (2004-2006) investigation period. From the 10 confirmed mutation carrier families identified during the whole investigation period 7 were Amsterdam positive and 3 Amsterdam negative. The complete evaluation procedure was performed in 19 patients from the two periods. From this patient group 9 families (47%) were Amsterdam positive and 10 (53%) Amsterdam negative. Some kind of pathogenic mutation could be detected in 77% of the fully evaluated, Amsterdam positive families. This rate proved to be 30% in the Amsterdam negative group.

Results and discussion of the pedigree analysis

Family 1

The young male patient underwent surgery at the age of 31 due to cancer of the splenic flexure. Colorectal cancer was found in his mother at the age of 43, in his grandfather on the mother's side at the age of 56 and in his cousin at the age of 34.

Pedigree analysis back to 3 generations made it clear that this family fulfilled several points of both the Amsterdam and the Bethesda criteria. On the mother's side 4 colorectal and 1 breast cancer was found in the first-degree relatives including the index person. On the father's side 1 gastric and 1 lung cancer was registered. Using DNA sequencing 2 genetic disorders could be identified in the hMSH2. The first mutation was a Glu → STOP change (nonsense mutation) in the exon 7, codon 442. The second alteration proved to be an Asp → Ser change (missense mutation) in exon 3, codon 127. 7 people on the mother's side carried the nonsense mutation in exon 7, 4 of these suffered from colorectal cancer. Mutation in exon 3 was found in 2 siblings on the father's side and also on the mother's side due to merging with another family. In 3 family members mutations in the exon 3 and 7 occurred simultaneously. Unfortunately we could not investigate the family members with breast, lung and gastric cancers because they deceased before the start of the study.

The two genetic alterations found in the hMSH2 gene were compared with the most comprehensive international database available (The Human Gene Mutation Database, Cardiff. International Society for Gastrointestinal Hereditary Tumors). The nonsense mutation in exon 7 has not yet been published anywhere, thus it is considered a new detection. The mutation with the STOP codon formation is undoubtedly a pathogenic alteration leading to the expression of a truncated protein which is functionally defective. Since this protein breaks

down very early, it was undetectable even with immunohistochemical examination. This hypothesis is supported by the fact that the mutation could be verified in all 4 patients with colorectal cancer.

The missense mutation in exon 3 – a non-pathogenic polymorphism with one amino acid exchange - has been published by several authors. According to the "National Institute of Environmental Health Sciences Genome Project" database, the allele frequency of this polymorphism is 0.02 which means that prevalence of heterozygosis is 4% in the American population.

The single presence of the mutation in exon 7 led to the development of colorectal cancer in 2 patients at ages of 43 and 56 years. When both genetic faults (mutations in exon 3 and 7) could be detected in a patient, colorectal cancer was presented at a much younger age (31 and 34 years). We found no colorectal cancer among those patients who carried only the exon 3 mutation. Our results and relevant international data suggest that the exon 3 polymorphism cannot induce a malignant disease by itself; however, in the presence of a pathogenic mutation it can advance the development of malignancies by several years. Naturally, due to the low number of cases all this remains a hypothesis.

Family 1 is a "standard" HNPCC family. The excellent compliance of the family members not only helped our pedigree analysis, but also made regular follow-ups possible. As a consequence, we have no knowledge of any further malignancies in the family.

Family 2

The proband was diagnosed with the synchronous occurrence of rectal adenocarcinoma and mucinous appendiceal cancer at the age of 25 years; the patient underwent a subtotal colectomy at our institute. The patient's family does not meet the Amsterdam criteria. This has family has a relatively poor history with only one case of colorectal cancer apart from the

proband (grandfather, at the age of 77). The 28-year old brother was diagnosed with a tubulovillous adenoma of the sigmoid bowel, which was removed endoscopically. The proband also meets 3 Bethesda criteria: synchronous colorectal cancer, diagnosed before 45 years of age, having a first-degree relative with colonic adenomatous polyp discovered before 40 years of age. During sequencing we found a mutation in exon 19 codon 716 (Val → Met change) in the hMLH1 gene and another one in intron 13 at nucleotide 2210+1 in the hMSH2 (G → C change). Mutation of exon 19 in the hMLH1 gene was confirmed in 9 persons on the mother's side including the proband. The hMSH2 mutation was found in 7 siblings on the father's side including the proband. The proband and his brother carries both mutations.

Hutter et al. published the exon 19 mutation in hMLH1 in a HNPCC family which was deemed pathogenic. Cederquist et al. considered this alteration only a rare variant (polymorphism). In the family we investigated the abovementioned mutation was found in 9 siblings on the mother's side including the proband. The amino acid change in the expressed hMLH1 protein is not conservative and is abnormal in at least 1% also in the control population. Based on these results, we are on the opinion that this alteration is a non-pathogenic, rare polymorphism.

The other mutation (hMSH2, c2210+1 G→C) has already been published by Kurzawski et al. in a Polish HNPCC family and was considered absolutely pathogenic. This alteration is a frameshift mutation on the exon-intron border. We identified this mutation in 5 siblings on the father's side including the proband. The grandfather who was diagnosed with colorectal cancer at the age of 77 years does not carry this mutation, thus his disease must be classified as sporadic tumor. The 28-year old brother of the index person carries both mutations. Colonoscopy revealed an adenomatous polyp on the sigmoid bowel which was removed endoscopically. We think that this genetic alteration is the pathogenic one, and it is also possible that the combined occurrence of the two mutations leads to a very early onset of

colorectal cancer. Although all family members were accurately informed on the nature of HNPCC, we could not manage to enroll them into a follow-up schedule. Three years after the proband was diagnosed and operated with HNPCC associated cancer, his father – who did not attend follow-ups at all – presented at our institute with mucinous adenocarcinoma of the ascending colon. It must be noted also that a simultaneous prostate cancer was also diagnosed in the same patient who was 52 years old at this time. Meanwhile, the brother of the proband's father (54) was treated with advanced rectal cancer at another institute, requiring the formation of a permanent ostomy. With regular follow-ups we could have diagnosed the malignancies at an early stage in both of these patients. Both carry the hMSH2 mutation identified in this family. This family is characteristic of the "HNPCC suspect family with poor family history" type.

Family 3

Our index person was diagnosed with endometrial cancer at the age 44 and adenocarcinoma of the descending colon at the age of 56. Her mother died at the age of 48 from endometrial, her father at the age of 69 from gastric cancer. Due to the small number of siblings, this family did not meet the Amsterdam criteria. Our noticed, however, a family member with the metachronous occurrence of colonic cancer and another HNPCC-related tumor at a relatively young age. With sequencing we found a pathogenic mutation (exon 13, codon 711 in the hMSH2 gene, Arg→STOP) which was already registered in the international databases, first reported by Kurzawski from a Polish Amsterdam positive HNPCC family. Kim et al. also identified this mutation in Korean families with hereditary gastric malignancies. He came to an interesting conclusion: opposed to the common experience that endometrial is the second most frequent malignancy after colorectal cancer in HNPCC, in the Korean HNPCC families gastric tumors had the second highest incidence.

With the targeted investigation of 7 more family members we could detect the abovementioned mutation in 3 persons who are healthy at the present and attend regular follow-ups. This family is the typical "small HNPCC family that does not meet the Amsterdam criteria".

Family 4

The proband suffered from adenocarcinoma of the hepatic flexure at the age of 49 years. Two sisters died in endometrium cancer at ages of 23 and 36 years. His mother was diagnosed with endometrium cancer at 49 years of age, his father with rectal cancer at 71. No more malignancies were found on the father's side but 3 instances of urinary tract, endometrial and colorectal tumors on the mother's side. Using MLPA technique we identified a long segment deletion in the hMLH1 gene, which mutation was not found in the international databases. Starting point of the mutation is at exon 31 codon 301 and endpoint is in the intron 11, leading to the loss of 1786 base-pairs (g.28756-g.305429). This large deletion (frameshift mutation) causes the malfunction of a domain responsible for the heterodimer formation with MutS homologues (hPMS2, hPMS1).

The 4th family is a real "cancer family" where – interestingly - not colorectal tumors dominate. Many HNPCC-associated malignancies are found in the family which undoubtedly fulfills the Revised Amsterdam criteria. Family members live very far from each other by now, making screening and follow-ups technically impossible.

Family 5

The 57-year old male patient was operated with the synchronous occurrence of a mucinous appendiceal adenocarcinoma and tumor of the head of the pancreas. The patient mentioned the previous removal of a malignant skin lesion from the thoracic region. Physical

examination revealed a protruding lesion on the neck of the patient; histology confirmed a "sebaceous" cancer. Pedigree analysis yielded two colon cancer (proband's mother, at age of 49 and daughter, at the age of 25), two gastric cancer (proband's father, at the age of 54) and a metachronous endometrial tumor (proband's sister, at the age of 54 years). A malignant skin lesion was also removed from the mother of the proband before the appearance of the colonic cancer, but at an undetermined age.

The Muir-Torre syndrome (MTS) is an autosomal dominant hereditary disease characterized by the combination of sebaceous skin lesions and gastrointestinal malignancies. The disease is considered a HNPCC subtype. It must be noted that the benign and malignant skin lesions in 60% precede the gastrointestinal tumors, which, on the other hand, have a significantly deeper impact on the survival of the individual. As a result, these skin lesions could serve as early markers for other HNPCC-associated malignancies.

DNA sequencing in this family revealed a G→C change (missense mutation) at exon 10 codon 794 in the hMLH1. This otherwise conservatively localized mutation - leading to the production of a proline instead of an arginine - has not been published so far, and no references was found in the international databases.

Connection of the gMLH1 and hPMS2 results a heterodimer (MutLα) which plays a key role in the repair of spontaneous DNA errors during replication. The MutLα interacts with the heterodimer MutSα - which is able to detect DNA errors - in an ATP-dependent manner and the newly formed complex can actually repair the errors. The 265. Arg is located in the MutS homologue–associated region of HMLH1 which is an evolutionally conserved region. The arginine is a basic amino acid with a polar side chain which makes it hydropyllic. Proline, on the other hand, is cyclic, aliphatic with an apolar side-chain which determines the secondary structure of the protein.

This genetic alteration could be confirmed in 5 family members. An interesting feature of this proband is the simultaneous occurrence of the colorectal and pancreas cancer as part of the Muir-Torre syndrome. This rare tumor combination – eventually leading to the death of the patient – could have been operated at a much earlier stage, provided the hereditary nature of the disease was discovered sooner. A thorough family history taking when the skin lesion was originally removed, subsequent genetic testing and a regular follow-up schedule could have altered the course of disease. Such a synchronous tumor occurrence has not yet been published in the literature in Muir-Torre syndrome.

Family 6

The 39-year old female patient underwent rectosigmoid resection at our institute due to an obstructing tumor on the sigmoid bowel. Her father suffered from colon cancer at the age of 53 and rectal cancer at the age of 68. The grandmother on the father's side was died of endometrial cancer at 43 years of age. On the mother's side 2 cases of central nervous system tumor (grandmother on the mother's side at 64 and her mother's sister at 70 years of age), 1 lung (her mother's sister at 62 years of age) and 1 endometrial cancer (her mother's other sister at 66 years of age) was reported.

We confirmed a long segment deletion in the hMLH1. Of the 4 patients we could investigate from the family members, the proband and her father also carries this mutation. The proband's mother and son carries the normal allele.

This deletion involves the whole length of the exon 11 (3800 base-pairs) in the hMLH1 gene. No such mutation has been reported in the literature.

Interestingly, from the various malignancies discovered in the family, tumors of the central nervous system and lungs are generally not considered HNPCC associated. This knowledge is supported by the fact that we could not detect any MMR gene mutations on the mother's side.

On the father's side, where the mutation could be identified, typical HNPCC-related cancers did occur. The index person herself, as the mutation carrier requires gynecological and gastrointestinal follow-ups to prevent the development of a metachronous malignancy. Her father, also a carrier, would have benefited from regular follow-ups as well. This family is a nice example of the "HNPCC suspect family with a misleading tumor profile", since the investigators may fail to recognize the few HNPCC-associated malignancies among the large number of non HNPCC-associated tumors.

Family 7

The proband has undergone rectosigmoid resection at our institute due to an obstructing mucinous adenocarcinoma (18-20 cm from the anal verge) at the age of 72. Pedigree analysis revealed 2 more colorectal cancers (proband's mother at the age of 90 and his brother at the age of 67), one central nervous system tumor (daughter of the proband's sister at 16 years of age) and one endometrial cancer (daughter of the proband's other sister, 19 years old) among the first-degree relatives. The colorectal malignancies occurred at relatively old ages, which is not characteristic to HNPCC. Our interest was focused on the endometrial cancer developed in the very young patient. Interestingly, the mother of this patent is completely healthy, thus the phenotype manifestation skipped one generation.

During DNA sequencing we identified two missense mutations in the hMLH1 gene (exon 14: L555V, exon 19: P747H) which were not previously registered in the international databases. The exon 14 mutation was found in 6, the exon 19 mutation in 7 family members.

We used three different pieces of software to evaluate the effect of exchanging these two amino acids. These software packages described the exon 19 mutation as "tolerable amino acid change" based on sequence homology and the physical properties of the amino acids. The exon 14 mutation was considered "tolerable amino acid change" by one and "probably

pathogenic" by the two other software package. The exon 19 mutation is located on the C-terminal end of the hMLH1 gene; we could identify it in neither of the cancer patients except the proband. We think this genetic alteration is probably a non-pathogenic polymorphism only. The exon 14 mutation is located in the hPMS3/hMLH3/hPMS1 "interaction" domain. Apart from the index person, this alteration was confirmed in the young female relative with cervix cancer and in both sons of the 67-year old patient with tumor of the transverse colon. This means that the proband is also a carrier. Based on these findings we consider the exon 14 mutation as probably pathogenic; however, the penetration of the mutation is questionable since it was also detected in 5 completely healthy relatives. Carriers of the exon 14 mutation were enrolled into our follow-up program and they seem to be free of any further malignant diseases ever since.

Family 8

The proband is a 47-year old male patient with mucinous adenocarcinoma of the transverse colon. His mother suffered from gastric cancer at the age of 46 and died of Klatskin tumor (biliary tract carcinoma) at the age of 59. The daughter of his mother died of an advanced colon cancer with hepatic metastases at the age of 41. The brother of his father suffered from bronchial cancer at the age of 68. The proband's sister was diagnosed and operated with synchronous colonic and sigmoid cancers at the age of 38, and underwent Wertheim's operation at the age of 52 due to an ovarian tumor. One of the proband's children required conization at the age of 21 for an early stage ovarian cancer. Based on the age distribution of the malignancies, the family easily fulfills both the Amsterdam and Bethesda criteria and can be classified as a "standard HNPCC family".

With DNA sequencing we found an A→C change (missense mutation) in the exon 2 codon 143 in hMLH2 (p.Q48). This genetic alteration has not been registered in the international

databases so far. The mutation is located in the ATP-ase domain of the hMLH1 gene. Pathogenicity is hard to assess, since the family refused to take part in the pedigree analysis for religious reasons. One of the abovementioned three different software packages indicated the mutation as “absolutely pathogenic”, the other two as “probably pathogenic”.

Family 9

The 44-year old female patient was operated with a mucinous adenocarcinoma of the large bowel; a left-sided hemicolectomy was performed at another institute. She was referred to our ward with a recurrent, advanced malignant process which required a Hartmann’s procedure alongside with excision of the tumor from the abdominal wall. Following the oncologic treatment her tumor marker levels improved significantly. One year later the temporary ostomy was reconstructed and no signs of recurrence were found at that time. Detailed family history revealed 4 more colorectal cancers with a relatively early onset, 1-1 skin and lymphoproliferative malignancy, and 1 malignant liver disease with undetermined origin. All the colorectal cancers developed on the mother’s side at ages of 31, 43, 50, 52 and 55.

During sequencing we found a small deletion of exon 6 in the hMSH2 affecting two nucleotides, which has not been previously published elsewhere (delTC c. 969-970). Another 6 family members were tested and in 4 of them the abovementioned mutation could be identified. From the 5 person carrying the mutation 4 suffered from colorectal cancer.

This “standard HNPCC family” undoubtedly meets the Amsterdam criteria and is a fine example that HNPCC-related colorectal malignancies generally respond well to 5FU-based chemotherapy and have a much better prognosis than the sporadic cases. Owing to the excellent compliance of the family, regular follow-ups showed no further occurrence of malignant diseases.

The small deletion which we identified first in exon 6 of the hMSH2 involving 2 nucleotides is located in the "DNA binding domain", leading to a frameshift mutation, and as such, it can be considered clearly pathogenic.

Family 10

The 59-year old proband was diagnosed with a constricting tumor on the sigmoid bowel; in his case a left-sided hemicolectomy was performed. His father suffered from an advanced colorectal cancer (transverse colon) at the age of 62. The brother of the proband's father was medically treated with rectal cancer at the age of 46 and he is free of disease ever since. The proband's sister was diagnosed with breast cancer at the age of 35. The proband has three daughters; one of them underwent conization due to an early-stage cervix tumor and one had a tubulovillous adenoma removed endoscopically from the descending colon. The early death of the proband's grandmother on the father's side (at the age of 37) was most probably caused by an advanced gastric malignancy but the exact details are not known. The proband's grandfather on the mother's side died of bronchial cancer but at a relatively old age.

With DNA sequencing we found a Glu→Asp change in exon 9 in the hMSH2 (missense mutation, c.1392 A>T).

This family clearly meets the Amsterdam criteria and also one Bethesda criterium (colorectal cancer in first-degree relatives under the age of 50, adenomatous polyp in the large bowel under the age of 40). The genetic alteration was identified in 5 family members including the proband; from these patients 2 suffered from colorectal cancer (at the ages of 46 and 59), one from breast cancer (at the age of 37) and one had a tubulovillous adenoma removed from her descending colon at the age of 29. In the sister of the proband's father – who was diagnosed with rectal cancer at a young age - we isolated the mutation, suggesting that the genetic transformation is inherited on the father's side.

This mutation has not been previously registered in the international databases. The Glu→Asp missense mutation involves the "interaction domain" of the hMSH2 gene, which is considered a conservative region. The family is a "standard HNPCC family". Unfortunately, due to social problems and geographical diversity we could not draw all the mutation carriers into the follow-up program.

Discussion of the results of the population screening

In the first 7-year period we could include fewer patients into our study than the length of the period would suggest, not only because many of them were dead or unreachable, but a large number of the received questionnaires had to be rejected due to misinterpretation or unreliable data. It must be noted the percentage of HNPCC suspect families (Amsterdam positive and negative) were lower in the first investigation period. In the second period, when all patients completed the questionnaires upon admission with the help of the investigation physician, our results improved significantly. From the shorter second investigation period we could enroll more patients into the study, and the percentage of the HNPCC suspect families also increased notably. Because of the great variety in the appearance of HNPCC and the often misleading replies from the patients, only a family history taken in the presence of an investigation physician can be judged reliable. Based on these considerations we think that the whole investigation period cannot be accepted as a representative sample for population screening. Results from the first period suggested that the prevalence of HNPCC in our region was much lower than it was in reality. The second investigation period much better represents the real-life percentage of HNPCC but the number of patients was low. Further population-wide investigation is needed for the proper statistical analysis of our data.

In the course of our study we identified 10 patients carrying a pathogenic mutation from the 809 screened patients with colorectal cancer, which amounts to an incidence rate of 1.2%.

When compared to the international data – other publications generally cite 3-5% incidence rates –, our results most probably under-represent the real-life occurrence of HNPCC. The difference may come from the fact that we were unable to test a significant portion of our Amsterdam positive and negative patients, and also, sequencing of hMSH6 was not available in our genetic laboratory. Moreover, we classified all mutations without confirmed pathogenic properties as polymorphisms. Previous literature references generally consider all families meeting the Amsterdam criteria as HNPCC families even if no pathogenic mutations were found. By following this approach we could have reached completely different results. In the more representative second investigation period (2004-2006) the proportion of the undoubtedly Amsterdam positive families was 6%,

As regards the whole investigation period, we performed DNA sequencing in 19 patients and from the 9 Amsterdam positive patients we identified a mutation in 7 cases (77%)! From the 10 Amsterdam negative patients genetic mutation was found in 3 (30%). Apparently, we could have missed a significant number of the mutation carrier families (family 2, 3 and 7) by applying only the strict Amsterdam criteria. Indeed, in those families perfectly meeting the Amsterdam criteria the proportion of the confirmed pathogenic mutations was higher, but we still think that genetic screening should be carried out in a more thorough manner, involving all the criteria systems.

The mutations we detected are scattered over the exons of the hMLH1 and hMSH2 genes. We found no repetitive mutations, neither sequences with accumulated mutations.

Conclusion, novel scientific contributions

1. We can consider only the 2nd investigation period as a representative sample. Knowing that patients from all over the Eastern-Hungarian region are often referred

to the Institute of Surgery of the University of Debrecen, the population we investigated can be judged as characteristic of the Eastern-Hungarian general population. In this period 6% of our patients perfectly met the Amsterdam criteria and a further 10.4% were classified as HNPCC suspect for some reason. These data make it clear that a significant portion of the colorectal cancer cases in Eastern-Hungary are of hereditary origin and the screening and care of these patients is far from being solved. No such results are published so far from Hungary and our results confirm that establishing a screening and follow-up program for these patients must be considered a significant preventive oncologic measure.

2. Regarding the application of the international criteria systems we can say that even if the Amsterdam criteria are by far the easiest to implement lead to the detection of the most mutation carrier families, their exclusive use may result in a 30% of missed families. For this reason, application of both criteria systems is advised to detect any possible HNPCC suspect families.

3. Regarding the fact that the combined use of the criteria systems increases the number of the HNPCC suspect patients and also that the most expensive and time-consuming investigation is the DNA sequencing, it must be preceded by other examinations which increase the efficacy of mutation searching. We developed a testing protocol in which this theory can be easily implemented: when taking the family history, an EDTA blood sample is obtained and frozen, as is later the fresh tumor tissue sample. From these samples the microsatellite and immunohistochemical investigation can be routinely performed. As a result, only those patients get to DNA sequencing in whom the possibility of HNPCC is already confirmed. All these measures lead to a significant degree of cost-saving. Should the patient die in the meantime, the examinations can be completed from the blood

sample taken at the first meeting, thus notification and screening of the family members will nevertheless be possible.

4. During the course of our research we found 10 mutations and several already known or unknown polymorphisms. Comparison of the identified pathogenic mutations with the international databases made it clear that our workgroup was the first reporter for 8 of these mutations. We found no repetitive mutations (e.g. characteristic to the investigated population). The mutations we identified are scattered in various exons of the affected MMR genes. This finding supports the hypothesis that there are no predilection spots on the MMR genes where mutations would accumulate. We are convinced that by continuing our research and by increasing the patient cohort we can get more accurate data on the Hungarian incidence rates of HNPCC and the various mutations involved.

5. The clinical value of our study is underlined by the example of family 1, 3, 5, 6, 7 and 9: in these families no new cancers developed after the follow-up schedule was implemented. On the contrary, we detected and treated several premalignant lesions (colonic adenomatous polyps, early skin tumors, etc.). We must emphasize that by identifying the carrier and non-carrier members of the screened families, we can decrease the mental burden of cancer risk in those who are not considered high-risk patients. Unfortunately, family 2 can be an example for the fate of the uncontrolled HNPCC families. These cases usually remain hidden because the different family members are treated at different hospitals. As a conclusion, identification of the HNPCC suspect families, their subsequent genetic testing and the establishment of a follow-up program in our patient cohort is necessary for the prevention of the newly developed malignancies.

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5. Damjanovich L, **Tanyi M**: A kóros elhízás sebészeti kezelése. Gastro Update (2007)
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