
Genotype-Phenotype Disturbances of Some Biomarkers in Colorectal Cancer

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1. Introduction

Colorectal carcinoma (CRC) is one of the most common human cancers. In 2008, 1,233,000 new CRC patients were diagnosed worldwide and about 608,000 deaths caused by colorectal cancer were estimated making it the fourth most common cause of death from cancer in the world. Five-year survival for CRC patients indicates a percent of 54.0% in Europe. Additionally, from the five-year survival, it was observed 74.0% of survival for patients with stage I, 66.5% for patients with stage IIA, 73.1% for patients with stage IIIA and only 5.7% for patients with stage IV disease (Stanczak, 2011). The success of colorectal cancer screening programs has resulted in an increasing number of biopsies of early neoplastic lesions with subtle histological features, making development of ancillary diagnostic testing for CRC essential. The incorporation of ancillary techniques, such as immunohistochemistry, cytochemical staining, electron microscopy, cytogenetic and, more recently, molecular testing, has made a significant impact in the diagnosis and management of solid tumors. Interpretation of hematoxylin-eosin stained slides by light microscopy remains the basic of anatomic pathology. However, an expanding menu of molecular assays continues to be implemented owing to their clinical utility in diagnosis, prognosis and risk assessment, therapy selection, as well as cancer screening and minimal residual disease detection. Carcinomas tend to carry multiple, complex, non-recurrent chromosomal and molecular aberrations, and they were not traditionally considered ideal candidates for molecular testing. However, this is changing with the discovery and implementation of new diagnostic, prognostic, and therapeutic molecular markers. Although single molecular biomarkers have proved useful, technical advances allowed performing the global genomic, epigenomic, or proteomic profiling of solid tumor malignancies. The research continues for more definitive molecular indicators that correlate with histological features and patient response to therapy and/ or survival.

Increasing understanding of cancer biology is beginning to explain the reasons for therapeutic failures. Signal transduction research have revealed that the receptors, enzymes and transcription factors that regulate cell fate are virtually all connected into a complex network of cross-regulatory interactions. The cell fate control system is not only interconnected but also highly redundant, such that if a gene or protein is disabled, another can perform a similar function (Rizzo, P, 2008). Key molecular mechanisms implicated in the genesis of CRC include chromosomal instability, DNA repair defects, and aberrant methylation. Chromosomal instability causes structural chromosomal anomalies, usually during DNA replication, with subsequent loss of tumor suppressor genes. DNA repair defects are caused by mutations in genes responsible for the repair of base-base DNA mismatches. These can be found as germline mutations or somatic methylation anomalies in acquired cases of CRC. A significant proportion of cases of CRC associated with mismatch repair anomalies occur on the right side of the colon and have a characteristic histological appearance. DNA repair defects can be detected indirectly by the associated epiphenomenon of microsatellite instability or unrepaired strand slippage within microsatellite regions.

Taking all these into account, we can conclude that study of colorectal carcinogenesis provides fundamental insights into the general mechanisms of cancer evolution. Now, it is believed that there are two patho-genetically distinct pathways for the development of colon cancer involving stepwise accumulation of multiple mutations. However, the genes involved and the mechanisms by which the mutations arisen are different.

The pathway, sometimes called the APC/ β -caterin pathway, is characterized by chromosomal instability that results in stepwise accumulation of mutations in a series of oncogenes and tumor suppressor genes. The molecular evolution of colon cancer along this pathway occurs through a series of morphologically identifiable stages. Initially, there is localized colon epithelial proliferation. This is followed by the formation of small adenomas that progressively enlarge, become more dysplastic, and ultimately develop into invasive cancers. This is referred to as the adenoma-carcinoma sequence. The genes that are correlated with this pathway are as follows:

Adenomatous Polyposis Coli (APC) - *APC* gene is located on chromosome 5 in 5q21 locus, and the mutations appearing at its level are responsible for the progression of CRC. Reported mutations in the *APC* gene include missense mutations and deletions, resulting in synthesis of truncated APC proteins. While "inherited" mutations are not clustered in a certain region of the gene but appear at the 5'-end or in nearby it, somatic mutations are clustered in the central region. The *APC* gene mutation is the genetic basis for FAP (Familial Adenomatous Polyposis) syndrome and fulfills the "first hit" concept advanced by Knudson in the 1970s. FAP patients have hundreds to thousands of colorectal adenomas and early onset carcinoma and allelic mutation of the *APC* gene followed by a loss of heterozygosity (LOH) is a common feature. Loss of this gene is believed to be the earliest event in the formation of adenomas. APC is involved in cell migration and adhesion and regulates levels of β -catenin (Senda T, 2005), an important mediator of the Wnt/ β -catenin signaling pathway. More than 80% of CRC have inactivated *APC*, and 50% of cancers without *APC*

mutations have β -catenin mutations (Muhammad WS, 2010). *APC* gene product, a 310kDa protein located both in the cytoplasm and in the nucleus, interacting with β -catenin on the signaling pathway of Wnt-1. At the N-terminus site, the APC protein contains Armadillo-repeat binding domains and oligomerization domain and at the C-terminus site there are EB1 and tumor suppressor protein DLG binding domains. The APC protein also contains three 15-amino acids and seven 20-amino acids repeat regions from which the second one was show to be involved in the negative regulation of β -catenin protein expression in cells. At the 5'-end *APC* gene we can found the mutation cluster region (MCR) which is responsible for most of the mutations in *APC* gene which create truncated proteins. The truncated proteins contain ASEF (APC-stimulated guanine nucleotide exchange factor) and β -catenin binding sites in the armadillo-repeat domain but loose the β -catenin regulatory activity which is located in the 20-amino acids repeat domain (Narayan S, 2003). The diverse effects of mutations in *APC* gene indicates that this molecule plays a key role in the regulation of cell growth in a number of colonic and extracolonic tissues.

β -Catenin is a member of the cadherin-based cell adhesive complex, which also acts as a transcription factor if the protein is translocated to the nucleus. When it is not bound to E-cadherin and participating in cell-to-cell adhesion, a cytoplasmic degradation complex (consisting of APC, Axin, GSK-3 β , and β -catenin) leads to β -catenin phosphorylation and degradation. When *APC* gene loss the normal function, β -catenin is not efficiently degraded and accumulates in the cytoplasm and is translocated to the nucleus where bind to a family of transcription factors called T-cell factor (TCF) or lymphoid enhancer factor (LEF) proteins and lead to transcriptional activation of certain target genes like c-Myc and Cyclin D. β -Catenine gene (*CTNNB1*) is located on the 3p chromosome and modifications in expression are associated with both early and tardive genetic events (Stanczak A, 2011). Most human cancers that involve *CTNNB1* mutations possess changes in exon 3 (amino acid residues in the N-terminus region), which provides loses binding affinity to GSK-3 β , the kinase that phosphorylates and degrades β -catenin, in normal cells (Samowitz W.S., 1999). *APC* mutations are present in 80% of sporadic carcinomas (Knudson AG, 2001). Mutations in the *CTNNB1* gene at various key phosphorylation sites have been identified in CRC and several other solid tumors and it seem to prevent destruction of β -catenin by the proteasome pathway, which then leads to constitutive activation of Wnt signaling.

The **E-cadherin** gene (*CDH1*) is located on chromosome 16q22.1 and it contains 2.6 kb of coding sequences with 16 exons. There are overwhelming genetic data to support the role of E-cadherin as a tumor/ invasion suppressor in epithelial cells, and loss of expression, as well as mutations, has been described in a number of epithelial cancers. The implication of the *CDH1* gene in the process of carcinogenesis was initially associated with the gastric cancer because at this gene level somatic mutations which were associated with different types of diffuse gastric cancer (Becker KF, 1994) were observed. Subsequent research showed the existence of some germline mutations of *CDH1* in the families with dominant autosomal susceptibility for the hereditary diffuse gastric cancer (Suriano G, 2005). The genetic studies up to the present are sustaining the suppressor invasive/tumoral role of E-cadenin in the epithelial cells, and the expression loss along with mutations were described in some types

of epithelial cancers (breast, colorectal, thyroid, endometrium, ovary cancer). Allelic imbalances of the LOH type were frequently observed in metastasizing malignancies derived from liver, prostate and breast. It is presumed that the loss of function contributes to the cancer progression by increasing the level of proliferation, invasion and/or metastasis. The E-cadherin phenotypic expression in carcinomas is very well known, but the studies on the appearance of allelic imbalances at the *CDH1* level are rare. E-cadherin expression modifications are frequently associated with a high tumoral level, like the disease of prostate, breast, bladder, pancreas, stomach and colon. The mature protein product belongs to the family of cell-cell adhesion molecules and it plays a fundamental role in the maintenance of cell differentiation and the normal architecture of epithelial tissues (Stanczak A., 2011, Handschuh G, 1999). As an epithelial cell adhesion molecule E-cadherin mediates the contact between neighboring epithelial cells, including the colorectal epithelial cells, and helps to establish the defined membrane domains and cell polarity (Goodwin and Yap 2004). The extracellular domain of E-cadherin is responsible for homotypic binding of adjacent cells, and the cytoplasmic domain of E-cadherin facilitates adhesion through interaction with catenin proteins (Bryant and Stow 2004). The ectodomain of this protein mediates bacterial adhesion to mammalian cells and the cytoplasmic domain is required for internalization. Identified transcript variants arise from mutation at consensus splice sites. E-cadherin expression in epithelial cells is crucial for the establishment and maintenance of epithelial cell polarity.

BRCA1 gene mapped on the long arm of chromosome 17 (17q12-21) was identified by positional cloning methods. Mutations at the level of this gene are responsible in part for inherited predisposition to ovary, breast, prostate and colon cancers. However, whether these mutations are a factor in sporadic forms of these tumours remains unclear. Loss of *BRCA1* heterozygosity represents a molecular alteration presented in colorectal cancer, with unfavorable consequence in survival rates and that can be considered an independent prognosis factor in steps I and II of colorectal cancer stages (Roukos D., 2010). *BRCA1* is a large gene with many functional domains, each with different biological features. The C terminal region is related to the transactivation region of the protein and residues 758–1064 to the domain binding to Rad51, thus working as a complex to repair double stranded DNA breaks. In relation to its repair role, *BRCA1* is also related to co-activation of p53. The relationship of truncating germline mutations in the *BRCA1* gene and breast and ovarian cancers is established. Mutations in this gene are responsible in part for the inherited predisposition to breast and ovarian cancers, and probably for one third of all site specific inherited breast cancer. In previous studies, researchers found a high percentage of LOH in the 17q21 region in sporadic CRC cases. BRCA proteins have a significant role in multiple pathways, signaling cell cycle delays for DNA lesions or leading to apoptosis for severe damage. BRCA proteins function in transcriptional regulation and chromatin remodeling, and they are required to repair double-strand breaks. Double-strand breaks in mammalian chromosomes stimulate the activity of recombination repair enzymes by more than 100-fold. In transformed colon cells of *BRCA1* mutation carriers, *BRCA1* functions are probably lost. In almost all colorectal cancers, the mutated *APC* gene, lead to *MYC* over-expression and as

consequence involve *BRCA1* over-expression. *BRCA1* directly link *MYC* at double-strand break repair and participate to the preserving genome integrity. When *BRCA1* is mutated and have only one normal allele, *MYC*-associated loss of homology - directed recombination repair should occur earlier than in individuals with two normal *BRCA1* alleles. *BRCA1* expression is reduced in at least some sporadic colon adenocarcinomas and somatic loss of one normal *BRCA1* allele is common not only in hereditary but also in sporadic CRC tumors (Friedenson B, 2004).

Group IIA PLA₂ is a 14-kDa enzyme found in a number of tissues and secretory products (Nevaleine TJ, 1993). The plasma concentration of the enzyme increases dramatically in severe infections and other diseases involving generalized inflammation and cancer (Ogawa M, 1991). In the gastrointestinal tract, expression of group IIA PLA₂ has been localized in Paneth cells of the small intestine (Nevaleine TJ, 1995), metaplastic Paneth cells of gastric (Nevaleine TJ, 1995) and colonic mucosa (Haapamaki MM, 1999) as well as columnar epithelial cells of inflamed colonic mucosa. Functional defects in PLA₂ in tumor cells may interfere with the regulatory mechanisms of tumor growth. The *PLA2G2A* gene function is relevant in tumorigenesis, and is a good candidate gene modifying the *Apc* gene in the Min (multiple intestinal neoplasias) mice. On the one hand, it has been suggested that a mutation resulting in splice variants of the *Pla2g2a* gene and in different truncated forms of its protein accounts for the increased number of polyps in mice carrying the Min mutation. Numerous studies suggested that *Pla2g2a* is a candidate gene for *Mom-1*. The analysis of a mouse/ human hybrid panel showed that the *PLA2G2A* gene, located on the human chromosome 1p, is a candidate gene for the MOM-1 locus, (Spirio LN, 1996; Ishiguro Y, 1999; Mounier CM, 2008). It was also observed that the *PLA2G2A* gene is intact, but an allelic imbalance (AI), or an allelic loss, was found at one of the alleles and a loss of heterozygosity (LOH) was identified on *PLA2G2A* regions (Mihalcea, A, 2009).

The **EGFR** is a member of the HER (human epidermal growth factor receptor) family, and includes HER1 (EGFR, ErbB-1), HER2 (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4) (Boss JL, 1989). The natural ligands for EGFR include EGF, transforming growth factor (TGF), amphiregulin, heregulin, heparin-binding EGF, and cellulin. Ligand binding induces receptor dimerisation and subsequent auto-phosphorylation that activates critical pathways for cellular survival and proliferation such as PI3K/Akt, Stat, Src and MAPK. EGFR mediates signaling by activating the MAPK and PI3K signaling cascades (Jhaver M, 2008). EGFR modifications have been described in many cancers as a consequence of mutations or gene amplifications that induce protein over-expression, structural rearrangements and autocrine loops. EGFR abnormalities may have a relevant role in both carcinogenesis and clinical progression of CRC. EGFR is differentially expressed in normal, premalignant, and malignant tissues, and over-expression of EGFR has been documented in up to nearly 90% of cases of metastatic CRC (Boss JL, 1989; Arteaga CL, 2001). In addition, EGFR is over-expressed in a wide range of solid tumors and is involved in their growth and proliferation through various mechanisms. Given the documented role of EGFR in the development and progression of cancers, this receptor signaling pathway represents a rational target for drug development (Vokes EE, 2006; Lee JJ, 2007). Recent clinical data have shown that advanced

colorectal cancer with tumor-promoting mutations of these pathways -- including activating mutations in KRAS, BRAF, and the p110 subunit of PI3K-- do not respond to anti-EGFR therapy.

The variability in clinical presentation, aggressiveness, and patterns of treatment failure suggests distinct genotypes and phenotypes identification, which can help future treatment strategies. A new concept called “personalized medicine” may be another beginning of a new era and it has been designed to offer every patient a suitable therapy. By this new approach, “Personalized medicine” can be defined as the tailoring of medical treatment to a specific subset of patients who are usually identified by genetic markers or other molecular profiling strategies. There is an increasing interest in this therapeutic strategy on the part of pharmaceutical and bio-pharmaceutical companies, consumers, and third party payers. Consequently, the level of clinical trial activity surrounding personalized medicines is intensifying as sponsors seek ways to target their therapies to patient populations that would most benefit from them. The aim of the present chapter is to elaborate an experimental model in order to improve the “personalized” therapeutically strategy, by evaluating some key gene expression involved into a crosstalk signaling, in colorectal cancer.

By our study design we have evaluated the comparative expression at proteic and genetic level of several key point proteins (*APC*, *PLA2G2A*, *CDH1*, *BRCA1*, and *EGFR*). Our *in vivo* experiment involved diagnosis testing of CRC patients and molecular biology testing on biological samples in order to clarify the cross-talk of interested genes and to better understand the CRC typology among Romanian patients.

The idea of applying such a model to our studies was generated during the research that we conducted in our projects. We have noticed that between different proteins and genes is a very close relationship, which depends on the tumor type, cell grade and staging. Following a study of a large number of articles published in the international databases we observed that other researchers have drawn the same conclusion.

2. Results and discussion

2.1. Tissue samples and blood

Samples were obtained with the consent of 93 patients, consisting of histopathologically confirmed colorectal adenomas. Samples were obtained during colonoscopy with biopsy forceps, by harvesting at least four fragments from all the quadrants of the pathological tissue. The surgical intervention for CRC treatment included radical and palliative techniques (right or left hemicolectomy, segmentary colectomy, low anterior rectal resection–Dixon, Milles operation, Hartmann operation). All tumors were histologically (HP) examined by pathologist in order to: (a) confirm the diagnosis of adenocarcinoma, (b) confirm the presence of tumor and evaluate the percentage of tumor cells in these samples, and (c) carry out pathological staging. The complete HP diagnosis included: degree of differentiation (well/ moderate/ poor), vascular, neural and lymphatic invasion, status of the

margins of resection (invaded/ noninvaded) and also TNM stadialisation. After surgical resection, tumor tissues were cut in small pieces, frozen immediately in liquid nitrogen and stored at - 80°C until they were analyzed.

For the initial patients group, only 75 patients who had at least 75% tumor cells were taken in consideration for molecular biology analyses. To perform immunohistochemistry by immunofluorescence (IHF) analyses, five micrometers thick tissue serial sections were incubated with primary antibodies diluted in BSA (bovine serum albumin) in PBS (phosphate buffered saline). After washing with PBS, FITC-conjugated secondary antibodies (Invitrogen) were applied and then the samples were washed again. The protein expression was evaluated by fluorescent microscopy. In order to analyze the mutational status, DNA was extracted from patients' venous blood (as control) and from tumours. DNA preparation was performed using the *Wizard® Genomic DNA Purification kit* (Promega) according to the manufacturer's recommendations. The extracted DNA was stored at -80°C until molecular biology analyses.

2.2. Clinicopathological characteristics

The medical records of all 93 patients provided their birth date and sex, and the following parameters: tumor location, tumor size, lymph node metastases, pathological stage, vascular and neural invasion and tumoral differentiation grading.

Out of 93 cases, there were 40 womens and 53 mens. The mean age was 50 years. The majority had T3 tumors (31.8%); T2 tumors (25.80%) according to tumor stage of the TNM classification of colon and rectum neoplasm and 53 patients (57%) had lymph node involvement (N+). In the study lot, 17 cases (18.27%) presented metastasis at the time at CRC diagnosis. These were predominantly localized in the liver (12 cases, 70.58%) and rarely in the lungs (4 cases, 23.52%).

Regarding the histopathological type of colorectal tumors, the vast majority was adenocarcinomas (ADK) with different grades of differentiation. Most of the tumors (42 cases: 45.16%) were well differentiated (G1) while 33 cases (35.48 %) were moderately differentiated (G2) and 18 cases (19.35%) poor differentiated (G3) tumors. Beside typical adenocarcinoma another histopathological type of tumors was rare and was localized: i) to the right colon - especially mucinous ADK (5 cases from a total of 9 cases in the all study lot) and 1 adenosquamous carcinoma; ii) to the left colon - 2 cases of mucinous ADK and 1 case of "signet-ring" cell carcinoma; iii) to the rectum - 2 mucinous ADK, 1 squamocellular carcinoma and 1 case of anaplazic carcinoma. Patients characteristic is summarized in Table 1.

Our study has not taken into consideration the diet, because most of the patients do not know the food properties or they use food with pro-carcinogen potential. Regarding the diet, we consider that the patient instruction is extremely useful and has to be done by the surgeon doctor after the surgical treatment and then by the family doctor. This approach allows both secondary prophylaxis and control of possible relapses/ recidivists. A monitoring of the patients included in the study will shows the efficiency of medical control

and the conscious of this mortal disease. In the studied lot of patients we have not registered cases with relapse, and we cannot predict their future behavior.

CLINICO-PATHOLOGICAL CHARACTERISTICS OF CRC TUMORS	No CASES n (%)
Age	
< 50	12
> 50	81
Gender	
Male	53
Female	40
Tumor localisation	
RC	13
LC	42
RECTUM	38
Stage	
I	23 (24,73%)
II	24 (25,80 %)
III	29 (31,18%)
IV	17 (18,27%)
Lymph nodes status	
N – (N0)	40 (43%)
N +(N1,2,3)	53 (57%)
Histopathological grading	
Well differentiated G1	42
Moderately differentiated G2	33
Poor differentiated G3	18
Total	93

Table 1. Clinico-pathological characteristics of CRC tumors in the study lot

2.3. Immunohistochemical expression by immunofluorescence of the studied proteins

Because the interpretation of immunohistochemistry analyses remains the basic of anatomic pathology, in our study we first evaluated the protein expression of the key point proteins that were taken in our study. Unlike the normal histopathological analyses, our evaluation was based on protein fluorescent signal which, from our point of view, is more specific than classical immunohistochemistry.

The expression of α -SM (smooth muscle) was included in our study as a positive control to prove the method accuracy and it is used as a typical marker for myofibroblasts. It is one of the four muscle actin isoforms, a protein involved in supporting basic contractile apparatus in muscle cells. This expression can be found in vascular cells, intestinal muscularis mucosae and muscularis propria, and in the stromal tissue. In normal tissue, the immunofluorescence signal is strong (+3) around tumor crypts, in the vessel walls and stromal smooth muscle

fibers. In the crypt epithelial cells the signal is absent (-). In CRC patients the α -SM expression decreases with increasing disease grade, and disappear in most of the advanced CRC, when the tissue is disorganized and a lot of tumor cells are present (Figure 1).

By labeling the APC C-terminus, there were observed changes of protein expression in tumor tissue compared with APC expression in normal tissues. In normal tissues, muscle tunic polyps analysis confirmed the expression of target protein in SM from blood vessels and fibers of the smooth muscle shell structure, where it is stored.

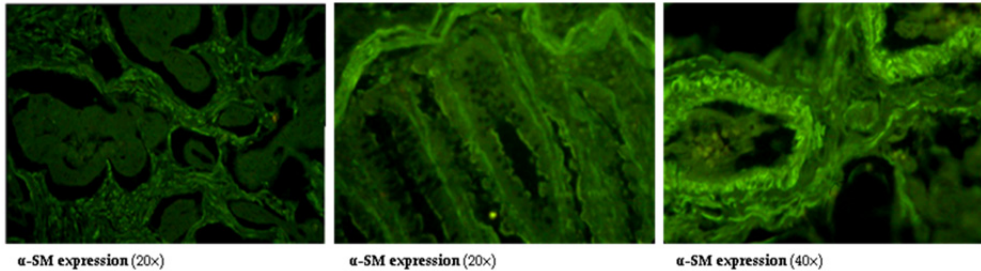


Figure 1. α -SM expression. Smooth muscle, used as a positive marker for immunofluorescence signal, have immunofluorescent signal in blood vessels, intestinal muscularis mucosae and muscularis propria, and in the stromal tissue.

With few exceptions, the intensity of fluorescent signal given by the expression of APC is strong (3+), fluorescent signal obtained overlapping fluorescent signal of α -actin expression given by smooth muscle cells (Figure 2). Adenocarcinomas of the colorectal mucosa analysis revealed APC expression changes. During tumorigenesis process, the mucosa is invaded by stromal tissue, the crypts become large, elongate, their architecture is destroyed and the fluorescent signal intensity of epithelial cells (CE) decreases becoming weak (1+).

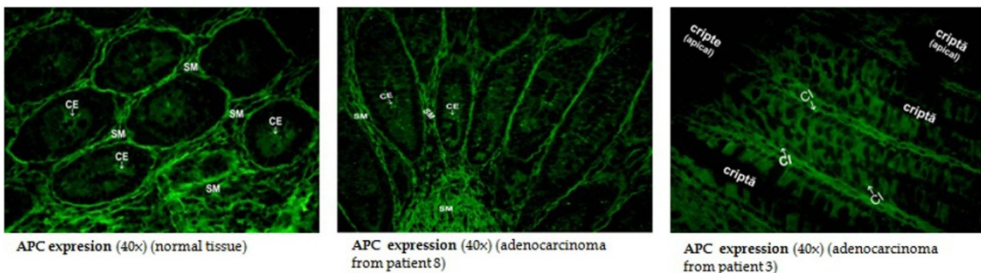


Figure 2. APC expression. A normal expression with immunofluorescent signal on the border of the crypts and in SM cells can be observed on 8 patient's section, like in normal tissue. On section obtained from patient 3 we can observe a weak intensity on the apical part of epithelial cells and loss of signal, too.

At the same time we observed an increase of its intensity in neoplastic infiltrated cells (CI). In the apical half of the fluorescent signal crypt, epithelial cells and infiltrated cells disappeared (-). The IHF expression pattern overlaps the APC sequential histopathological

changes occurring in the colorectal carcinogenesis, in which β -catenin and APC play the role of so-called "Second Hit".

In normal colorectal tissue, **β -catenin** expression appears on the membrane of epithelial cells. In tumor tissue, can occur either over-expression of β -catenin in the nucleus where it is translocated from the cytoplasm as a result of APC mutation, or signal absence when β -catenin changes. In our study, 33.33% (25/ 75) of patients show a similar β -catenin expression to that of normal tissue because the fluorescent signals were obtained on the membrane of epithelial cells. In 33 CRC patients, the β -catenin target protein expression was changed compared with normal tissue (Figure 3).

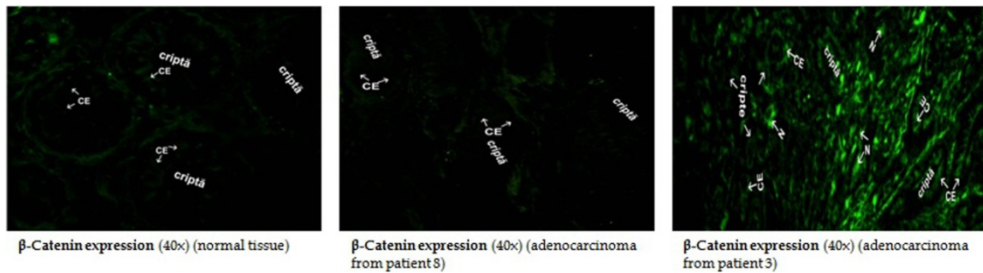


Figure 3. β -Catenin expression on patient 8. A normal expression with immunofluorescent signal on cytoplasm and on the border of crypts can be observed on the section from patient 8. On section from patient 3 we can observe an over-expression in the cytoplasm/ nucleus of epithelial cells and loss of expression in the membrane.

We can observe how the fluorescent signal on the membrane of epithelial cells gradually decreases in intensity during the tumor progression, along with increased fluorescent signal by over-expression in cytoplasm (in 28 patients) and in the nucleus (in 5 patients).

Regarding **E-cadherin** expression, colorectal tumors showed a heterogeneous type of expression compared to the normal colorectal epithelium in which E-cadherin expression is present on the basolateral membrane to the whole length of the glandular crypts and on the intercellular membranes. An abnormal pattern of expression is observed on CRC tumor sections: i) a reduced expression (2+, 1+) at the membrane level was observed in 20% (15/ 75) of patients; ii) cytoplasmatic expression was observed in 37.33% (28/ 75) of patients and the expression is similar to that observed for β -catenin; iii) loss of expression (-) was observed in 12% (9/ 75) of patients. In 30.66% (23/ 75) of patients, the E-cadherin expression was similar with that observed in normal colon epithelium, in the cell membrane, with strong immunofluorescent signal (3+) and is co-localized with membrane β -catenin (Figure 4).

Comparative analyses of E-cadherin protein expression for CRC tumors with various histological differentiation grades (G1, G2, G3), showed an almost similar expression pattern for all G1, G2 and G3 tumor grades, although the majority of the well differentiated G1 tumors indicated strong membranous signal; the moderately differentiated tumors (G2) showed a heterogeneous membranous signal and some of the poorly differentiated tumors (G3) had no membranous expression for E-cadherin. In the case of lymph nodes analyses,

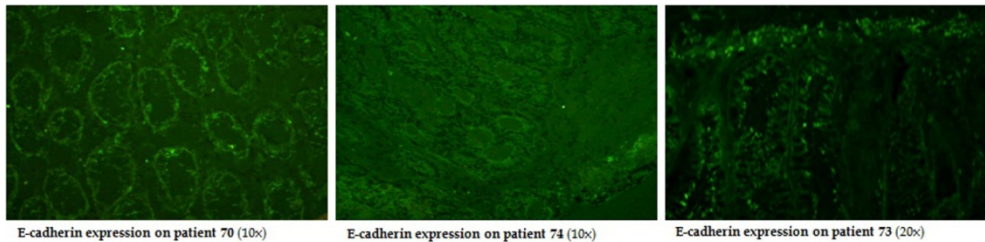


Figure 4. E-cadherin expression. A normal expression with immunofluorescent signal on the membrane of epithelial cells can be observed on section from patient 70. In the case of patient 74 we can observe a reduced/ loss of expression in the epithelial cell membranes. On patient 73 an over-expression in the cytoplasm of epithelial cells and in some infiltrating cells was noticed.

there is a strong correlation between the presence of the lymph node invasion status and protein expression of E-cadherin. From a total number of 75 cases of CRC, we observed that patients with lymph node invasion N + (N1, N2, N3) have low or no expression of E-cadherin. Thus E-cadherin could be considered a biomarker that can help to determine the risk in patients with CRC, and a strong indicator of the lymph node status. In the group of N0 CRC tumors from 27 cases, only 77.77% (21/ 27) of patients presented E-cadherin membrane expression in different staining grades, scored as 0, 1+, 2+ , while in the group of lymph node invasion N+ tumors (48 cases) only 35.41% (31/ 48) of patients were positive for membranous staining (0,1+, 2+).

In normal colon mucosa the **sPLA₂ type IIA** enzyme was detected by a strong staining in muscularis mucosae in a large fraction of SM cells (recognized by α -SM actin antibody) and vascular SM cells (Figure 5). In lamina propria, the PLA₂ type IIA enzyme was detected with a weaker staining (2+), surrounding the crypts (as determined by morphological and histological evaluation), and in vascular smooth muscle. These results show that PLA₂ type IIA enzyme is expressed only in smooth muscle cells from normal colon mucosa. An abnormal pattern for PLA₂ type IIA expression was observed in 27 of the 75 CRC cases (36.00%), which were examined. In muscularis externa and submucosa, the SM cells express PLA₂ type IIA with a strong intensity (3+). The presence of PLA₂ type IIA was not observed (-) in other types of cells.

Beginning with mucosa, the PLA₂ type IIA expression started to be modified. Thus, near the submucosa, the immunofluorescence signal for PLA₂ type IIA was observed in SM cells from lamina propria, but only around crypts, and with a weak signal comparative with the normal pattern (1+). As the crypts get longer with more ramifications, the number of SM cells that express PLA₂ type IIA decrease, although we had a positive signal for α -SM actin from all the SM cells. In this area, PLA₂ type IIA expression was found in epithelial cells, on the border of Lieberkühn crypts. The number of epithelial cells that express PLA₂ type IIA increases during the crypts growing. The immunofluorescence signal is also stronger (3+) than fluorescent signal observed in SM cells. No immunoreaction for PLA₂ (type II) was found in all 11 patients' sections (14.66%) that were analyzed. This may suggest that the malignant cells lose their ability to express PLA₂ type IIA, when invasive carcinoma develops in the adenoma.

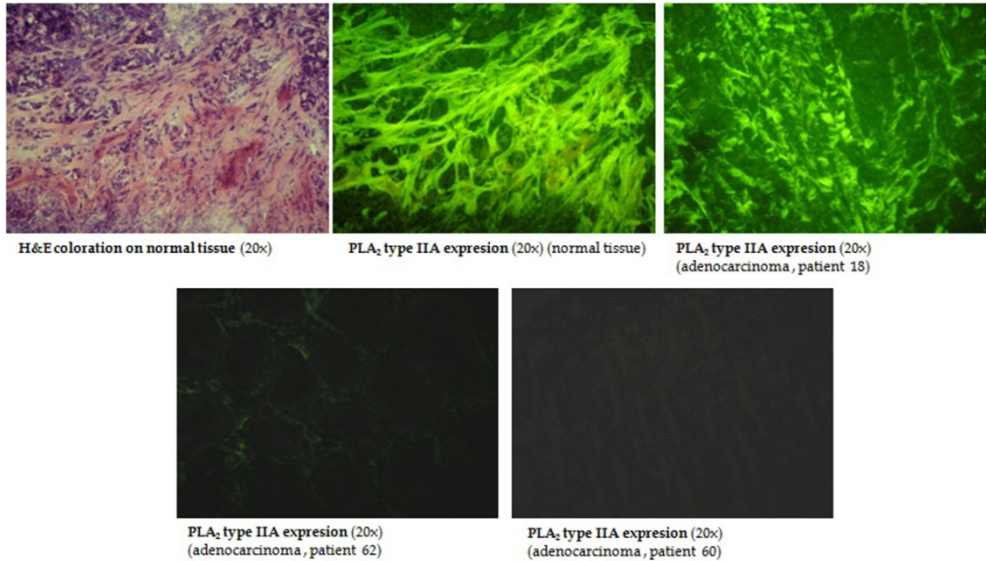


Figure 5. PLA2 type IIA expression. A normal expression with immunofluorescent signal in SM cells can be observed on section from patient 12. On section from patient 18 we observe an over-expression in infiltrated cells. Patient 62 shows a weakly signal on SM cells around the crypts and on vascular smooth muscle. In the case of patient 60 the loss of signal is remarked.

We characterized the expression of **BRCA1** in 75 sporadic colorectal carcinomas. It was found an increased BRCA1 expression in the apical cell pole of epithelial malignant cells and a significant increase in BRCA1 nuclear foci in tumor colorectal specimens in comparison with the corresponding normal tissues, in 10 cases out of 75 (13.33%). These increases in BRCA1 expression may be explained by the fact that colorectal tissue is subject to very active proliferation and differentiation. In 14 cases out 75 (18.66%) we observed the loss of BRCA1 expression (Figure 6).

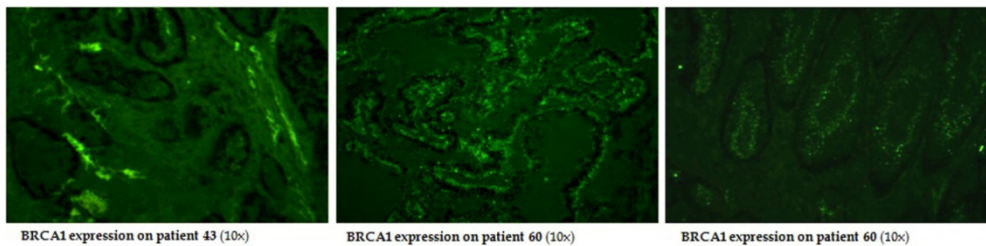


Figure 6. BRCA1 expression. Patient 43 showed loss of expression in nucleus of epithelial cells. On patient 60 we can observe an over-expression on the epithelial cells from the crypt foci. On other sections from patient 60 over-expression was observed only on the apical pole of epithelial cells.

The epidermal growth factor receptor (EGFR) expression had an abnormal pattern in 41.33% (31/ 75) of patients. Out of these, the signal intensity was weak (1+) in 22.58% (7/ 31)

of patients and moderate (2+) in 32.25% (10/ 31) of patients. Moreover, in both cases EGFR expression was observed in cytoplasm of tumoral cells (Figure 7). Complete strong circumferential expression (3+) was found in 45.16% (14/ 31) of patients. Normal expression, like signal absence was observed in 58.67% (44/ 75) of patients. In our study (2+) and/ or (3+) were defined for those cases with EGFR expression in 50% or more tumoral cells on the section. By our study we observed that EGFR expression was significantly associated with higher rates of cell proliferation. EGFR activation and intracellular signal can be a result of its roles in transcription, up-regulation, degradation and gene amplification. Our results demonstrate that EGFR over-expression is correlated with higher tumor stage (III and IV) as compared with weaker EGFR expression. Due to the knowledge of EGFR expression in CRC, now it is possible to apply targeted therapy with cetuximab-EGFR monoclonal antibodies in the treatment algorithm of the CRC at the EGFR-positive patients identified by IHC examination. Also, the observed differentiated association between EGFR expression, ganglion EGFR status – N and tumor differentiation degree - G, could significantly assign to the EGFR the role of prognostic marker for disease recurrence. Determination of EGFR status may be used to identify cases of CRC, which could benefit from anti-EGFR therapies and on the other hand would have the potential to be a rigorous mean for monitoring efficacy of anti-EGFR therapy in CRC (Mendelsohn, 2003). Although EGFR remains a controversial prognostic factor, the association between EGFR over-expression and tumor stage may have an important role in the anti-EGFR therapy of patients with CRC.

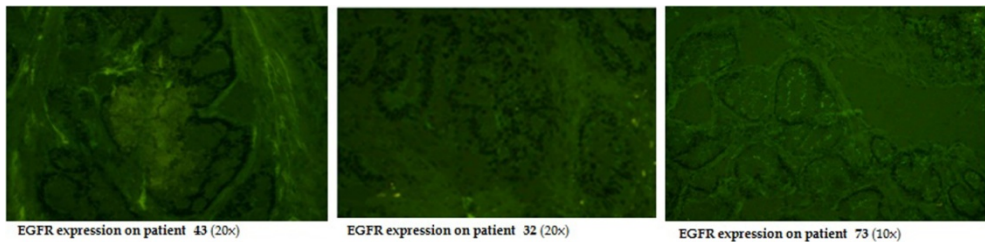


Figure 7. EGFR expression. On patient 43 we can observe an over-expression on the membrane of epithelial cells from the crypt foci. In the case of patient 32 we remarked loss of expression. Patient 73 presented expression in cytoplasm of tumoral cells.

2.4. Deletion/duplication evaluation for the interested genes (MLPA)

MLPA analysis detects large deletions or duplications in the gene. This is a semi quantitative reaction based on PCR identifying copy number variations and contributes for assessing predictive genetic markers giving an intra-individual variation spectrum of the genes included in this study. It is also a useful tool for the diagnosis of genetic diseases characterized by large genomic rearrangements. In order to perform the test on blood and tissue samples in the first step of our analyses we optimized the procedure for the specific genes. For each gene we optimized the range of DNA concentration in order to have a good signal and to obtain the most suitable mix of primers that we have to use. After protocol optimization we went through the technique and in each run we used three DNA samples from blood and tissue for each patient.

According to the microsatellites alteration assay we performed the MLPA analysis of *APC* and *BRCA1* genes and two other genes (*EGFR* and *CDH1*) were included.

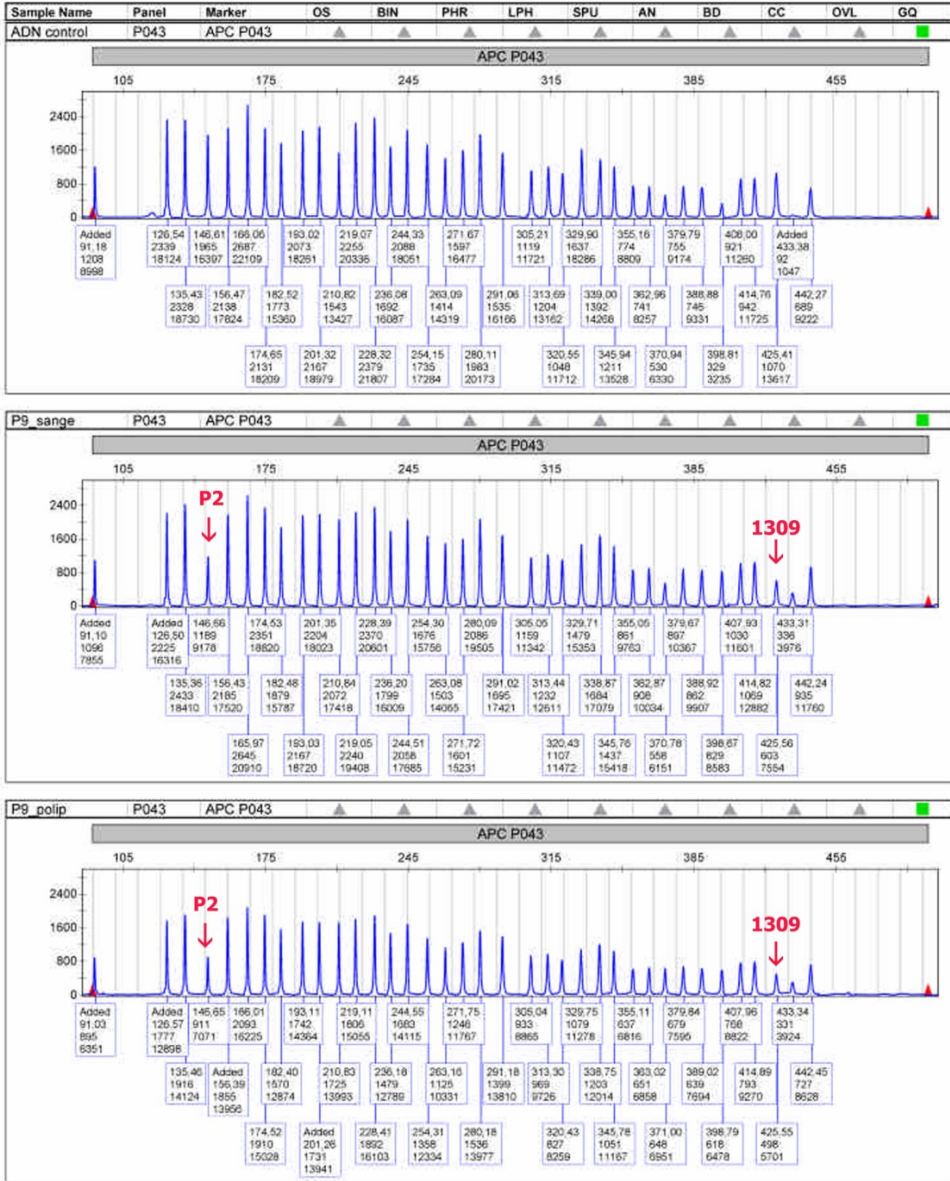


Figure 8. MLPA chromatograms for patient with FAP (patient 15).

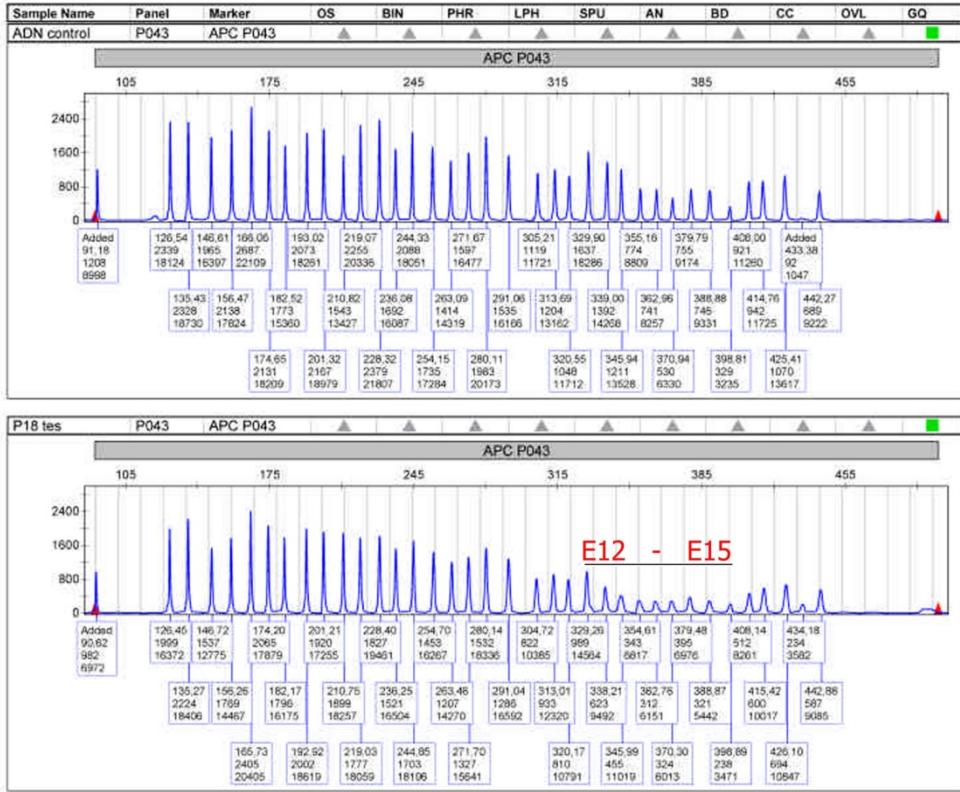


Figure 9. MLPA chromatograms for the patient 31.

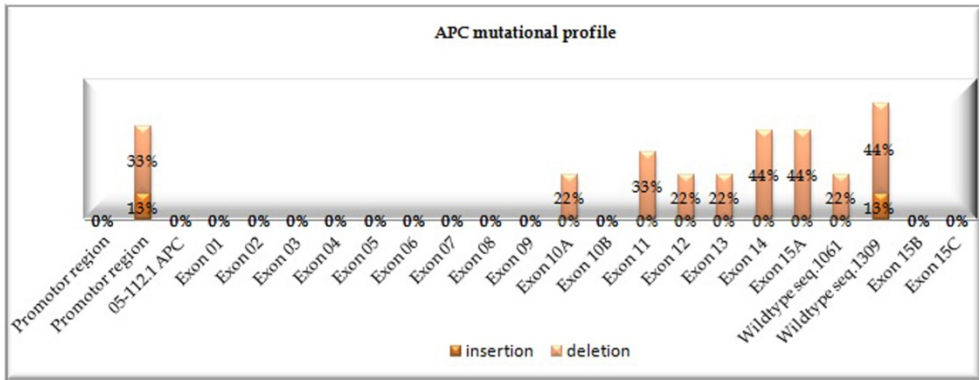


Figure 10. Mutational profile of APC by MLPA

The interpretation of the results was made by the help of a specific soft that assesses the reaction products in accordance with their molecular weight and quantitative expression. The GeneMapper results were exported in Coffalyzer software for normalization and the relative probe signals were calculated by dividing each measured peak area by the sum of all peak areas of the sample. A value of 1.0 indicated the presence of two alleles, and values of 0.5 and 1.5 represented a heterozygous deletion or duplication at that locus, respectively.

The mutational analyses at *APC* gene indicate that patient 15 diagnosed with FAP (Familial Adenomatous Polyposis) had deletion at the promoter region and also constitutional mutation 1309 (Figure 8) and no positive cases were found in the blood DNA samples.

This patient showed two deletions, in blood and in the tumour, in the promoter 2 and mutation 1309 region, although the individual did not show microsatellite loci alteration. Another example is patient 31 who presents a large deletion in between exon 12 – exon 15 (Figure 9) and by immunohistochemistry we found *APC* loss of expression in epithelial cells. In all studied cases we observed that 12% (9/ 75) of patients had a mutational profile. Deletions appeared frequently at the E12 - E15 level (11.9%) and in 3/ 9 cases in the promotor region 2 (33%); in E15 in 44% (4/ 9) of cases. Insertions were observed in 13% of cases (10/ 75) of cases in the promoter region and 13% (10/ 75) of patients have shown presence of wild type mutation 1309 (Figure 10).

Regarding the *CDH1* mutational status we observed that mutational profile appear in 30% (20/ 75) of patients. Insertion was observed at exon 4 in 30% (6/ 20) of patients and in 20% (4/ 20) of patients at exon 10. Loss of heterozygosity was observed at exons 08 and 13 in 20% (4/ 20) of patients for each exon (Figure 11). Without making microsatellite instability analyze, at the *CDH1* gene locus, loss of heterozygosity that was found by MLPA analysis was not necessary overlapped with results of E-cadherin protein expression studied by IHF in the tumors samples.

Mutational analyses at *BRCA1* gene indicate that 20% (15/ 75) of patients have mutations like duplication or loss of heterozygosity. Duplication at exon E13B was observed in 40% (6/ 15) of patients and at exon 20 was observed in 20% (3/ 15) of patients. As well as duplication, loss of heterozygosity was observed in principal to exon 13B in 40% (6/ 15) patients (Figure 12).

EGFR mutational status analyzes indicate that mutational profile appears like insertion, in 18.66% (20/ 75) of patients. Out of these, in 50% (10/ 20) of patients we observed insertion at the exon 3, in 20% (4/ 20) of patients at the exon 08, in 40% (8/20) of patients at the exon 17, in 40% (8/ 20) of patients at exon 25 and in 30% (6/ 20) of patients at exon 28 (Figure 13). For each of the following exons 02, 09 – 16, 18 – 24, 26 and 27 we have found insertions in 10% (2/ 20) of patients.

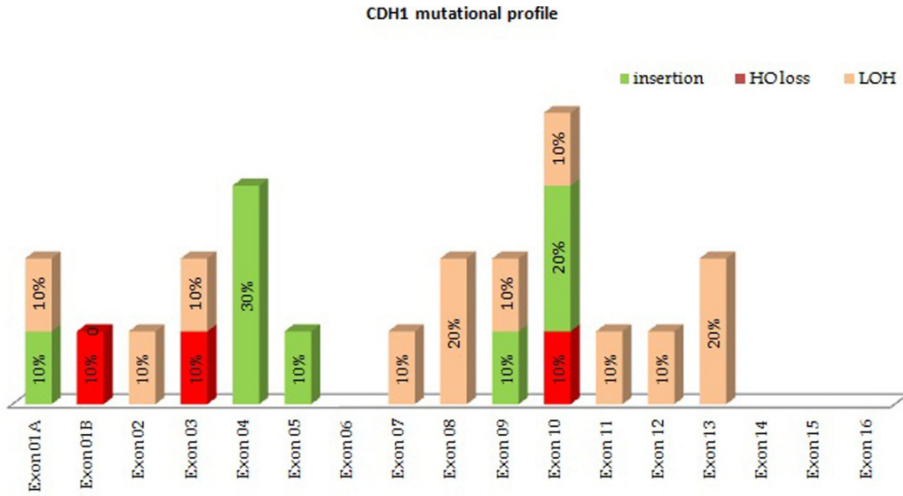


Figure 11. Mutational profile of *CDH1* by MLPA

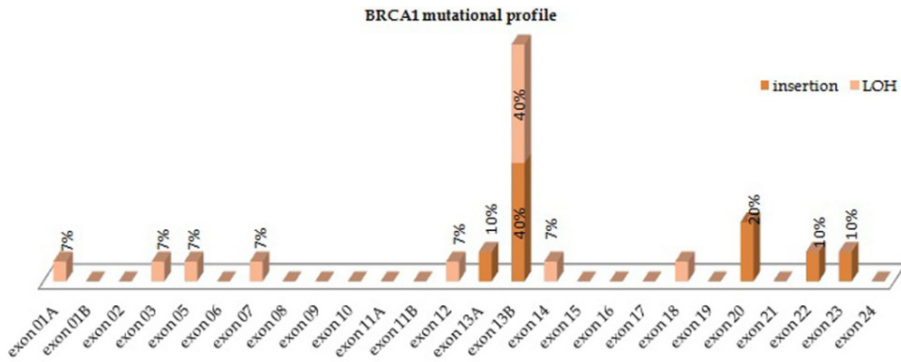


Figure 12. Mutational profile of *BRCA1* by MLPA

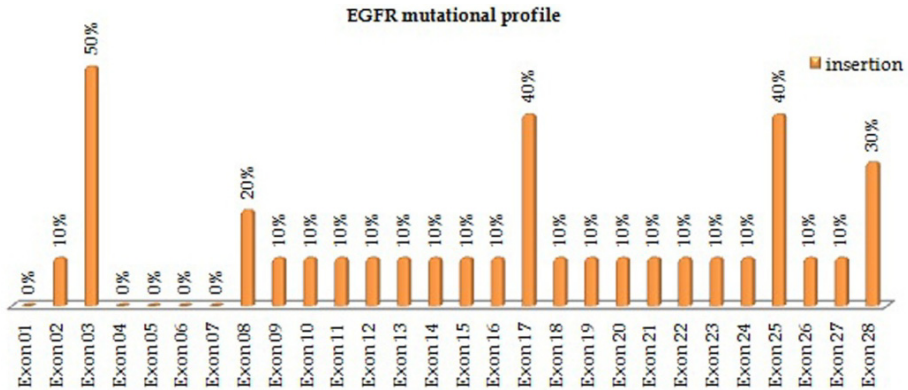


Figure 13. Mutational profile of *EGFR* by MLPA

2.5. Microsatellite instability correlation on *APC*, *BRCA1* and *PLA2G2A*

During tumorigenesis, loss of wild-type alleles (inherited from the non-mutation-carrying parents) is frequently observed. Loss of heterozygosity (LOH) on tumor suppressor genes play a key role in colorectal cancer transformation, and LOH analysis of sporadic colorectal cancers could help discover unknown tumor suppressor genes (Ahmed B, 2011). For those patients who presented deletion/ duplication at the interested genes, in order to have a more accurate mutational analysis we decided to analyze the microsatellite instability. A panel of microsatellite markers, labeled with FAM, HEX, TET, were used to amplify DNA from normal and tumour tissues for LOH and MSI analyses of chromosomal loci specifics for *APC*, *PLA2G2A*, and *BRCA1*.

In order to analyze the polymorphic microsatellite markers, a PCR reaction was carried out for 10 ng DNA from normal and tumour tissue. The fluorescent specific-marker amplification PCR products were separated on ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). Resulted electrophoregrams were analyzed with GeneMapper ID v3.1 software for molecular size and peak heights. Data analysis was done with Sequencing DNA Analysis Software. The allelic imbalance can appear as loss of heterozygosity (LOH) or as microsatellite instability (MSI). LOH was determined using the following ratio: $(T_1:T_2)/(N_1:N_2)$, where 1 and 2 are the first and the second peaks of alleles identified in the tumour/ blood DNA samples from patients with colorectal cancer. When the ratio is lower than 0.67 or higher than 1.5, this is revealing the loss of one of the alleles (LOH). The presence of a novel allele in the tumour sample was interpreted as microsatellite instability (MSI).

In case of homozygosity, the two alleles are identical as dimension, and the corresponding picks are overlapped. Thus we cannot make distinction between the two alleles and their height.

Highly polymorphic markers were designed for AI analysis. The designed microsatellite markers for *PLA2G2A* located on chromosome 1, were D1S199, D1S2843, D1S2644 which are located around the gene and D1S234 from the coding region of the gene. For *APC* gene we selected D5S82, D5S489 microsatellite markers which are surrounding the gene, D5S656 which partial overlaps the gene and D5S421 which are localized on the coding region. Another panel of microsatellites loci was used for *BRCA1* gene: D17S855, D17S1322, D17S1323 which are localized on the introns 20, 12 and 19 of the gene and, D17S250, D17S800, D17S856, D17S1327 on chromosome 17q, surrounding the gene.

At the microsatellite loci designed on chromosome 1, LOH/ MSI was observed in 28% (21/ 75) of patients and 68% (17/ 21) of these have had allelic imbalance at the D1S234 locus which covers the *PLA2G2A* locus (Figure 14, Figure 15). MSI was observed in only 6.66% (5/ 75) of patients (Figure 16) and that, make us to suggest that MSI is very rare in sporadic adenocarcinomas and routine screening such lesions for MSI may not be a high priority. Previous studies showed that the 1p36 region frequently present allelic loss in various cancers, such as colon cancer, neuroblastoma, hepatocellular carcinomas, lung cancer, and breast cancer. However, only NB (neuroblastoma) gene was confirmed to be the tumor suppressor

gene of neuroblastomas. In 1993, Tanaka *et al.* believed that a normal chromosome 1p36 might contain a tumor suppressor gene of colon carcinogenesis. Due to many genes located in the region of 1p36.33-36.31, additional analyses are necessary in order to confirm our hypothesis.

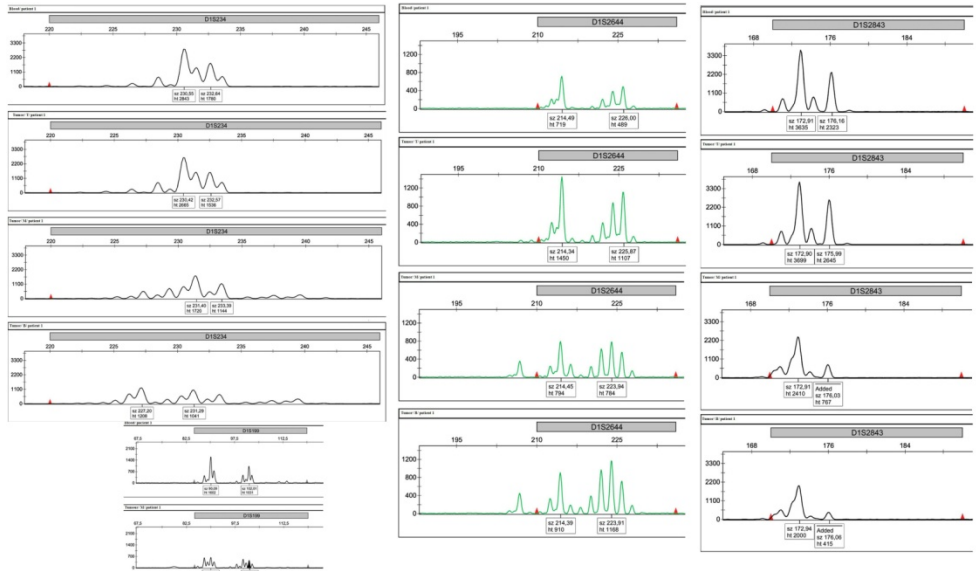


Figure 14. Microsatellite alteration for *PLA2G2A* gene in patient 1. D1S234, D1S 264 and D1S2843

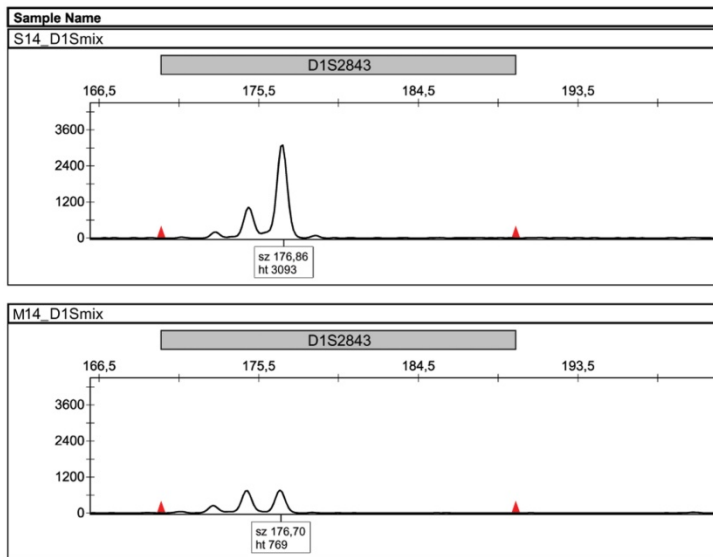


Figure 15. Microsatellite alteration for *PLA2G2A* genes in patient 14. D1S2843 - S14 – Blood (considered as normal); D1S2843 – M14 –MSI with low amplitude signal;

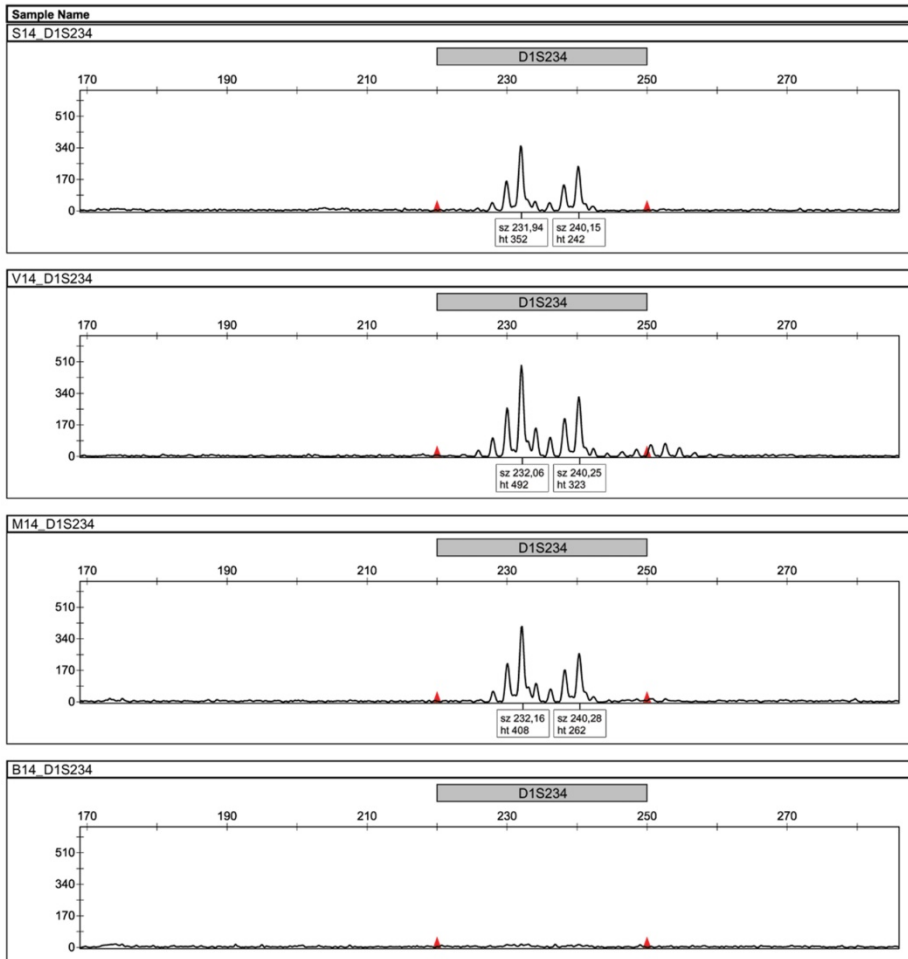


Figure 16. Microsatellite alteration for *PLA2G2A* genes in patient 14. D1S234 - S14 – Blood (considered as normal); D1S234 – Vf14 – with MSI; D1S234 – Mj14 –MSI with low amplitude signal; D1S234 – B14 – the signal could not be detected and was considered not measurable.

On chromosome 5 LOH/ MSI was observed in 38.66% (29/ 75) of patients (Figure 17) and 51.72% (15/ 29) of these have had allelic imbalance at the D5S421 locus which overlap the *APC* locus. MSI was observed only in 6.66% (5/ 75) of patients (Figure 17), similar with the results obtained for *PLA2G2A*. Allelic imbalance/ loss of heterozygosity appear to be a more frequent alteration than microsatellite instability in adenocarcinomas.

Microsatellites loci alterations corresponding to *BRCA1* gene have been found in 29.33% (22/ 75) of patients where D17S855 was the most affected (11 AI). Allelic imbalance analyses at the microsatellite loci D17S1323, D17S1322, and D17S855, which localize to introns 12, 19, and 20, respectively, indicates that 86.36% (19/ 22) of patients have LOH/ MSI in these

regions (Figure 18). Another observation is that for microsatellite marker D17S1327, all individuals have a homozygote profile.

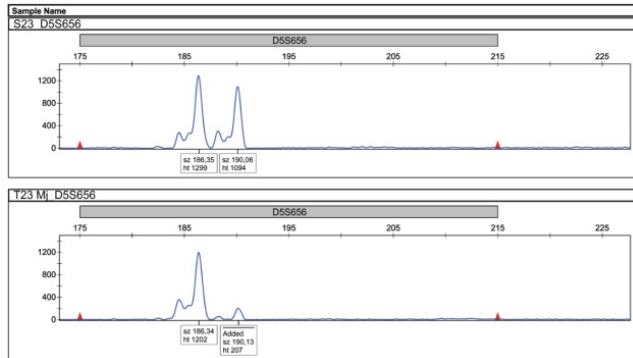


Figure 17. Microsatellite alteration for APC genes in patient 23. D5S656 - S23 – Blood (considered as normal); the report between D5S656 – T23_Mj is (1202:207)/(1299:1094) = 5 which is interpreted as LOH.



Figure 18. Microsatellite alteration for APC and BRCA1 genes at patient 1.

By examining the allelic imbalance analyses for the three genes included in this study and for all the patients, we can conclude that instability variation was: a) 29.63% on the short arm of chromosome 1; b) 55.56% on the long arm of chromosome 5; c) 37.10% on the long arm of chromosome 17 (Figure 19, Table 2). Because MSI was observed only in 13 patients (14.81%) we suppose that this type of instability is no specific for sporadic colorectal cancer and appears to be a relatively specific pointer for HNPCC. As MSI is very rare in sporadic adenomas, routine screening of such lesions for MSI is not a high priority (Xue-Rong C, 2006). However, MSI analysis in adenomas is likely to be useful in the cases where clinical features or family history suggest hereditary predisposition (Jesus V, 2011). Consequently, these results can be associated with sporadic colon cancer and not with hereditary cancer, like in HNPCC.

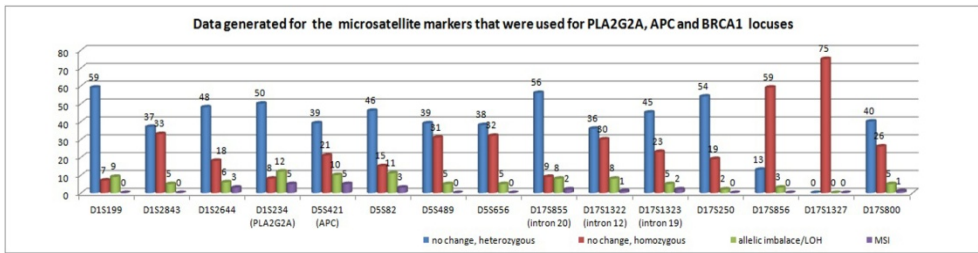


Figure 19. Comparative analyses of the fifteen microsatellites markers

By comparative analysis of all 15 microsatellite markers, we found that: a) 7/ 93 patients have instability on all three genes (7.52%); b) 20/ 93 patients on both *PLA2G2A* and *APC* genes (21.50%); c) 23/ 93 patients on both *APC* and *BRCA1* genes (24.73%); d) 7/ 93 patients on both *PLA2G2A* and *BRCA1* genes (7.52%) (Table 3).

	PLA2G2A microsatellite markers				APC microsatellite markers				BRCA1 microsatellite markers						
	D1S199	D1S2843	D1S2644	D1S234 (PLA2G2A)	D5S421 (APC)	D5S82	D5S489	D5S656	D17S855 (intron 20)	D17S1322 (intron 12)	D17S1323 (intron 19)	D17S250	D17S856	D17S1327	D17S800
no change, heterozygous	59	37	48	50	39	46	39	38	56	36	45	54	13	0	40
no change, homozygous	7	33	18	8	21	15	31	32	9	30	23	19	59	75	26
allelic imbalance/LOH	9	5	6	12	10	11	5	5	8	8	5	2	3	0	5
MSI	0	0	3	5	5	3	0	0	2	1	2	0	0	0	1
total samples	75	75	75	75	75	75	75	75	75	75	75	75	75	75	72
% Informativity	90.67	56.00	76.00	89.33	72.00	80.00	58.67	57.33	88.00	60.00	69.33	74.67	21.33	0.00	63.89

Table 2. The instability variation at the fifteen microsatellite loci

The frequencies of instability observed at *PLA2G2A* (89.33%) locus makes us not to exclude the possibility that *PLA2G2A* gene plays a key role in colorectal tumorigenesis. Similar to other studies we observed that the region where *PLA2G2A* gene is located is frequently modified in colorectal cancer, and encourages us not to exclude the possibility that it may represent a tumour suppressor gene.

On chromosome 5q, in the region where *APC* gene is located, the informative percent was 72.00%. Despite the construction of D5S421 microsatellite marker, in our analyses we

observed that the informative percent of the larger D5S82 (5q15 – 5q21) marker is at higher level (80.00%), and makes us to suppose that, probably, other genes around *APC* can be also mutated in colorectal cancer. According to our expectation, the other two markers located under D5S82 marker, have also a good informative percent: 58.67% for D5S489 (5q21) and 57.33% for D5S656 (5q21.3). On the other hand, the higher percentage of modifications encountered at the level of the microsatellites in the *PLA2G2A* gene region, demonstrates that the alterations at its level are much more frequent than those of the *APC* gene.

By comparing *APC* and *PLA2G2A* genes with the allelic imbalance observed at the *BRCA1* locus, the informative percent was 69.33%. Among all 7 microsatellites designed for the *BRCA1* gene, only one marker – D17S1327 is non-informative because it constantly appears as homozygote meaning that it has no variable number repeat. The most altered microsatellite marker was D17S855 (17q21), designed for intron 20 of *BRCA1* gene, for which the informative percent was 88.00%. For the other two markers designed into the *BRCA1* gene, namely D17S1322 for intron 12 and D17S1323 for intron 19, the informative percent was 56.00% and 64.00% respectively.

3. Conclusions

In order to improve the “personalized” therapeutic strategy in CRC, by our study we have comparatively evaluated the protein and gene expression for several key point biomarkers (*APC*, *PLA2G2A*, *CDH1*, *BRCA1*, and *EGFR*). Our *in vivo* experiment involved diagnosis testing of CRC patients and molecular biology testing on biological samples in order to clarify the cross-talk of interested genes and to better understand the CRC typology among Romanian patients.

We observed a close relationship in between different proteins and genes, which depends on the tumor type, cell grade and staging. For LOH/ MSI evaluation, our investigations were undertaken at the chromosomal regions where *APC*, *PLA2G2A* and *BRCA1* genes are located. We used microsatellite markers, in a series of sporadic CRCs with unknown status with respect to mutations in germline *PLA2G2A*, *APC* and *BRCA1*. Mutational status of 1p35-36.1, 5q and 17q21 chromosomal regions was evaluated and correlated with immunohistochemical and MLPA expression.

Regarding the *APC* MLPA analyses, our results are in accordance with those obtained by Sieber and Lamlum (2000), according to which, occasionally, in certain tumors in patients with germline mutations at the level of codon 1309, either the MCR (mutational cluster region) locus or the 3' and 5' region of *APC* gene, do not associate with the allelic loss at the level of adenomas. This same fact is observed in the case of patient 19 whose deletion, detected through MLPA at the E12 - E15 level, a region also including the MCR situ, is not supported by an allelic loss in any of the other microsatellite markers assayed. Although in this case no germline mutations were identified, we could extrapolate the same argument as Lamlum, starting from the premise that *APC* is often cited as the first tumor suppressor

gene affected both by familial and sporadic tumours. Regarding the PLA₂ type IIA expression our results suggest that the malignant cells lose their ability to express PLA₂ type IIA when invasive carcinoma develops in the adenoma. Our results are in line with the findings of Avoranta et al., who reported elevated gene and protein expression of PLA₂ type IIA in colorectal adenomas from FAP patients. The lack of PLA₂ type IIA expression is very common among colorectal cancer patients and, accordingly to the other studies, it seems that during tumor progression, malignant cells lose their ability to express PLA₂ type IIA. These patients have a better prognosis than the patients with positive tumours (Buhmeida A., 2009) in contrast to normal mucosa. Most of the cell types that over-express PLA₂ type IIA are apoptotic and necrotic, and this expression can be associated with the role of PLA₂ type IIA in promoting death of cancer cells. Regarding BRCA1 expression, previous studies indicate a higher rates of CRC in families linked to the *BRCA1* gene than in other families (Porter D.E., 1994) and mutations on this gene in stomach and colon cancers are associated with the microsatellite mutator phenotype. After several studies in which controversial importance of BRCA1 expression and mutator phenotype is still in debate, in 13.33% (10/ 75) of patients we observed a correlation between IHF and AI analyses. Considering that 3/7 microsatellites are intragenic to *BRCA1*, hypermethylation of *BRCA1* can be an event that has been described in breast and ovarian tumours. Because LOH was not observed in the microsatellites surrounding the *BRCA1* locus, the loss of the large part of chromosome 17q is not necessary to be considered. Somatic mutation can be taken in account because by MLPA analyses in 13.33% (10/ 75) of patients we observed deletion at different exons, especially on exon 13B. Our results suggest that BRCA1 can be an independent prognostic factor in patients with CRC, and it may be used to identify patient subgroups at high risk that might benefit from adjuvant chemotherapy. In conclusion, the comparative analyses between immunohistochemical expression and mutational status of *APC*, *PLA2G2A* and *BRCA1* genes suggest that at the *APC* level, 10% (7/ 75) samples have loss of heterozygosity without any presence of a deletion on MLPA. A complete loss is correlated with reduction of APC protein expression. The mutational status of the studied genes correlated with the protein and MLPA expression provides us useful data about the most common type of modification that can appear in individuals with colorectal cancer and how they can be group in order to receive a proper therapy.

Without making microsatellite instability analyze, at the *CDH1* gene locus, loss of heterozygosity that was found by MLPA analysis was not necessary overlapped with results of E-cadherin protein expression studied by IHF in the tumors samples. We can suppose that abnormal E-cadherin protein expression could be a result of some type of mutation at *CDH1* level or to others genes that are involved by association in its regulatory functions (some members of ECCU complex such α -cadherin or β -catenin), probably, as a consequence of tumor progression status. At the locus of *EGFR* gene, the mutational profile indicates only the presence of insertions, which can be interpreted as frame-shift mutations. The insertions founded at the exons E18, E19 and E21 are in relation with the catalytic domain of the *EGFR* gene. Future analyzes have to be done in order to reveal some specific somatic mutations that are generally associated with the target therapy in CRCs.

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