Chapter 16

COL11A1 — Genetic Biomarker Targeted in Stool Samples for Early Diagnosis of Colorectal Cancer in Patients at Risk

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Additional information is available at the end of the chapter

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1. Introduction

Colorectal cancer (CRC) is the third most frequently diagnosed cancer in both men and women and the second leading cause of cancer death after lung cancer. Even though CRC is considered to be 90% curable if detected in early stage, the majority of patients are diagnosed with advanced stages, III or IV [1].

Screening tests applied according to well known strategies make the early diagnosis of CRC possible and there is is strong evidence evidence that screening lowers mortality and incidence rates of cancer, if recommended at proper time in people at risk [2]. Still, the existing tests practiced on a large scale in CRC screening do not fully accomplish the goal of best specificity and sensitivity or either an optimal cost/efficiency ratio. A more effective screening test may significantly decrease disease burden.

Extensive research over the past two decades provided large information about genetic aberrations underlying CRC and revealed complex and heterogeneous mechanisms in the occurrence of the disease [3, 4]. Genetic changes occurred in normal colonic epithelium cells promoting the neoplastic transformation into benign adenomas and subsequently malignant adenocarcinomas were the essence for understanding the disease behavior and related clinical outcome, and created a perspective for future improvements in diagnosis, treatment and survival rates.

2. Colorectal cancer genesis — Gene mutations and underlying stool testing

A cancer cell develops through a collection of gene mutations. Mutations left uncorrected by cell cycle regulation before division are fixed in that cell and in its future progenitors. A cell undergoes full carcinogenic transformation once a sufficient number of genes are mutated and the cell can no longer respond to the external signals that act as brakes on cell growth. Comparing with breast cancer, in which a single gene is required for disease initiation, in CRC there are 7 to 10 genes responsible for neoplastic transformation and thereforee, the genetic-based screening for CRC is a more laborious task than screening breast cancer [5].

Although a smaller subgroup can arise as a result of inherited mutations or previous inflammatory bowel disease (Crohn's disease or ulcerative colitis), the great majority of CRC arise sporadically. This means that mutated genes are present only in precancerous and cancerous lesions in the colon and rectum, and are not present in all cells of the body. Therefore, CRC cannot be detected by a blood test, as breast cancer does [6].

Over the time, mutations of three different classes of genes have been described in colon cancer etiology: oncogenes, suppressor genes, and mismatch repair genes [7]. Knowledge of many of the specific mutations responsible for colon carcinogenesis allowed understanding the phenotypic manifestations and provided a large field for genetic testing from stool cell's DNA [8]. Although genetic testing is possible and available, it is not yet clear what battery of genetic tests are more accurate to use as an alternative diagnostic tool instead present widely accepted stool test.

Until now, the genetic changes targeted in the stool cell's DNA involved in the development of some colorectal cancers included: activating mutations of the K-RAS oncogene, inactivating mutations of the adenomatous polyposis cancer (*APC*) and *TP53* tumor suppressor genes [9] and germline or somatic mutations of mismatch repair genes (*MMR*) [10, 11].

A much less studied biomarker targeted in stool samples for early diagnosis of CRC in patients at risk is *COL11A1* gene, mutations of which have been first described in Marshall's syndrome and Stickler's syndrome [12]. The normal function of this gene is the production of collagen type XI, which participate to build the structure and the resistance of conjunctive tissues. Beside its main role in the assembly, organization and development of cartilage, *COL11A1* was found to be expressed at low level in a wide variety of normal adult human tissues, including lung, parotid gland and colorectal cells.

Few studies have found overexpression of the *COL11A1* gene in various types of cancers, such as non-small cell lung cancer (NSCLC), ovarian, oral cavity and colorectal cancers. In particular, overexpression of the *COL11A1* gene was found to be correlated with invasive and metastatic potential of these cancers [13-16].

This gene is located on chromosome 1, arm p, site 21, between the 103.055.015 and 103.286.072 pair of bases, and is composed of 231 kbase. It contains 68 exons, not yet wholly sequenced [17]. A major contribution to the *COL11A1* gene sequencing knowleddgee, especially with the purpose of detecting mutations, is *Annune S* research and results [18]. Recent extented studies

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demonstrated that some polymorphysms of *COL11A1* are associated with different types of adenocarcinoma [19].

3. AIM

This study was designed to analyze *COL11A1* gene mutations identified in the DNA of exfoliated epithelial cells of the colon in the stool of the patients diagnosed with CRC through screening and to demonstrate the perfect similarity between the detected mutations in tumor samples and in exfoliated stool cells, in order to prove the reliability of the method as a diagnostic tool for early CRC diagnosis.

4. Patients and method

We selected 250 patients diagnosed in the Endoscopy Department of Emergency Hospital of Constanta with adenomatous polyps and CRC using colonoscopy and biopsy and confirmed by histopathological exam, during screening programme or admitted and investigated for intestinal disturbances such as chronic diarrhea or recent exacerbated constipation, stool bleeding or association of the above symptoms in their recent history.

We collected samples biopsied from tumors during colonoscopy and stool sample from each patient.

Colonoscopy and biopsies were performed with Olympus Exera equipment.

The bowel preparation was done according to guidelines and its quality was noted.

The colonoscopist documented the presence, size, location and extension of colonic tumors.

Biopsy or surgical resection samples were examined histopathologically and genetically.

Subjects were instructed prior stool collection. No dietary or medication modifications were required. Until shipping samples to genetic lab, these were disposed in a coded container into a refrigerator, between 0 and 4°C. Specimens were required to arrive within 3 days after collection.

The minimum quantity of stool sample required was 30 g. Samples were stored at –80°C until genetic analysis.

DNA was extracted from biopsy and feaces samples for mutation analysis:

- from stool, with QIAmp stool extraction kit (QIGene, Germany);
- from biopsy sample, with IQ-DNA Extraction Kit (Promega USA).

Primers for PCR amplification were provided by TIB MOLBIOL, Germany.

The *COL11A1* gene, examined in the present study, produces a component of the colagen type XI, named pro-alpha1 chain, an important factor for connective tissue structure and resistance.

The method used for *COL11A1* mutations was polyacrylamide gel electrophoresis method for the heteroduplex analysis (HA).

Investigation of *COL11A1* gene is made sequentially by setting fragments to be analyzed. To do this, fragments that usually carry most mutations are identified. Each fragment of *COL11A1* gene that is proposed for investigation is amplified by PCR. For this, primers flanking each particular fragment are used.

PCR conditions are dependent on the characteristics of each pair of primers. Each program contains a PCR initial denaturing step, lasting for 3 minutes at 94°C, followed by 30-35 reaction cycles (depending on the length of the fragment of interest and the size and composition of the primers), each cycle comprising: denaturation, alignment, elongation (conditions are established for each pair of primers used), and the final elongation step, lasting for 5 minutes at 72°C. Obtained amplicons are subjected to additional steps of forced denaturation-renaturation (to encourage heteroduplex formation), and then migrated in a 6% polyacrylamide gel.

The heteroduplex is represented by a fragment of double-stranded DNA in which the two strands do not express perfect complementarity. When DNA is denatured, the two strands are separated. Through renaturation, complementary chains come together to form a homoduplex. If there is a mutation in one of the two strands, heteroduplex is formed (figure 1).

Heteroduplex analysis was imagined by *Ziemmermann et al* in 1993 [20] and has been used to enhance the sensitivity of denaturing gradient gel electrophoresis (DGGE) in the detection of point mutation [21-23]. DNA fragments for HA can be visualized via a variety of methods including bromide staining, labelling with radioisotopes and silver staining. The mutations detection rate of HA under ideal conditions is near 90%.

Heteroduplex differ from homoduplex by electrophoretic migration speed in polyacrylamide gel. Mutational alteration of a single base pair is sufficient to produce changes in mobility. Electrophoretic mobility of heteroduplex is lower than that of homoduplex, and it can be detected as a slower migrating band. This method can detect insertions, deletions and substitutions of even a single base pair in fragment lengths smaller than 200 bp.

The working protocol for this technique is very simple and quick and consists of a denaturation-renaturation step for which we set the following conditions: 94°C – 1 minute, 72°C – 1 minute, 65°C – 1 minute, 40°C – 1 minute and thermal shock at 4°C. Each stage is covered by one cycle. The existence of deletion mutations will result in the formation of four bands, two heteroduplex and two homoduplex (heterozygous condition) (Figure 1).

Migration occurs differently based on molecular weight. Samples from patients and healthy individuals (control samples) are migrated in the same gel, in order to analyze the difference in migration. If the investigated individual is a normal homozygous or a homozygous for analyzed mutation, a single band will be displayed in each case. The difference between these conditions is based on different migration and reported to the molecular weight marker used. If there is a substitution mutation, two bands are visualized on polyacrylamide gel: a band representing heteroduplex and a band representing homoduplex. In this case, the difference in migration is explained on the basis of different chemical composition of the four DNA

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strands. For optimal view of amplification products, we used 6% polyacrylamide gel, containing 0.5 μg ethidium bromide, migrated 6 V/cm. The gel is observed and photographed on UV transilluminator.

In the latter stages of a PCR amplification the polymerase is limiting, so that during the final annealing and synthesis steps, a proportion of the single stranded products spontaneously reanneals without primer extension. When amplifying from individuals heterozygous for any sequence difference, the single strands do not necessarily rehybridize exactly with the complementary strand. They can alternatively form a DNA hybrid (heteroduplex) consisting of a sense strand with one sequence variant and an antisense strand with another variant.

As a consequence, the heteroduplex DNA has a region of at least one base pair mismatch. The region of mismatch can elicit a mobility shift by altering the conformation adopted by the heteroduplex DNA, probably by causing it to bend at the location of the mismatch. The mobility shifts are usually small but can be visualized after prolonged electrophoresis on native polyacrylamide gels.

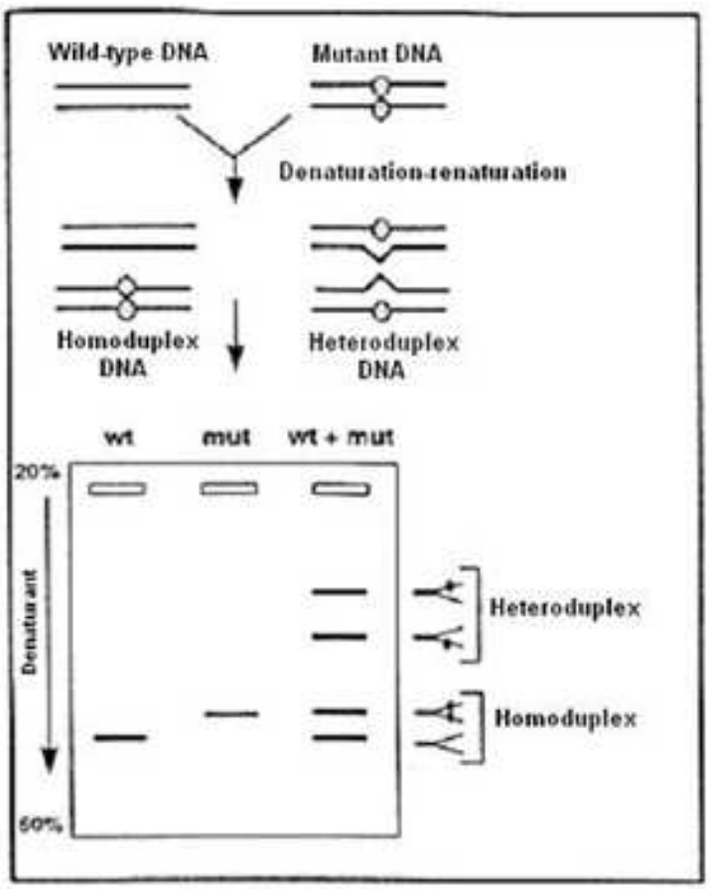

Figure 1. Denaturation and renaturation of normal and mutated DNA fragments in order to generate four types of fragments: two heteroduplex and two homoduplex. Fragments were migrated parallel on denaturating gradient gel. "Melting" heteroduplex are modified in the sense that they denaturate at a lower concentration of denaturant, allowing their visualization.

1. Detection of *COL11A1* gene mutations included the following steps:
2. Genomic DNA extraction from the analyzed samples: tumor tissue obtained through biopsy and exfoliated cells from feces;
3. Amplification of the interested gene amplicons through PCR reaction;
4. Amplification check up through electrophoresis in agarosis gel and bromide ethidium staining;
5. Mutations identification through DGGE technique and silver or ethidium bromide staining.

5. DNA extraction from proposed samples

DNA extraction from stool has been made by a specific kit for stool extraction [24, 25]. DNA tissue extraction from the colorectal biopsy has been performed using DNA IQ (TM) System kit [26]. DNA IQ (TM) System kit uses the principle of DNA extraction based on a paramagnetic resine. In addition, the kit contains a series of denaturating agents ("lysis buffer"), having the role of disintegrating the biologic product that is the DNA source.

An important advantage of this kit is that it provides extraction of an optimal DNA quantity for PCR reaction (100 ng/μl).

6. DNA extraction from stool

DNA extraction from stool was performed with a kit designed for extraction from faeces (QIAGEN GmbH, Hilden Germany). The technique included the following steps:

1. 200 mg of faeces were suspended in 2 ml of ASL buffer by vortexing for 1 minute.
2. 1.6 ml of this lysate was transferred to a new tube.
3. Suspension was boiled for 5 minutes.
4. Centrifuged at maximum speed for 1 minute.
5. Transfered 1.2 ml of supernatant into a new tube containing an InhibitEX tablet.
6. Vortexed the tube for 1 minute and incubated for 1 minute at room temperature.
7. Centrifuged the tube for 3 minutes.
8. Transfered 200 μl of supernatant into a new tube containing 15 μl of proteinase K.
9. Added 200 μl of Buffer AL and vortex.
10. Incubated at 70°C for 10 minutes.

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11. Added 200 μl of ethanol to lysate and vortex.

12. Content was applyed in a column of centrifugation and centrifuged at 10,000 x g for 1 minute.

13. The column was washed once with 500 μl of Buffer AW1 at 10,000 x g for 1 minute and then with 500 μl of Buffer AW2 at 10,000 x g for 1 minute.

14. DNA was eluted from the column at 10,000 x g, 1 minute, with 100 μl heated buffer AE.

7. DNA tissue extraction from colorectal biopsy

DNA extraction from biological product was performed with DNA kit IQTM System, manufactured by Promega, USA. DNA IQTM System is a kit that uses a new DNA extraction principle based on the use of paramagnetic resin. In addition, the kit contains a number of denaturating agents ("lysis buffer") which are designed to disintegrate biological product that is the source of DNA.

For some biological products (hair, tissue) which are resistant to this type of disintegration, an additional pretreatment with proteinase K is used, an enzyme that produces sample lysis.

A considerable advantage of this kit is that it provides an optimal quantity of DNA extraction for PCR reaction, respectively 100 ng/μl, regardless of used biological product.

Used magnetic resin binds only a limited amount of DNA even if it is in excess. Finally, DNA is eluted from the resin with 100 μl of eluent solution yielding a final concentration of 1 ng/μl. Thus, it is no longer necessary to quantify the amount of extracted DNA.

In principle extraction kit use the following steps:

- Extraction of the sample and its lysis;
- Resin-capture DNA;
- Magnetic resin-washing;
- Elution of DNA from the resin.

8. Purification of DNA from a tissue sample

1. We place about 1 mg of tissue in a 1.5 ml tube.

2. We added 50-100 μl of incubation buffer solution/freshly prepared proteinase K and incubated at 56°C for 2 hours. Usually all tissue is digested after 2 hours, and if it doesn't occur, incubation is extended.

3. We removed the source sample incubation and added 2 volumes of lysis buffer.

4. We added 7 μl of magnetic resin. The sample was vortex 3 seconds and left at room temperature for 5 minutes.
5. Then the simple was vortex for 2 seconds and left the tube on the magnetic stand. Separation occured instantly.
6. Carefully we aspirated all the solution without disturbing the resin at the bottom of the tube.
7. We add 100 μl of prepared lysis buffer. We removed the tube from the magnetic stand and vortex 2 seconds.
8. Placed the tube again on the magnetic stand and vacuum lysis buffer.
9. Added 100 μl of Wash Buffer preparation. We removed the tube from the magnetic stand and vortex 2 seconds.
10. We replaced the tube in the magnetic stand and aspirated the solution.
11. We repeated steps 9 and 10, 2 times to make a total of three washes.
12. Tubes were allowed in the magnetic stand with the lid open for 5 minutes to dry.
13. We added 25-100 μl of elution buffer, depending on the amount of biological material used.
14. Then we closed the lid and vortexed 2 seconds. We incubated at 65°C for 5 minutes.
15. Removed the tube from the heating device, vortexed 2 seconds and immediately puted the tube on the magnetic stand.
16. Finally, we aspirated DNA containing solution and left the tube in conservation.

9. Validation of human origin of DNA extracted from stool by STR loci typing

The analysis of the nuclear DNA extracted from stools is a recent new method for CRC diagnostics.

In the preliminary phase of our study, we comparatively analyzed the DNA extracted from the biopsy samples of the patients with the DNA extracted from the stool samples of the same patients. This comparative analysis was performed by investigating a number of 9 human STR loci, frequently used in the DNA typing techniques of forensic medicine.

The STR type loci ("short tandem repeat" or "microsatellite repeats") contain 4 bases of segments that repeat 5-50 folds, depending on the loci. These STR are of a very small size (100-400 bases) and are very useful for the degraded DNA analysis. These repetitive sequences are largely spread in the human genome, being a rich source of polymorphic markers that may be detected through PCR.

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For the DNA typing in our cases, we used a number of 9 STR loci. Determination of the 9 loci can be made by using kits of molecular biology, forming GenePrintSTR Systems of Multiplex type.

10. Amplification of the interested gene amplicons through PCR reaction

Primers the analysis of all the 68 exons of *COL11A1* gene are not yet available. We managed to obtain sequence for primers of two groups of amplicons containing amplified segments in exons where most frequently mutations in various cancers were found.

Amplification groups are:

- Group 1:
  - Amplicon 38;
  - Amplicon 41;
  - Amplicon 16.
- Group 2:
  - Amplicon 54;
  - Amplicon 55;
  - Amplicon 56;
  - Amplicon 57.

PCR reactions were done simultaneously for each of the two groups above. We used PCR amplification kit manufactured by Promega (USA) called "PCR Core System". It was designed to enhance any type of amplicon, by usind standard type Taq polymerase.

Materials required:

- Thermal cicler for 0.2 ml tubes;
- microcentrifuge;
- Taq DNA polymerase;
- Nucleases-free water;
- Mineral Oil;
- 0.2 ml Amplification tubes;
- 1.5 ml microcentrifuge tubes;
- Anti-aerosol pipette tips;
- Ice.

11. Thermal cycling protocol

Manufacturing company recommends several types of thermal cycling protocols and the choice depends on the thermo-cicler and optimized version that has been established. We have optimized the following protocol – protocol *COL11A1*:

- Step 1: 94°C, 1 minute;
- Step 2: 52°C, 1 minute;
- Step 3: 72°C, 1 minute;

Repeated successive steps 1, 2 and 3, in 5 cycles.

- Step 4: 94°C, 1 minute;
- Step 5: 50°C, 1 minute;
- Step 6: 72°C, 1 minute;

Repeated successive steps 4, 5 and 6, in 5 cycles.

- Step 7: 94°C, 1 minute;
- Step 8: 48°C, 1 minute;
- Step 9: 72°C, 1 minute;
- Step 10: 72°C, 3 minutes;
- Step 11 (rest): 4°C.

12. Amplification setting

To prevent contamination it is strongly recommended the use of gloves and anti-aerosol pipette tips. Maneuvers that must be cosidered are as follows:

1. Defrost kit components and pairs of primers and then put them on ice.

2. Mark each 0.2 ml amplification tube and place it in the stand.

3. Determine the number of reactions to be performed. This number must include the positive and negative control reaction, respectively. Add to this number another 1-2 reactions in addition, to compensate for pipetting errors.

4. Prepare the amplification (PCR Master Mix) solution, according to the table below (table 1). Multiply the volume per sample (μl) with the total number of reactions, to obtain the final volume.

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PCR Master Mix Component	Volume per sample (µl)
$MgCl_2$ 25 mM sol.	1.5
10X Buffer Taq DNA Polymerase	2.5
PCR Nucleotide mix, 10 mM	0.5
"Primer Upstream", 15 µM	1.65
"Primer "Downstream", 15 µM	1.65
Taq DNA Polymerase (at 5 u/µl)	0.12
Distilled water without nucleases	17.08
Total volume	22.5

Table 1. Preparation of PCR Master Mix.

1. In order from above table lay the final volume of each reagent in a sterile tube. Shake gently (not vortex) and place the tube on ice.
2. Add 22.5 µl of PCR Master Mix to each reaction tube and place tubes on ice.
3. Pipette 2.5 µl of each DNA sample to respective tubes containing 22.5 µl of PCR Master Mix.
4. Pipette 2.5 µl (5 ng) of K562 DNA (diluted to 2 ng/µl) in a reaction tube containing 22.5 µl of PCR Master Mix, which is a positive control.
5. Pipette 2.5 µl of sterile distilled water (instead of DNA) in a reaction tube containing 22.5 µl of PCR Master Mix, which is a negative control.
6. Add 1 drop of mineral oil to each tube. Close the tubes.
7. Centrifuge tubes to bring the contents to the bottom of the tube.
8. Assemble the tubes in thermal triggers cicler and start amplification.
9. After amplification, the tubes must be kept at –20°C.

12.1. Electrophoresis of amplified samples for evidence heteroduplex

For this complex electrophoresis technique, we used a device type "DCode Universal Mutation Detection System" manufactured by Bio-Rad (Germany).

12.2. Formation reaction of heteroduplex

Protocol consists of a denaturation-renaturation step of PCR sample obtained from normal witness mixed with PCR sample from analyzed patient was established under the following conditions:

- 94°C – 1 minute;
- 72°C – 1 minute;

- 65°C – 1 minute;
- 40°C – 1 minute;
- heat shock at 4°C.

Heteroduplex are generated by adding to the same PCR reaction the mold of mutant and normal DNA, or by PCR product mixing, denaturation and ultimately their renaturation. A heteroduplex contains a mismatch base in the double chain, causing a distortion in its conformation; bands containing heteroduplex always migrates more slowly compared to bands containing homoduplex.

12.3. Preparation of reagents

Acrylamide concentration used generally depends on the sample to be analyzed, and we used a 40% stock solution containing acrylamide and bis-acrylamide.

- Acrylamide/Bis – 40% (37.5:1);
- Acrylamide – 38.9 g;
- Bis-acrylamide – 1.07 g;
- Water dist. – ad. 100 ml.

In the table below (table 2) we present the concentration of acrylamide/bis used to separate different DNA molecules:

Gel concentration	Separation of base pairs
6%	300-1000 bp
8%	200-400 bp
10%	100-300 bp

Table 2. Concentration of acrylamide/bis used to separate DNA molecules.

We worked with a solution of acrylamide/bis 6%, given the length of amplified fragments.

- Acrylamide/Bis solutions, 6% (1.25 x TAE, 6M urea);
- Acrylamide/Bis 40% 6.0 ml;
- 50X TAE buffer 1 ml;
- Urea 14.4 g;
- TEMED 40 μl;
- Ammonium persulfate 10% 400.0 μl;
- Total volume 40 ml.

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We added water to 40 ml. Pour gel immediately after adding TEMED and ammonium persulphate.

- 50X TAE buffer:
  - Tris base 242.0 g;
  - glacial acetic acid 57.1 ml;
  - 0.5M EDTA, pH 8.0, 100 ml;
  - water dist. ad. 1000 ml.
- Ammonium persulfate 10%:
  - ammonium persulfate 0.1 g;
  - water dist. 1 ml;
- Staining solution DCode;
  - bromophenol blue 0.05 g;
  - xylene cyanol 0,05 g;
  - 1X TAE buffer 10 ml;
- Solution for implementing samples:
  - bromophenol blue 0.25 ml 2%;
  - xylene cyanol 0.25ml 2%;
  - glycerol 7.0 ml;
  - water dist. 2.5 ml;
- 1.25 X TAE buffer migration:
  - 50X TAE buffer 175 ml;
  - water dist. 6825 ml.

13. Sample preparation

1. It is important that PCR is optimized to decrease the formation of artifact products that may interfere with test itself. PCR products should be assessed for purity by agarose gel electrophoresis before being used for electrophoresis.
2. On gel we applied 180-300 ng of amplified DNA per well. On each gel and for each amplicon there were joined migration of the sample and normal DNA.
3. At each sample, we added a volume of 2X sample application solution.

13.1. Preheating migration buffer

1. Electrophoresis tank was filled with a quantity of 7 L of 1X TAE buffer.
2. We placed the temperature control module above the electrophoresis tank.
3. Then we adjusted the temperature to 60°C. To achieve this temperature 1-1.5 hours wereneeded. If the buffer is preheated in the oven, this time can be reduced.

13.2. Assemble gel sandwich

Casting procedure is extremely laborious and is was done by strict electrophoresis guidelines provided by equipment manufacturer. A system of 16x16cm plates was used and the prepared gel was "sandwich" type.

1. "Sandwich" gel is mounted on a clean surface. We have placed large plate first, then we have set the spacers on the short edges of this plate.
2. Lower plate was disposed over the large plate so the bottom was flush with large plate edge.
3. We loosen the black screw of the two sandwich cutters. We placed plates in these pliers so that the arrows were facing upwards.
4. We tightened the clamps so that the glass plates were well fixed.
5. Sandwich assembly was inserted into the alignment (without clips into place) so short board was facing forward. We loosen the clips and clamps easily inserted between the plates alignment plate which serves to align the spacers.
6. We aligned the plates and spacers by moving laterally and obliquely claws. We must ensure that the spacers are perfectly parallel and the lower edge of the two plates was perfectly aligned. We tightened the screw clamps for immobilizing overall assembly.
7. We removed the plate alignment between glass plates. Then we removed the sandwich from the stand and check the lower edges of the plates and spacers are aligned perfectly.

13.3. Casting the gel

1. We placed the gray foam in the space provided for pouring the gel. Pins of the base were completely relaxed. Placed plates mounted on the lower plate to the front pad. After it was placed correctly by turning the cam, pressing the lower edge of the foam boards was performed.
2. In a 50 ml tube we puted the required amount of gel solution. Ammonium persulfate and TEMED were added to a final concentration of 0.09% (v/v). Stopped the tube and mixed by inversion.
3. We inserted the comb into sandwich and positioned it so that it was slightly bent (angle) to the edge boards. This prevented the formation of air bubbles between the gel and the comb teeth.

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4. Poured the gel solution into the sandwich until comb teeth were covered. Then pressed the comb in its correct position. Solution was added to the filling.

5. We allowed the gel to polymerize for 60 minutes. After polymerization we carefully removed the comb.

13.4. Migrating samples

1. Electrophoresis tank must contain 7 liters of buffer migration.

2. After the temperature was reached by the migration buffer (60°C) we disconnected temperature maintenance system.

3. Removed the temperature module from the electrophoresis tank. Gel fitting with gel electrophoresis was introduced into the tank and the temperature module was placed again into position.

4. Filled the volume of migration buffer until the level mark on the camera, and added also into the anode upper chamber.

5. Before applying the samples we left the machine running again to reach a migration temperature of 60°C.

6. Applied the samples after each well was previously rinsed with buffer. Sample application was made through a specially designed device that is provided.

7. Samples prepared as described above were applyed by automated pipetting carefully so that they do not spread outside the wells.

8. Closed the device and connected to the source. Migration was 5 hours at a voltage of 5 V/cm.

13.5. Gel staining

Electrophoresis was performed after the gel is removed from the tank, and glass plates carefully unfold. The gel sticks to the glass plate. Staining can be done by two procedures: simple procedure with Ethidium Bromide and fluorescence examination or staining procedure Argent. We opted for the second.

Protocol described below is an adaptation of that offered by the company with the Promega kit "DNA Silver Staining System".

A kit contains the following ingredients required for 10 stains:

- 500µl Bind silane;
- 20 G Silver Nitrate (10 x 2g);
- 60 Ml Formaldehyde, 37% (20 x 3 ml);
- 10 Ml Sodium thiosulfate, 10 mg/ml (10 x 1 ml);

- 600 G Sodium Carbonate (10 x 60g).

13.6. Materials required

- Fixing solution/stop:
  - 200 ml glacial acetic acid;
  - 1800 ml distilled water;
- Coloring solution:
  - silver nitrate (AgNO3) 2 g;
  - 3 ml 37% formaldehyde;
  - 2000 ml distilled water;
- Developing solution:
  - 3 ml 37% formaldehyde;
  - Sodium thiosulfate 10 mg/ml, (Na2S2O3 x 5H2O) 400 μl ;
  - 2000 ml distilled water;
  - sodium carbonate (Na2CO3) 60 g.

We prepared the solution just in time to use it, cooled at 4-10°C before use.

14. Technique used

1. Gel plates were placed on a flat surface. With a plastic "feather" glass was removed. The gel was caught on the short board.
2. The gel attached on short plate was placed in a plastic tray.
3. Argent coloring followed few steps:
   - **a.** fixing /stop solution – 20 minutes;
   - **b.** distilled water – 2 minutes;
   - **c.** Repeat step "b" 2 times 2 x 2 minutes;
   - **d.** staining solution – 30 minutes;
   - **e.** distilled water – 10 seconds;
   - **f.** developing solution (4-10°C) up to 5 minutes (to become visible Ladder allele);
   - **g.** fixing/stop solution* – 5 minutes;
   - **h.** distilled water – 2 minutes;

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*Solution was added directly above solution developer to stop the developer reaction.

4. We placed the gel upright and dry overnight.

15. Mutations identification through DGGE technique and silver or ethidium bromide staining

After amplification the samples were checked to confirm successful amplification by agarose gel electrophoresis. For migration were applied two tests, one that was considered the normal type (same in all cases) and the other representing the analyzed case.

To see if amplifyed amplicons in the studied cases presented mutations, we used samples examined by agarose gel electrophoresis, for electrophoresis on polyacrylamide 6%.

16. Statistical analysis

Statistical analysis was performed using Graph Pad InState and Graph Pad State Mate.

17. Results

Demographic and clinical characteristics of patients examined during study period were as follows (table 3).

Histopathological classification and localization of tumors in patients investigated through colonoscopy and biopsy referred to genetic analysis of *COL11A1* mutations can be seen in table 4.

From the total of 250 patients genetically explored, 178 (71.20%) were diagnosed with adenocarcinoma and TNM staged after hiistopathological and imagistic examinations. Most of the patients were staged as stage II or III (18.80%, respectively 23.60%).

We analyzed 51 patients diagnosed with advanced adenomatous polyps. Polyps were classified according to hiistopathological features in: 26 polyps with high-grade dysplasia (10.40%), 17 villous adenoma (6.80%), and 8 tubular adenoma bigger than 1 cm (3.20%).

Among the 250 patients studied, 178 had adenocarcinomas, 51 had advanced adenomas, and the rest 21 had minor polypspolyps.

All samples analyzed for fecal *COL11A1* mutations were processed in a single laboratory.

The plan for *COL11A1* analyses in feces or biopsy has been described previously and is shown in figure 2.

Characteristic	No.	%
Age		
Mean (yr)	67.44 ± 8.97	20.40
40-49	51	31.60
50-59	79	34.80
60-69	87	9.20
70-79	23	4.00
≥ 80	10	
Sex		
Male	189	75.60
Female	61	24.4
Ethnicity		
Caucasians	203	81.20
Other	47	18.80
Family history		
APC (adenomatous polyposis coli)	16	6.40
CRC	49	19.60
Other cancer	37	14.80
Without family history of cancer/polyps	148	59.20

Table 3. Demographic characteristics of patients enrolled in the study.

Histopathological feature	No./%	Localisation – no./%				
		Ascendinging Colon	Transvers	Descendinging colon	Sigmoid	Rectumum
Adenocarcinoma	178/250 [71.2]	41/178 [23.03]	21/178 [11.79]	62/178 [34.83]	44/178 [24.71]	10/178 [4.00]
Stage TNM I	33 [13.20]	6 [3.37]	5 [2.80]	12 [6.74]	7 [3.93]	3 [1.68]
Stage TNM II	47 [18.80]	7 [3.93]	6 [3.37]	18 [10.11]	12 [6.74]	4 [2.24]
Stage TNM III	59 [23.60]	14 [7.86]	8 [4.49]	22 [12.35]	12 [6.74]	3 [1.68]
Stage TNM IV	39 [15.60]	14 [7.86]	2 [2.23]	10 [5.61]	13 [7.30]	0 [0.00]
Advanced adenoma	51/250 [20.40]	12/51 [23.52]	7/51 [13.72]	16/51 [31.72]	14/51 [27.45]	2/51 [3.92]
High-grade dysplasia	26 [10.40]	8 [15.6]	4 [7.84]	7 [13.72]	7 [13.72]	–
Villous adenoma	17 [6.80]	3 [5.88]	1 [1.96]	5 [9.80]	6 [11.76]	2 [3.93]
Tubular adenoma ≥ 1cm	8 [3.20]	1 [1.96]	2 [3.93]	4 [7.84]	1 [1.96]	–
Minor polyps	21/250 [8.40]	3/21 [14.28]	5/21 [23.80]	5/21 [23.80]	6/21 [28.57]	2/21 [9.52]
Tubular adenoma <1cm	9 [3.60]	1 [4.76]	2 [9.52]	2 [9.52]	3 [14.28]	–
Hiperplastic	10 [4.00]	1 [4.76]	3 [14.28]	3 [14.28]	2 [9.52]	2 [9.52]
Unspecified	2 [0.8]	1 [4.76]	–	–	1 [0.56]	–

Table 4. Hiistopathological classification and tumor localization.

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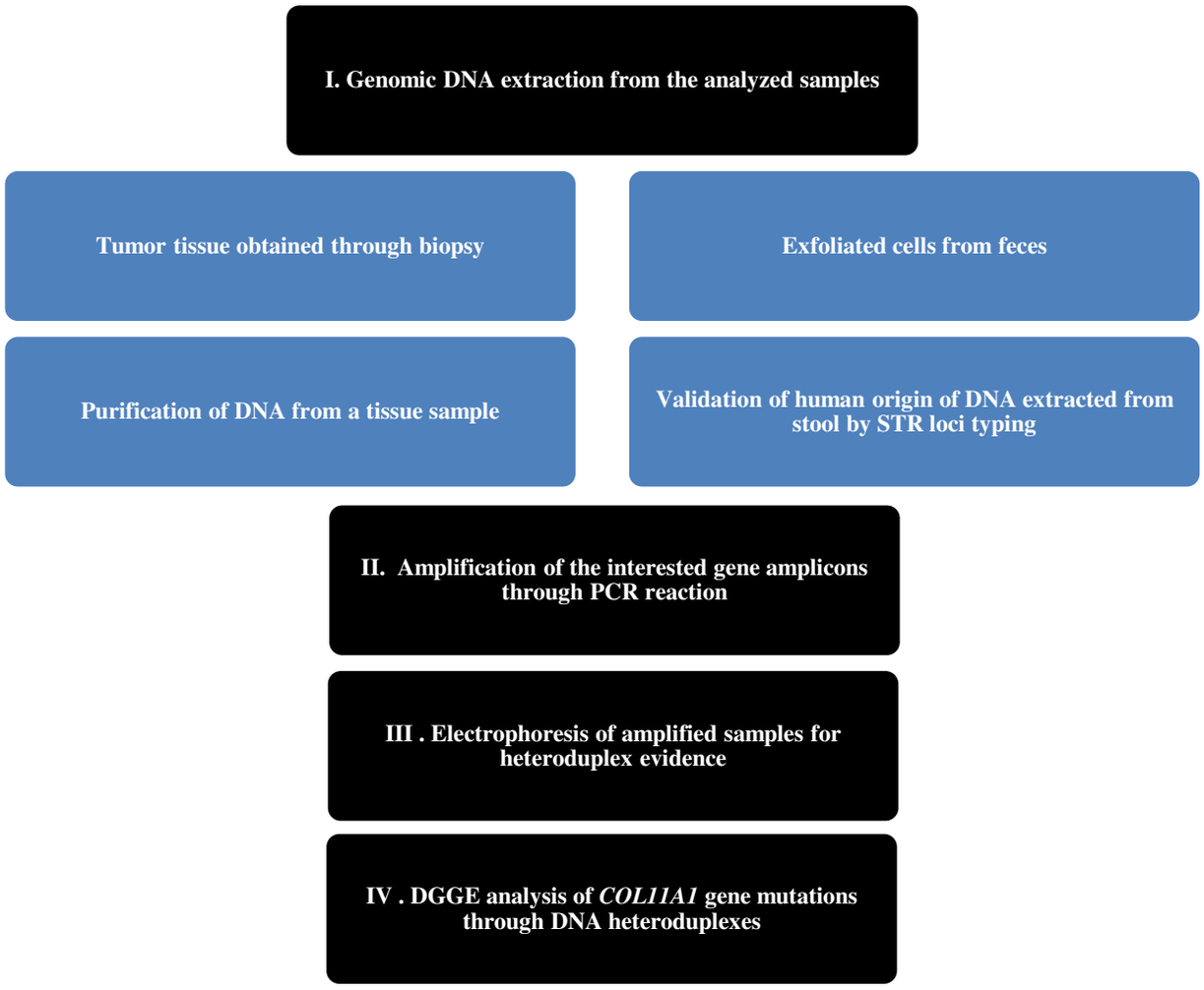

Figure 2. Approach to Extraction and Analysis of Fecal and Tumor DNA analysis.

Also laboratory handling of all samples was fully described above.

Each exon of gene *COL11A1* studied was assessed independently.

We considered a positive result any modified component of the study gene, and we noted any mutation as a positive fecal DNA test.

18. DGGE analysis of *COL11A1* gene mutations through DNA heteroduplexes

COL11A1 is located on chromosome 1p21 and consists of 232,030 bases. It contains 68 exons, yet not wholly sequenced, of which exons 38, 41, 16, 54, 55, 56 and 57 were until now studied.

HE analysis for exons 38, 41 and 16

For all 3 analyzed amplicons in all 250 studied cases, the migration speed was identical for both control and samples.

HE analysis for exons 54, 55, 56 and 57

HE analysis of exons 54 clearly demonstrated in 52 cases (20.80%) the presence of the same displaced bands pattern in the biopsy and stool extracted cells samples compared with the control. This pattern of mutation was correlated positively with male gender, TNM stage II/III tumors, vegetative pattern, descendent and sigmoid localization – table 5.

No.	Pacient no.	DNA sample	Mutations/amplicons						
			38	41	16	54	55	56	57
1	1	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
2	4	Biopsy	-	-	-	-	-	+	-
		Faeces	-	-	-	-	-	+	-
3	7	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	+
4	8	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	+
5	11	Biopsy	-	-	-	+	-	-	+
		Faeces	-	-	-	+	-	-	+
6	13	Biopsy	-	-	-	+	-	-	+
		Faeces	-	-	-	+	-	-	-
7	14	Biopsy	-	-	-	-	-	+	-
		Faeces	-	-	-	-	-	+	-
8	16	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
9	17	Biopsy	-	-	-	-	-	+	-
		Faeces	-	-	-	+	-	+	-
10	20	Biopsy	-	-	-	-	-	+	-
		Faeces	-	-	-	-	-	+	-
11	21	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	+
12	26	Biopsy	-	-	-	+	-	+	-
		Biopsy	-	-	-	+	-	+	-
13	29	Faeces	-	-	-	+	-	+	-
		Biopsy	-	-	-	+	-	+	-
14	34	Biopsy	-	-	-	+	-	+	-
		Faeces	-	-	-	+		+	-
15	38	Biopsy	-	-	-	+	-	-	-

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No.	Pacient no.	DNA sample	Mutations/amplicons						
			38	41	16	54	55	56	57
		Biopsy	-	-	-	+	-	-	-
16	46	Faeces	-	-	-	+			-
		Biopsy	-	-	-	+	-	+	-
17	48	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+			-
18	56	Biopsy	-	-	-	-	-	+	-
		Faeces	-	-	-			+	-
19	59	Biopsy	-	-	-	+	-	+	-
		Faeces	-	-	-	-	-	+	-
20	74	Biopsy	-	-	-	-	-	+	+
		Faeces	-	-	-	-	-	+	-
21	78	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+			-
22	84	Biopsy	-	-	-	+	-	-	-
		Biopsy	-	-	-	+	-	-	-
23	89	Faeces	-	-	-	+			-
		Biopsy	-	-	-	+	-	-	-
24	93	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+			-
25	96	Biopsy	-	-	-	+	-	+	-
		Biopsy	-	-	-	+	-	-	-
26	104	Faeces	-	-	-	+			+
		Biopsy	-	-	-	+	-	-	+
27	107	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
28	109	Biopsy	-	-	-	-	-	+	-
		Faeces	-	-	-	-	-	+	
29	116	Biopsy	-	-	-	+	-	-	-
		Biopsy	-	-	-	+	-	-	-
30	119	Faeces	-	-	-	+	-	-	-
		Biopsy	-	-	-	+	-	-	-
31	121	Biopsy	-	-	-	+	-	+	-
		Faeces				+	-	-	-
32	129	Biopsy	-	-	-	+	-	-	+
		Faeces	-	-	-	+	-	-	+
33	132	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-

No.	Pacient no.	DNA sample	Mutations/amplicons						
			38	41	16	54	55	56	57
34	134	Biopsy	-	-	-	+	-	+	-
		Faeces	-	-	-	+	-	-	-
35	138	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
36	143	Biopsy	-	-	-	+	-	+	-
		Biopsy	-	-	-	+	-	+	-
37	146	Faeces	-	-	-	-	-	+	-
		Biopsy	-	-	-	-	-	+	-
38	147	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
39	159	Biopsy	-	-	-	-	-	+	-
		Biopsy	-	-	-	-	-	+	-
40	166	Faeces	-	-	-	+	-	+	-
		Biopsy	-	-	-	+	-	+	+
41	168	Biopsy	-	-	-	+	-	+	-
		Faeces	-	-	-	+	-	+	-
42	171	Biopsy	-	-	-	-	-	+	-
		Faeces	-	-	-	-	-	+	-
43	177	Biopsy	-	-	-	+	-	-	-
		Biopsy	-	-	-	+	-	-	-
44	179	Faeces	-	-	-	+	-	-	-
		Biopsy	-	-	-	+	-	-	-
45	182	Biopsy	-	-	-	+	-	-	+
		Faeces	-	-	-	+	-	-	+
46	189	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
47	201	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
48	203	Biopsy	-	-	-	+	-	-	-
		Faeces				+	-	-	-
49	208	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
50	211	Biopsy	-	-	-	+	-	-	-
		Biopsy	-	-	-	+	-	-	-
51	220	Faeces	-	-	-	-	-	+	+
		Biopsy	-	-	-	-	-	+	+
52	224	Biopsy	-	-	-	+	-	-	-

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No.	Pacient no.	DNA sample	Mutations/amplicons						
			38	41	16	54	55	56	57
		Faeces	-	-	-	+	-	-	-
53	228	Biopsy	-	-	-	-	-	-	-
		Biopsy	-	-	-	-	-	-	-
54	230	Faeces	-	-	-	+			
		Biopsy	-	-	-	+	-	-	-
55	236	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
56	237	Biopsy	-	-	-	+	-	-	+
		Faeces	-	-	-	+	-	-	-
57	211	Biopsy	-	-	-	-	-	-	+
		Biopsy	-	-	-	-	-	-	+
58	220	Faeces				+			-
		Biopsy	-	-	-	+	-	-	-
59	224	Biopsy	-	-	-	+	-	-	+
		Faeces				+			+
60	228	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
61	230	Faeces	-	-	-	-	-	-	+
		Biopsy	-	-	-	-	-	-	+
62	236	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
63	237	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
64	238	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
65	241	Biopsy	-	-	-	-	-	-	+
		Biopsy	-	-	-	-	-	-	+
66	244	Faeces	-	-	-	+	-	-	+
		Biopsy	-	-	-	+	-	-	+
67	248	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	-	-	-	-
68	249	Biopsy	-	-	-	+	-	-	-
		Faeces	-	-	-	+	-	-	-
69	250	Faeces	-	-	-	+	-	-	-
		Biopsy	-	-	-	+	-	-	-

Table 5. Patients with *COL11A1* gene mutations

We also noticed the same pattern of different speed migration in case of HE analysis of exon 56 in 18 patients (7.20%) and exon 57 in 11 patients (4.40%).

Statistic analysis revealed that the last two kind of mutations were correlated with tumor stage IV, male gender and advanced age (> 70 yrs old) – table 6.

Histopathological and clinical features	*COL11A1 mutations (No./%)*
Adenocarcinoma Stage TNM I Stage TNM II Stage TNM III Stage TNM IV	2 [2.89] 18 [26.08] 27 [39.13] 3 [4.34]
Advanced adenoma High-grade dysplasia VillousVillous adenoma Tubular adenoma ≥1cm	0 1 [1.44] 1 [1.44]
Minor polyps Tubular adenoma <1cm HyperplasticHyperplastic Unspecified	0 0 0
Age (only for exon 56, 57)	> 70 yrs (p=0.0478, 95%CI 11.781-49.552)
Gender ratio	M/F=3.72 (p=0.021, 95%CI 26.330-49.312)
EthnicityEthnicity	CaucasianCaucasian/other (p=0.0037, 95%CI 14.114-49.226)
Localisation	Descendent/Sigmoid (p=0.02, 95%CI 29.481-50.227)
TNM classification	Stage II/III (p=0.009, 95%CI 7.336-39.386)

Table 6. Correlation between hiistopathological examination and genetic analysis.

The migrating front presented two bands, out of which the slowest part generates the hetero-duplexes obtained through denaturation-renaturation, and the faster one, the homoduplexes (figure 3).

The samples were different from the wild type due to the fact that they contain amplified mutant type DNA, which through electrophoresis leads to speed migration modification.

The mutation detected by us is a substitutive type one as a series of 2 bands was evident on the migration front.

There were no seen mutations for the rest of the analyzed cases for the exon 55.

All detected mutations can be observed in table no. 5

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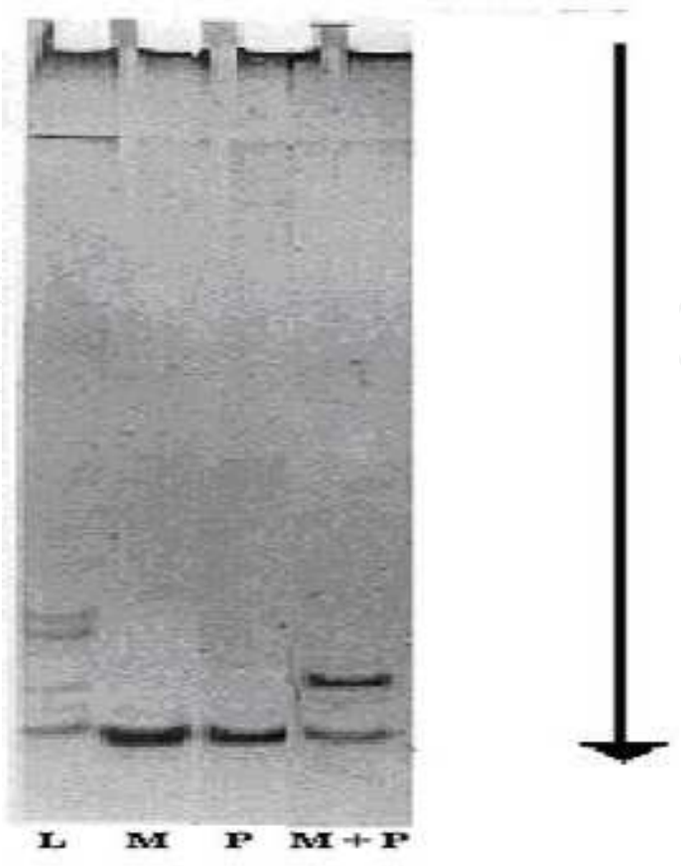

L – molecular weight ladder. M – healthy individual allele (control). P – unprocessed allele for exon 54. M+P – denatured-renatured sample.

Figure 3. Electrophoresis in 6% polyacrylamid gel of the processed samples in order to point out heteroduplexes for exon 54, using silver staining.

Among 229 patients with advanced neoplasia (tubular adenoma 1 cm in diameter or larger, villous polyp, polyps with high-grade dysplasia, or cancer), 69 patients (27.60%) presented mutations in *COL11A1* gene in at least 1 exon (table no. 5 and 6).

Regarding benign polyps, none of patients presented *COL11A1* mutations.

19. Disscusions and conclusions

COL11A1 gene overexpression has been implicated as a candidate marker of various types of cancers [26]. Previous studies have found overexpression of the *COL11A1* gene in different types of cancers, such as non-small cell lung (NSCLC), ovarian, oral cavity and colorectal cancers [13-16]. In particular, overexpression of the *COL11A1* gene was found to be correlated with invasion and metastasis of these cancers [13-16].

As we previously detected in a pilot study regarding genetic mutations related to *COL11A1* gene in exfoliated epithelial cells in the stool, we found mutations involving exon 54 [27].

Our present study confirmed the presence of *COL11A1* mutations in patients with colorectal adenomas or cancer. Polyacrylamide gel electrophoresis method for the heteroduplex analysis (HA) was a sensitive genetic method to diagnose mutations of *COL11A1*.

Mutations detected in biopsy cells were present in exfoliated cells from feaces, proving the usefulness of this genetic approach for noninvasive early diagnosis. Genetic alterations were detected at the level of exons 54, 56 and 57.

Our results are similar with the results of other studies which have previously shown that *COL11A1* is upregulated in the majority of sporadic colorectal cancer [28], emphasizing the fact that the expression of *COL11A1* could be the primary change giving rise to a tumorigenic response in epithelial cells [28].

1. Another study found a statistically significant overexpression of *COL11A1* in polyps from a patient with FAP [29]. The results from this study suggested that the expression of COL11A1 could directly contribute to tumorigenesis in fibroblasts in FAP and explain osteomas and desmoids, or indirectly to polyp-formation and tumor progression in sporadic CRC [29].
2. The study of Croner R et al [30] also showed up-regulation of *COL11A1* in CRC versus normal colonic mucosa ($p<0.001$). The same result was shown by Lascorz et al study, in which extracellular matrix receptor interaction and focal adhesion shared nine genes (*COL1A1, COL1A2, COL3A1, COL4A1, COL11A1, FN1, ITGA2, SPP1*, and *THBS2*) were upregulated in colorectal cancer [31].

Joined by other well known genetic tools already examined in the stool cell's DNA involved in the development of some colorectal cancers, *COL11A1* could be a feasible genetic biomarker targeted in stool samples for early diagnosis of colorectal cancer at risk patients.

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