

Original Research

Sanger sequencing of MMR genes in a one-plate system

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Abstract

Both incidence and mortality of colorectal cancer (CRC) in Romania have shown a continuous increase during the last decades. Hereditary Non-Polyposic Colorectal Cancer (HNPCC), also known as Lynch syndrome, is mainly attributable to mismatch repair (MMR) genes MSH2, MSH6, and MLH1. Individuals carrying germ-line mutations of these genes present high lifetime risk of colorectal and other cancers, compared to non-carriers. Oncogenetics is developed worldwide nowadays, for identifying hereditary predisposition to cancer and offering appropriate clinical follow-up to patients and mutation carriers in Lynch families. Molecular oncogenetic diagnosis in Lynch syndrome is based on complete Sanger sequencing of entire MMR genes, which is time and resources consuming, therefore needing an appropriate and adapted optimization. Conventional sequencing requires a sufficient number of available samples to be processed simultaneously, which increases the waiting time for diagnostic results. Complete analysis for only one patient meets difficult technical problems due to the complex co-amplification of all gene regions of interest within the same conditions, therefore increasing the costs and reducing the cost-effectiveness of the test. Here we present an original and robust technical protocol for sequencing the entire MSH2, MSH6, and MLH1 coding sequence for one patient in a single PCR plate. Our optimized and verified system overcomes all technical problems and offers a quick, robust, and cost-effective possibility to personalize molecular oncogenetic diagnosis in Lynch syndrome.

Keywords: Lynch syndrome; MMR genes; Sanger sequencing; molecular diagnostic; cost-effectiveness.

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Introduction

Colorectal cancer (CRC) is the third most common cancer and cause of cancer death worldwide (1-3). Lately, an alarming CRC incidence increase (75% fold) was reported for Romania (4), one of the few countries worldwide where increases in mortality rates by CRC are still occurring (2,9% per year). Up to 30% of CRCs have evidence of a familial component (5) and about 5% are thought to be due to inherited mutations in known genes (2), the rest being due to low-penetrance alleles (6). Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is characterized by early-onset CCR occurring in many members of the same family line, increased frequency of multiplicity for CRC, and is mainly attributable to germline mutations in the mismatch repair (MMR) genes *MSH2* (OMIM

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609309, 2p21), *MLH1* (OMIM 120436, 3p22.2) and *MSH6* (OMIM 600678, 2p16.3), and to a lesser extent to PMS2 or EPCAM genes. The more appropriate name of Lynch syndrome (LS) – named after Dr. Henry Lynch (7,8) – has been proposed (9,10) because the syndrome also involves predisposition to other sites. MMR genes cause LS with a penetrance of approximately 80% for CRC, 60% for endometrial cancer, and well below 20% for the other cancers (stomach, ovaries, small bowel, hepatobiliary epithelium, urogenital epithelium, and brain) (11). The overall lifetime risk of CRC for patients with LS is estimated to be 80% (12), while only 5.5% in the general population (13).

MSH2 mutations are responsible for about 35% of LS families, while MLH1 and MSH6 contribute to 25 and 15% respectively (11). Mutations in MLH1 and MSH2 are located in all regions of these genes, without obvious hot spots (14). Mutation-detection strategies must therefore cover the entirety of these genes. The identification of a germline mutation is the gold standard for the diagnosis of Lynch syndrome (15) and is performed by complete Sanger sequencing of MSH2, MLH1, and MSH6 genes (16), exons and exon-intron boundaries. This is a huge work to do, as the genes are very large (MLH1-19 exons on 100 kb genomic DNA, MSH2-16 exons on 73 kb, MSH6-10 exons on 24 kb) (17). Patients with LS are included in molecular testing according to inclusion criteria such as Amsterdam I and II (18), or Bethesda (19). However, MMR mutations are detected in only 60% of Amsterdam criteria fulfilling families, while up to 20% of families not fulfilling these criteria carry deleterious mutations and are therefore excluded from genetic counselling (20).

The main problems associated with molecular oncogenetic diagnosis are raised by elevated costs of human and material resources involved for entire gene sequencing and interpretation, as well as by the long period of time the patients have to wait for the result (21). This period of time (6-8 months for LS diagnostic) is due on the one hand to the laborious interpretation step of the many sequence variants identified by sequencing (22), and, on the other hand, to the limited number of samples available for analysis at one time (23). Also, recurrent MMR mutations, detectable by simple procedures and facilitating diagnostic approaches (24,25), have rarely been observed worldwide for MMR genes. While classical Sanger sequencing is performed in 96-well plated and would demand 95 available samples for maximum efficiency, performing the entire sequencing on one only sample is a challenging work, due to the difficulty of co-amplifying a great number of gene regions within the same PCR conditions. Such a system would, once implemented, allows a rapid MMR screening for a limited number of patients (down to 1), with reasonable cost-effectiveness, and especially in a very short period of time. We present the development of our optimized protocol, with its 2 variants adapted to 1 or 2 patients simultaneously.

Patients and methods

Patients

We identified and recruited HNPCC families at Sf. Spiridon University Emergency Hospital and the Oncology Institute of Iaşi, Romania. Family inclusion on Lynch syndrome was based on Amsterdam II criteria (18), i.e. at least 3 CRC or Lynch-related cancers in the same family line affecting at least 2 generations, with at least one case diagnosed before age 40. All patients agreed by written informed consent. Personal and family cancer histories were obtained from patients and participating relatives. This study was approved by the local Ethical Committee, UMF Iasi.

Molecular analysis

We performed genomic DNA extraction using the WizardTM Genomic DNA purification

kit (Promega[™] Inc, Madison, WI, USA). Spectrophotometric evaluation of DNA quantity and purity was used.

Sanger dideoxy sequencing was performed on 56 amplicons covering the whole coding sequence of MSH2, MSH6, and MLH1, including junctions with introns. Primers were designed using Primer ExpressTM Software v3.0.1 (Life technologies) and Primer3 web version 4.1.0 (26). PCR was performed in 20 µl reaction, containing one unit ApliTaq® Polymerase with appropriate Buffer (Applied Biosystems[™] Inc, Foster City, CA, USA), 0.4 mM each dNTP, 0.4 µM of each primer, 100 ng genomic DNA. We generally performed PCR reaction on a GeneAmp® Dual PCR System (Applied BiosystemsTM Inc, Foster City, CA, USA) using a common PCR program for all amplicons (94°C/5min, 35 cycles of 94°C/20 sec $-54^{\circ}C/20 - 72^{\circ}C/30$ sec, 7 min/72°C).

After gel electrophoresis evaluation, amplicons were purified by ExoSAP-ITTM (Applied Biosystems), following the manufacturer's instructions. Amplicons ware sequenced both in forward and reverse reactions using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems). Sequencing reaction was performed on a 96-Well GeneAmp[®] PCR System 9700 (Applied Biosystems), 94°C/1 min, 25 cycles of $94^{\circ}C/10 \text{ sec} - 52^{\circ}C/5 \text{sec} - 60^{\circ}C/3 \text{min}$. Sequence products were purified using BigDye XTerminator[™] Purification Kit (Applied Biosystems), and migrated by capillary electrophoresis on a Life Technologies 3500 Series Genetic Analyzer (Applied Biosystems). We performed analysis of raw data using Segman[®] (DNA StarTM Inc, Madison, WI, USA) and Variant Reporter[™] Software v2.0 (ThermoScientific). Mutations were systematically confirmed on an independent different DNA sample.

Data interpretation and analysis

All mutations and sequence variants are described according to the recommendations from the Human Genome Variation Society (HGVS), with first nucleotide of DNA numbering being the A from initiator translated ATG (27). We used reference sequences NM_000251.2 (coding), NG_007110.2 (genomic) and NP_000242.1 (proteic) for *MSH2*, NM_000179.2 (coding), NG_007111.1 (genomic) and NP_000170.1 (proteic) for *MSH6*, NM_000249.3 (coding), NG_007109.2 (genomic) and NP_000240.1 (proteic) for *MLH1* gene respectively. For bioinformatic prediction of variants, we used Alamut[®] (Interactive SoftwareTM) (28).

Results

Although the whole genomic region covered together by MMR genes represents almost 200 kb DNA, the coding region represents overall 9166 nucleotides to sequence. However, sequencing for diagnosis should equally cover exon/intron boundaries, as well as promoter and other regulating regions situated 5' or 3' outside the exons, as mutations in regulatory regions may be deleterious and affect the overall final protein (22). We calculated 18000 nucleotides to decrypt for a molecular diagnostic test to be validated for MMR genes in Lynch syndrome. All the regions of interest need to be double-strand sequenced and lectured, while the reading sequences need to be accurate enough to avoid any false positive or false negative ones possibly interfering with the final diagnosis.

We defined amplicons covering the coding regions of each exon, as well as exon/intron boundaries. The final coverage area is represented by 16 exons in *MSH2* gene (about 5,7 kb), 10 exons in *MSH6* gene (about 6,5 kb), and 19 exons in *MLH1* gene (about 5,4 kb). This total of 45 exons does not exactly correspond to the actual number of amplicons. The length of concerned exons varies between 200 and 600 bp, the efficiency of the PCR and subsequent sequencing reactions being optimum between 250 and

400 bp. While the majority of exons can generate amplicons within this range, there still are a few examples that need particular adaptation. Exon 4 of MSH6 is exceptionally large (2543 bp), several contiguous amplification fragments being necessary for the entire coding region to be covered. We calculated a total of 8 contiguous amplicons (named 4-1, 4-2, etc.), ranging between 370-580 bp and representing together over 3,6 kb sequence. Exon 12 of MLH1 (371 bp) is particularly complex to amplify and requires 2 contiguous amplicons measuring together 579 bp. Exons 8, 9 and 10 of MSH6 are particularly close to each other on genomic DNA, which hardens primer definition within the region, and brings the necessity of covering the sequence with 4 different amplicons. Nonetheless, some exons prove to be difficult to amplify or to sequence on both strands, due to repetitive regions causing enzyme slippage. In order to ensure an accurate lecture on the entire exon region, we imagined for those cases at least 2 independent amplicon variants.

Developing a one-plate PCR system for each patient

According to the description above, an overall amount of 56 amplicons is needed for covering the entire MMR sequence of interest (Figure 1C). As each amplicon has theoretically different PCR amplification conditions, efficiency and limitations, the classical and most efficient system consists of amplifying each region independently, for a given number of available DNA samples, with a maximum efficiency for 95 samples (in this case, an entire plate of 96 wells, including a no template control, would be used for each amplicon - Figure 1A). Amplifying each exon separately allows using different annealing temperatures, PCR program, and even different amounts of template DNA or primers for each reaction, which is beneficial for PCR and sequencing accuracy, and for overall cost-effectiveness.

Unfortunately, 95 samples are rarely available in molecular oncogenetic diagnosis for common laboratories covering limited regions or populations. Also, the period of time necessary for 95 samples to be available considerably delays the duration of each diagnosis. For those reasons, an intermediate amplification system can be imagined, with two or more exons amplifiable in one same plate (Figure 1B) for an adequate number of samples (i.e. 47 samples for 2 amplicons, 31 samples for 3 amplicons, or 23 samples for 4 amplicons in the same plate). However, the choice of the amplicons to be co-amplified in the same plate should take into account the different amplification and sequencing conditions.

As such, an extreme challenge is represented by the co-amplification of all MMR exons in a single plate when only one DNA sample is available (Figure 1C). Of course, such a system would answer the question of duration, but would raise problems linked to efficiency, cost-effectiveness, or even accuracy of the final result. In such a system, all exons are co-amplified in a single PCR reaction, which means the amplification conditions for each amplicon should be similar. We defined PCR primers for the amplification of each region of interest, taking into account the particularity of each amplicon in terms of repetitive or GC-rich regions, but we also tried to design primers with appropriate Tms, in order to ensure a common annealing temperature of 54°C for each amplicon, as well as the possibility of being amplified in a common 35-step PCR reaction. Obviously, these requirements are almost impossible to meet entirely for all 56 amplicons, but for each amplicon we tried to be close enough to the common reaction conditions. Since the same PCR primers were also used for forward and reverse sequencing, we also took into account the correspondence of each primer to a common sequencing reaction.

After multiple steps of optimization regarding primer sequences and relative concentra-

Н	G	F	Е	D	С	B	Α	
S8	S7	S6	S5	S4	S3	S2	S1	1
E1								
S16	S15	S14	S13	S12	S11	S10	S9	2
E1								
S24	S23	S22	S21	S20	S19	S18	S17	3
E1								
S32	S31	S30	S29	S28	S27	S26	S25	4
E1								
S40	S39	S38	S37	S36	S35	S34	S33	5
E1								
S48	S47	S46	S45	S44	S43	S42	S41	6
E1								
S56	S55	S54	S53	S52	S51	S50	S49	7
E1								
S64	S63	S62	S61	S60	S59	S58	S57	8
E1								
S72	S71	S70	S69	S68	S67	S66	S65	9
E1								
S80	S79	S78	S77	S76	S75	S74	S73	10
E1								
S88	887	S86	S85	S84	S83	S82	S81	11
E1								
NTC	S95	S94	S93	S92	S91	S90	S89	12
E1								

Figure 1A. Amplification of 95 DNA samples for a single PCR amplicon (S=sample, E=amplified exon, NTC=no template control)

Α	B	С	D	E	F	G	Η
S1	S2	S3	S4	S5	S6	S7	S8
E1							
S9	S10	S11	S12	S13	S14	S15	S16
E1							
S17	S18	S19	S20	S21	S22	S23	S24
E1							
S25	S26	S27	S28	S29	S30	S31	NTC
E1							
S33	S34	S35	S36	S37	S38	S39	S40
E2							
S41	S42	S43	S44	S45	S46	S47	S48
E2							
S49	S50	S51	S52	S53	S54	S55	S56
E2							
S57	S58	S59	S60	S61	S62	S63	NTC
E2							
S65	S66	S67	S68	S69	S70	S71	S72
E3							
S73	S74	S75	S76	S77	S78	S79	S80
E3							
S81	S82	S83	S84	S85	S86	S87	S88
E3							
S89	S90	S91	S92	S93	S94	S95	NTC
E3							

Figure 1B. Example of amplification of 31 DNA samples for three PCR amplicons (S=sample, E=amplified exon, NTC=no template control)

	Α	B	С	D	Е	F	G	Н
1	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2
	el	e2	e3	e4	e5	e6	e7	e8
2	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2
-	e9	e10	e11	e12	e13	e14	e15	e16
3	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6
Ŭ	e1	e2	e3	e4-1	e4-2	e4-3	e4-4	e4-5
4	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6
	e4-6	e4-7	e4-8	e5	e6	e7-var1	e7-var2	e8-var1
5	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1
Ŭ	e1	e2	e3	e4	e5	e6	e7	e8
6	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1
ľ	e9	e10	e11	e12-1	e12-2	e13	e14	e15
7	S1-M1	S1-M1	S1-M1	S1-M1	S1-M6	S1-M6	S1-M6	S1-M6
· ·	e16	e17	e18	e19	e8-var2	e9-var1	e9-var2	e10
8								
9								
10								
11								
12								

Figure 1C. Amplification of one only DNA samples for all PCR amplicons (S=sample, E=amplified exon)



Figure 2A (up). Different attempts to optimize co-amplification of all 56 exons (in this figure, for 2 patients). Figure 2B (down). Efficient co-amplification of all 56 exons (in this figure, for 1 patient)

tions, we obtained an acceptable result presented in figure 2B. A comparison between figures 2A and 2B demonstrates the importance of template DNA quality on the overall amplification efficiency.

In figure 2B one can observe most of the exons being correctly and specifically co-amplified in one reaction, in the presence of a single patient DNA template. We can observe lack of amplification for exon M2-e1 (difficult amplification proven in several different reactions) or for exon M1-e17 (accidental random loss, not reproducible). Also, exons like M6-e1 (special conditions) or M2-e16 (accidental) present additional non-specific bands, while exons M1-e6, M1-e7 or M1-e12 show poor amplification with less intense PCR bands. Some of these disadvantages disappeared because they were compensated for in efficient sequencing reactions, while some others directly influenced the accuracy of the final sequence lectured. After testing at least 10 DNA samples in this system, we concluded to the problems commonly appearing in every reaction. Here are some of our main conclusions:

Me-e1 is a very particular exon and its amplification demands a separate touch-down PCR reaction. However, we observed its possible amplification in our common conditions in 20% of the reactions.

Exons M2-e5, M2-e2, M1-e1, M1-e4, M1-e9 can only be sequenced on one strand (either forward or reverse), due to complex repetitive regions 5' or 3' of the exon

Exons M6-e1, M6-e7, M6-e8, M1-e5 are the most common to be missed in efficient amplification. However, each one of them appears correctly amplified in more than 50% of the samples. Using several different amplicon variants for those exons generally overcomes the problem.

Up to 5 exons (<10% of the total) are randomly missed from amplification due to general random pipetting or other laboratory environment errors. The missed exons are different for each sample.

The general efficiency widely varies with the quality and quantity of starting DNA. Extracted DNA to be used should always be double-measured and correctly diluted before being used for PCR reactions.

We can conclude that with our system, only 5-10% of the amplicons should be re-amplified in complement reactions, which is comparable with the amount of complements needed in all laboratory sequencing reactions.

Developing a one-plate PCR system for two patients

As one can observe in figure 2A, PCR products from 2 different plates can be simultaneously migrated in the same electrophoresis system. This aspect raised the question whether 2 DNA samples could be entirely co-amplified in the same plate. The system described above uses for a patient 7 out of 12 columns of a PCR plate, while some of the exons need re-amplification due to PCR loss. Also, some of the exons are amplified in several variants, which may become obsolete (e.g. M1-e12 can easily be covered by the first amplicon variant). Therefore, we decided to keep the amplicons "working" together in the same plate, while excluding difficult or iterative amplicons from the plate, which can be amplified separately. The novel system we managed to develop is presented in Figure 3A, while the electrophoretic profile from the whole plate (2 patients) can be seen in Figure 3B.

Practically, we pulled out from the plate 8 out of 56 amplicons, and covered 6 columns with the remaining amplicons from Sample 1, the rest of the plate being filled by corresponding amplicons from Sample 2. The eliminated amplicons were M1-e1, M6-e1, M6-e7, M6-e8, M1-e5, M1-e11, M1-e12 (variant 2). These amplicons can be amplified together in a separate system or even in separate reactions each (Figure 1A and

	Α	В	С	D	E	F	G	Η
1	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2
2	e1	e2	e3	e4	e5	eo	e7	e8
	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2	S1-M2
	e9	e10	e11	e12	e13	e14	e15	e16
3	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6
	e2	e3	e4-1	e4-2	e4-3	e4-4	e4-5	e4-6
4	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6	S1-M6
	e4-7	e4-8	e5	e6	e8-var2	e9-var1	e9-var2	e10
5	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1
	e2	e3	e4	e6	e7	e8	e9	e10
6	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1	S1-M1
	e12-1	e13	e14	e15	e16	e17	e18	e19
7	S2-M2	S2-M2	S2-M2	S2-M2	S2-M2	S2-M2	S2-M2	S2-M2
	e1	e2	e3	e4	e5	e6	e7	e8
8	S2-M2	S2-M2	S2-M2	S2-M2	S2-M2	S2-M2	S2-M2	S2-M2
	e9	e10	e11	e12	e13	e14	e15	e16
9	S2-M6	S2-M6	S2-M6	S2-M6	S2-M6	S2-M6	S2-M6	S2-M6
	e2	e3	e4-1	e4-2	e4-3	e4-4	e4-5	e4-6
10	S2-M6	S2-M6	S2-M6	S2-M6	S2-M6	S2-M6	S2-M6	S2-M6
	e4-7	e4-8	e5	e6	e8-var2	e9-var1	e9-var2	e10
11	S2-M1	S2-M1	S2-M1	S2-M1	S2-M1	S2-M1	S2-M1	S2-M1
	e2	e3	e4	e6	e7	e8	e9	e10
12	S2-M1	S2-M1	S2-M1	S2-M1	S2-M1	S2-M1	S2-M1	S2-M1
	e12-1	e13	e14	e15	e16	e17	e18	e19



Figure 3A (up). Co-amplification of the most efficient 48 exons for 2 patients in the same PCR plate (S=sample, E=amplified exon). Figure 3B (down). Co-amplification of the most efficient 48 exons for 2 patients in the same PCR plate



Figure 4. Example of detection of the MSH2 c.1277-118G>A variant, in forward and reverse sequencing, in heterozygous state (bottom), homozygous (middle) or wild-type (upper)

1B), depending on the number of available samples. However, one should notice that the main plate we imagined covered more than 85% of the entire region of interest, and the probability of finding deleterious mutations in MMR genes is accordingly inside this plate. In a diagnostic system, when identifying a deleterious pathogenic mutation, one has not to consider continuing the sequence of the other exons.

The accuracy of our implemented system can be easily observed in figure 4. We chose a distant intronic variant upstream exon 8 of *MSH2* (c.1277-118G>A), detectable either in homozy-gous or heterozygous state, both on forward and reverse strands.

Discussion

Improving molecular diagnosis for Lynch syndrome in Romania is essential. Both incidence and mortality are increasing in our country, so early diagnosis and prevention should become national health priorities. The observed epidemiologic trends are similar with those recorded in Western Europe in the early 90s, where the increase awareness about CRC and methods of screening and early diagnosis determined a stabilization of incidence and mortality due to CRC in the late 90s (29). This moment also corresponds to the emergence and development of Oncogenetics as the medical and diagnostic follow-up of patients and their families presenting a hereditary monogenic risk to cancer. The prognosis for patients with CRC heavily depends on the stage on diagnosis (6): 5-year survival is over 90% for early stages, but only 5% for late ones, which emphasizes early diagnosis.

Although overall diagnosis in LS includes germ-line and somatic analysis, the oncogenetic approach is mainly based on germ-line mutation detection by entire MMR gene sequencing. While promising progress has been recently reported by next-generation sequencing, the gold standard in oncogenetic molecular diagnosi remains Sanger sequencing (30). As shown earlier, this methodology is not only expensive and complex, but also time consuming, therefore a patient should wait a minimum of 8 months for a diagnosis, a period which increases with the limited number of available samples. The challenge we took in this context is to entirely sequence the MMR genes, for a single patient, on a single PCR plate, without losing any of the accuracy of the general sequencing procedure.

We developed an integrated system able to rapidly cover the entire coding region of *MSH2*, *MSH6* and *MLH1* by Sanger sequencing. This can be performed for absolutely all 56 needed amplicons in a single PCR amplification plate, followed by separate forward and reverse corresponding sequencing plates. Alternatively, this can be performed for 2 DNA samples simultaneously, in the same plate, for a total of 48/56 amplicons, the remaining ones being amplified separately. In both systems presented, the overall efficiency achieves 95% (maximum we observed – i.e. the number of amplicons not needing re-amplification after the final sequence lecture). However, we have to notice that this efficiency is directly correlated with the quality of template DNA, as well as with the overall accuracy of laboratory workflow. Nonetheless, the systems described are optimized to function on local conditions and equipment, each reproduction in different conditions needing separate optimization.

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Conflicts of interest

The authors declare no conflict of interest

References

- Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOB-OCAN 2012. Int J Cancer. 2015 Mar;136(5):E359-86. DOI: 10.1002/ijc.29210
- Patel SG, Ahnen DJ. Familial colon cancer syndromes: an update of a rapidly evolving field. Curr Gastroenterol Rep. 2012 Oct;14(5):428-38. DOI: 10.1007/s11894-012-0280-6
- Center MM, Jemal A, Smith RA, Ward E. Worldwide variations in colorectal cancer. CA Cancer J Clin. 2009 Nov-Dec;59(6):366-78. DOI: 10.3322/caac.20038
- Valean S, Armean P, Resteman S, Nagy G, Muresan A, Mircea PA. Cancer mortality in Romania, 1955-2004. Digestive sites: esophagus, stomach, colon and rectum, pancreas, liver, gallbladder and biliary tree. J Gastrointestin Liver Dis. 2008 Mar;17(1):9-14.
- Risch N. The genetic epidemiology of cancer: interpreting family and twin studies and their implications for molecular genetic approaches. Cancer Epidemiol Biomarkers Prev. 2001 Jul;10(7):733-41.
- de la Chapelle A. Genetic predisposition to colorectal cancer. Nat Rev Cancer. 2004 Oct;4(10):769-80. DOI:

10.1038/nrc1453

- Lynch HT, Krush AJ. Cancer family "G" revisited: 1895-1970. Cancer. 1971 Jun;27(6):1505-11. DOI: 10.1002/1097-0142(197106)27:6<1505::AID-CN-CR2820270635>3.0.CO;2-L
- Lynch HT, Guirgis HA, Lynch PM, Lynch JF, Harris RE. Familial cancer syndromes: a survey. Cancer. 1977 Apr;39(4 Suppl):1867-81. DOI: 10.1002/1097-0142(197704)39:4+<1867::AID-CN-CR2820390820>3.0.CO;2-Q
- Schlussel AT, Gagliano RA Jr, Seto-Donlon S, Eggerding F, Donlon T, Berenberg J, et al. The evolution of colorectal cancer genetics – Part 1: from discovery to practice. J Gastrointest Oncol. 2014 Oct;5(5):326-35.
- Schlussel AT, Gagliano RA Jr, Seto-Donlon S, Eggerding F, Donlon T, Berenberg J, et al. The evolution of colorectal cancer genetics – Part 2: clinical implications and applications. J Gastrointest Oncol. 2014 Oct;5(5):336-44.
- Lynch HT, de la Chapelle A. Hereditary colorectal cancer. N Engl J Med. 2003 Mar;348(10):919-32. DOI: 10.1056/NEJMra012242
- Vasen HF, Wijnen JT, Menko FH, Kleibeuker JH, Taal BG, Griffioen G, et al. Cancer risk in families with hereditary nonpolyposis colorectal cancer diagnosed by mutation analysis. Gastroenterology. 1996 Apr;110(4):1020-7. DOI: 10.1053/gast.1996.v110. pm8612988
- Giardiello FM, Allen JI, Axilbund JE, Boland CR, Burke CA, Burt RW, et al. 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. 2014 Aug;109(8):1159-79. DOI: 10.1038/ajg.2014.186
- Peltomaki P, Vasen HF. Mutations predisposing to hereditary nonpolyposis colorectal cancer: database and results of a collaborative study. The International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer. Gastroenterology. 1997 Oct;113(4):1146-58. DOI: 10.1053/gast.1997.v113.pm9322509
- Ransohoff DF. Rules of evidence for cancer molecular-marker discovery and validation. Nat Rev Cancer. 2004 Apr;4(4):309-14. DOI: 10.1038/nrc1322
- Bedeir A, Krasinskas AM. Molecular diagnostics of colorectal cancer. Arch Pathol Lab Med. 2011 May;135(5):578-87.
- Cunningham JM, Tester DJ, Thibodeau SN. Mutation detection in colorectal cancers: direct sequencing of DNA mismatch repair genes. Methods Mol Med. 2001;50:87-98.
- 18. Vasen HF, Watson P, Mecklin JP, Lynch HT. New

clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology. 1999 Jun;116(6):1453-6. DOI: 10.1016/ S0016-5085(99)70510-X

- Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Ruschoff J, et al. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst. 2004 Feb;96(4):261-8. DOI: 10.1093/jnci/djh034
- Genuardi M, Anti M, Capozzi E, Leonardi F, Fornasarig M, Novella E, et al. MLH1 and MSH2 constitutional mutations in colorectal cancer families not meeting the standard criteria for hereditary nonpolyposis colorectal cancer. Int J Cancer. 1998 Mar;75(6):835-9. DOI: 10.1002/(SICI)1097-0215(19980316)75:6<835::AID-IJC4>3.0.CO;2-W
- Negură A, Matei M, Negură L, Carasevici E, Uhrhammer N, Bignon Y.-J, et al. Ethical implications in communicating molecular tests results to hereditary cancer predisposition families. Revista Romana de Bioetica. 2010 Dec;8(4):74-83.
- Negură L, Negura A, Coneac A, Neagoe IB, Azoicai D, Irimie A. Pathogenic intronic and deleterious benign variants: two extremes in cancer predisposition molecular diagnosis. Rev Romana Med Lab. 2012;20(4):317-26.
- Negură L, Uhrhammer N, Negură A, Artenie V, Carasevici E, Bignon Y-J. Complete BRCA mutation screening within breast and ovarian cancer predisposition families in North-Eastern Romanian population. Familial Cancer 2010 Dec;9(4):519-23. DOI: 10.1007/ s10689-010-9361-6
- 24. Negură L, Carasevici E, Negură A, Uhrhammer N, Bignon Y-J. Identification of a recurrent BRCA1 mutation in two breast/ovarian cancer predisposition families with distinct phenotypes, by allele-specific multiplex-PCR. Rev Romana Med Lab. 2010;18(2):53-61.
- Negură L, Azoicăi D, Matei M, Popoiu G, Negură A. Screening of a novel BRCA2 mutation by rapid in-house PCR-RFLP. Rev Romana Med Lab. 2011;19(4):333-9.
- 26. http://primer3.ut.ee/
- 27. http://www.genomic.unimelb.edu.au/mdi/mutnomen/
- 28. http://www.interactive-biosoftware.com/alamut.html
- Jemal A, Ward E, Hao Y, Thun M. Trends in the leading causes of death in the United States, 1970-2002. JAMA. 2005 Sep;294(10):1255-9. DOI: 10.1001/ jama.294.10.1255
- Mardis ER. Genome sequencing and cancer. Curr Opin Genet Dev. 2012 Jun;22(3):245-50. DOI: 10.1016/j. gde.2012.03.005